Folate Receptor β Is Expressed by Tumor-Associated Macrophages and Constitutes a Marker for M2 Anti-inflammatory/ Regulatory Macrophages

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Abstract

Macrophage activation comprises a continuum of functional states critically determined by cytokine microenvironment. Activated macrophages have been functionally grouped according to their response to pro-Th1/proinflammatory stimuli [lipopolysaccharide, IFNγ, granulocyte macrophage colony-stimulating factor (GM-CSF); M1] or pro-Th2/anti-inflammatory stimuli [interleukin (IL)-4, IL-10, M-CSF; M2]. We report that folate receptor β (FRβ), encoded by the *FOLR2* gene, is a marker for macrophages generated in the presence of M-CSF (M2), but not GM-CSF (M1), and whose expression correlates with increased folate uptake ability. The acquisition of folate uptake ability by macrophages is promoted by M-CSF, maintained by IL-4, prevented by GM-CSF, and reduced by IFNγ, indicating a link between FRβ expression and M2 polarization. In agreement with *in vitro* data, FRβ expression is detected in tumor-associated macrophages (TAM), which exhibit an M2-like functional profile and exert potent immunosuppressive functions within the tumor environment. FRβ is expressed, and mediates folate uptake, by $CD163^+$ $CD68^+$ $CD14^+$ IL-10–producing TAM, and its expression is induced by tumor-derived ascitic fluid and conditioned medium from fibroblasts and tumor cell lines in an M-CSF–dependent manner. These results establish FRβ as a marker for M2 regulatory macrophage polarization and indicate that folate conjugates of therapeutic drugs are a potential immunotherapy tool to target TAM. [Cancer Res 2009;69(24):9395–403]

Introduction

Macrophages exhibit a continuum of functional activation states under homeostatic and pathologic conditions (1, 2). Depending on the stimulus, activated macrophages acquire microbicidal, pro-inflammatory, and antitumor activities, but might also contribute to tissue repair, resolution of inflammation, and tumor cell growth and metastasis (1). These two extremes of the spectrum of macrophage activation have been coined as "classic"/M1 and "alternative"/M2 (3) and play opposing roles during immune and inflammatory responses. Although granulocyte macrophage colony-stimulating factor (GM-CSF) and M-CSF contribute to macrophage differentiation, each cytokine promotes the acquisition of distinct pathogen susceptibility (4) and inflammatory functions (5–8). GM-CSF–derived macrophages (M1) are proinflammatory and potentiate Th1 responses, whereas M-CSF–driven macrophages (M2) secrete IL-10 in response to pathogens and do not activate Th1 responses (8).

Tumor-associated macrophages (TAM) are abundant immunosuppressive cells recruited into the tumor microenvironment by cytokines such as M-CSF and CCL2 (9). The relevance of M-CSF and TAM in tumor progression and metastasis is now well established (10, 11). TAM represent a unique type of M2-polarized macrophages, as they promote angiogenesis, tissue remodeling, and repair (2, 12). In fact, clinical studies have revealed a correlation between high tumor macrophage content and poor patient prognosis. Because TAM are potential targets for anticancer therapy (13, 14), identification of TAM-specific markers constitutes a very active area of research.

The folate receptor gene family includes four members (FRα or *FOLR1*, FRβ or *FOLR2*, FRγ or *FOLR3*, and FRδ or *FOLR4*), whose encoded products bind folic acid with high affinity (15). *FOLR1* and *FOLR2* encode glycosyl phosphatidylinositol–anchored endocytic receptors expressed in certain epithelial tissues and various tumors (*FOLR1*; refs. 16, 17) or in normal myeloid cells and acute myelogenous leukemias (*FOLR2*; refs. 18–20). Within the myeloid lineage, folate receptor β (FRβ) is expressed in a nonfunctional state in $CD34^+$ bone marrow cells (21, 22) and neutrophils (18), whereas it mediates folate binding in activated synovial macrophages from rheumatoid arthritis (23) and in ovarian cancer–associated murine macrophages (24). The high affinity of FRα and FRβ for folate binding, their endocytic capacity, and their restricted expression have prompted the evaluation of the potential therapeutic value of folate-drug conjugates in cancer and inflammatory pathologies (25, 26).

In the present article, we describe that functional FRβ is specifically expressed by M-CSF–polarized (M2) macrophages as well as by *ex vivo* isolated TAM, and that tumors induce its expression in an M-CSF–dependent manner, thus supporting folate-drug conjugates as valuable tools to target TAM in tumor immunotherapy protocols.

Materials and Methods

Cell culture and treatments. Human monocytes were purified by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech) as described (27).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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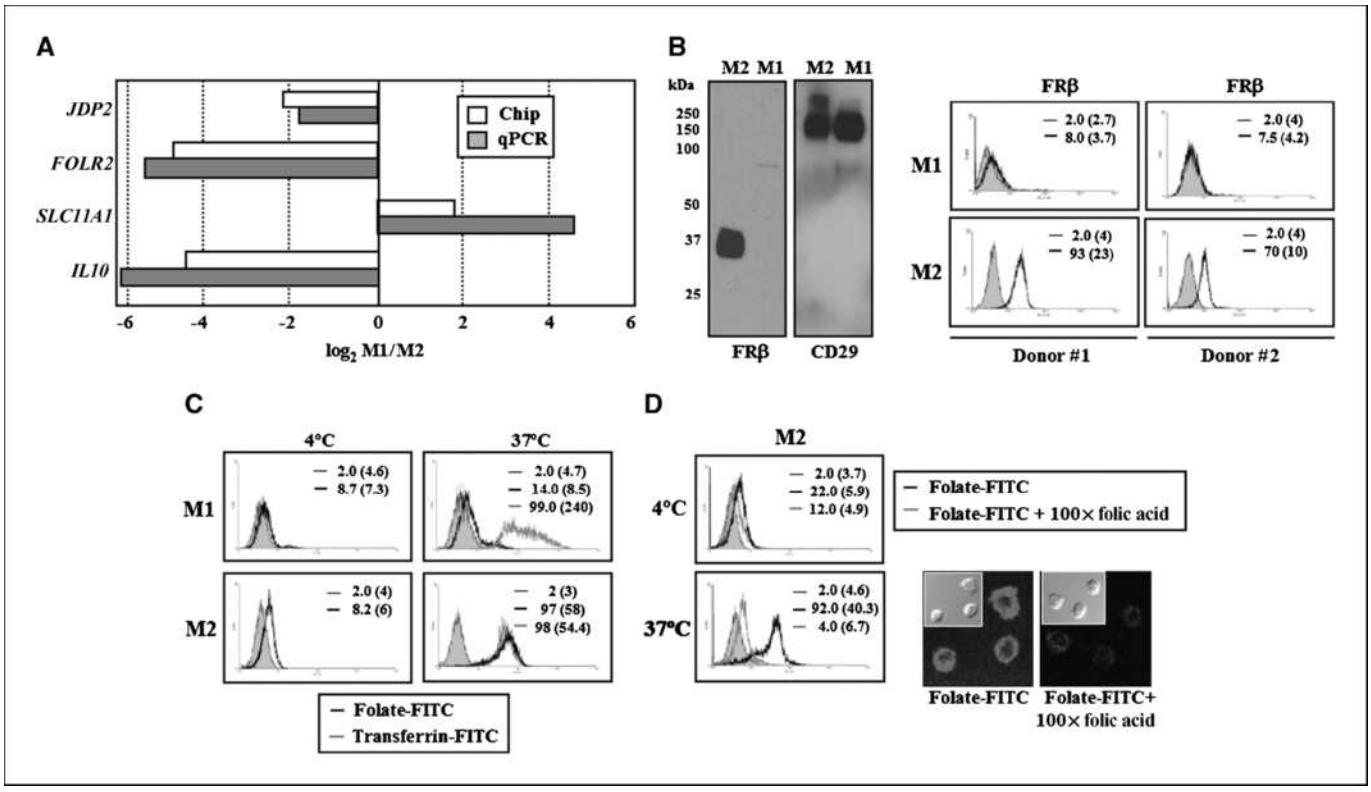

Figure 1. FOLR2 mRNA and FRβ protein expression and function in M1 and M2 macrophages. *A, FOLR2, JDP2, SLC11A1*, and *IL10* are differentially expressed in M1 and M2 macrophages, as determined by microarray DNA analysis and quantitative RT-PCR. *B, right,* FRβ expression in cell membrane extracts, as determined by Western blot using an antihuman FRβ polyclonal antiserum (18). As a control, CD29 expression levels were determined in parallel. *Left,* cell surface expression of FRβ on M1 and M2 macrophages, determined by flow cytometry using a polyclonal antiserum against human FRβ (ref. 18; *empty histogram*). As a control (*filled histogram*), a previously described rabbit preimmune antiserum (29) was used. *C,* FRβ function in M1 and M2 macrophages, as shown by binding (4°C) and uptake (37°C) of folate-FITC (*empty histogram, black line*). Transferrin-FITC internalization (*empty histogram, gray line*) was determined in parallel on both macrophage types. Each experiment was done three times, and a representative experiment is shown. *D,* binding (4°C) and internalization (37°C) of folate-FITC by M2 macrophages, in the absence (*empty histograms, black line*) or the presence (*empty histograms, gray line*) of a 100 mol/L excess of folic acid. The experiment was done four times, and one of the experiments is shown. Representative confocal sections of M2 macrophages incubated with folate FITC for 1 h at 37°C, and their corresponding differential interference contrast images, are shown. The percentage of marker-positive cells and the mean fluorescence intensity (in parentheses) are indicated in flow cytometry experiments (*B–D*), and filled histograms indicate cell autofluorescence (*C* and *D*).

M1 or M2 monocyte-derived macrophages were generated in the presence of GM-CSF (1,000 units/mL, ImmunoTools GmbH) or M-CSF (10 ng/mL), respectively. When indicated, macrophages were treated for 72 h with IL-6 or IL-10 (50 ng/mL), and anti-M-CSF blocking monoclonal antibody (Abingdon) was used at 0.5 μg/mL. For activation, macrophages were treated with IL-4 (1,000 units/mL), IL-10 (50 ng/mL), IFNγ (500 units/mL), or lipopolysaccharide (LPS; 50 ng/mL; *E. coli* 055:B5, Sigma) for 48 h. Human tumor cell lines (JAR, JEG-3, NIH-OVCAR-3, and Colo320) were cultured in DMEM containing 10% FCS. Cultures of tumor-associated fibroblasts were established from primary melanoma according to standard procedures.

Human TAM were obtained from melanoma and breast adenocarcinoma patients after obtaining written informed consent and following Medical Ethics committee procedures (Hospital General Universitario Gregorio Marañón). Histopathologic diagnosis was confirmed for each specimen. TAM were isolated by Ficoll gradient cell separation and subsequent magnetic cell sorting using CD14 microbeads. Phenotypic analysis was carried out by indirect immunofluorescence (28) using rabbit polyclonal antisera anti-human FRβ (18). Folate-FITC binding and endocytosis assays were done as reported (26). Flow cytometry on permeabilized *ex vivo* isolated TAM was done using phycoerythrin (PE)-labeled anti-CD68 monoclonal antibody (clone Y1/82A, Biolegend), Alexa Fluor 647–labeled anti-CD163 monoclonal antibody (clone RM3/1, Biolegend), and a polyclonal antiserum against human FRβ followed by incubation with FITC-labeled goat anti-rabbit affinity-purified antibody. The presence of Tie2-positive FRβ-positive macrophages was evaluated using a PE-labeled anti-Tie2 monoclonal antibody (clone 33.1, Biolegend). Isotype-matched monoclonal antibodies (PE-Control, Alexa 647-Control) and a preimmune rabbit antiserum (29) were used as negative controls.

Western blot. Western blot was carried out with 10 μg of lysates from crude plasma membranes (30). Protein detection was done with a polyclonal antisera against FRβ (18) or a monoclonal antibody against CD29. For control purposes, a previously described rabbit pre-immune antiserum was used (29).

PCR. Total RNA from solid tumor tissue and TAM was extracted (RNAeasy kit, Qiagen), retrotranscribed, and amplified using standard procedures. Oligonucleotides specific for *FOLR2, MAFB, IL10, ESR1, MAGEA3*, and *GAPDH* were as follows: FRBs, 5′-AGAAAGACATGGTCTGGAAATGGATG-3′, and FRBas, 5′-GACTGAACTCAGCCAAGGAGCCAGAGTT-3′ (21); Maf-Bs, 5′-CCCGGCTGGCCCGCGAGAGAC-3′, and Maf-Bas, 5′-CTAGGAGGCGGCGCTGGCGT-3′ (31); IL10s, 5′-ATGCCCCAAGCTGAGAACCAAGACCCA-3, and IL10as, 5-TCTCAAGGGGCTGGGTCAGCTATCCCA-3; ESR1s, 5′-TCAGATAATCGACGCCAGG-3′, and ESR1as, 5′-GGCTCAGCATCCAACAAGG-3′; MAGEA3s, 5′-GAAGCCGGCCCAGGCTCG-3′, and MAGEA3as, 5′-GGAGTCCTCATAGGATTGGCTCC-3′; and GAPDHs, 5′-GGCTGAGAACGGGAAGCTTGTCA-3′, and GAPDHas, 5′-CGGCCATCACGCCACAGTTTC-3′. Amplified fragments (783 bp for *FOLR2*, 347 bp for *MAFB*, 352 bp for *IL10*, 511 bp for *ESR1*, 457 bp for *GAPDH*, and 423 bp for *MAGEA3*) were resolved by agarose gel electrophoresis. For quantitative reverse transcription-PCR (RT-PCR), oligonucleotides for *FOLR1, FOLR2, FOLR3, JDP2, NRAMP1*, and *IL10* were designed according to the Roche software for quantitative real-time PCR, and RNA was amplified using the Universal Human Probe Roche library (Roche Diagnostics). Assays

were made in triplicates and results normalized according to the expression levels of 18S RNA and GAPDH. Results were obtained using the ΔΔCT method for quantitation and expressed as normalized fold expression.

Confocal microscopy and immunohistochemistry. Human melanoma tissues (subcutaneous tissue, lymph node, and lung metastasis) were obtained from patients with primary and metastatic lesions undergoing surgical treatment. Thick sections (4 μm in depth) of cryopreserved tissue were first blocked for 10 min with 1% human immunoglobulins and then incubated for 1 h with a rabbit polyclonal antiserum against human FRβ (18), anti-CD163 or HMB-45 monoclonal antibodies, or isotype-matched control antibodies. All primary antibodies were used at 1 to 5 μg/mL, followed by incubation with FITC-labeled antimouse and Texas red–labeled antirabbit secondary antibodies. Samples were imaged using a confocal scanning inverted AOBS/SP2 microscope (Leica Microsystems) with a 63× PL-APO NA 1.3 immersion objective. Image processing and colocalization analyses (scatter plots) were assessed with the Leica Confocal Software LCS-15.37. Tissue microarrays (TMAH-MTC-01, RayBiotech) were processed according to the manufacturer's recommendations.

Results

FRβ is expressed in macrophages generated in the presence of M-CSF. Gene expression profiling on macrophages generated in the presence of GM-CSF (M1) or M-CSF (M2) resulted in the identification of more than 250 differentially expressed genes (>2-fold differences, $P < 0.05$; data not shown). Among them, *FOLR2*, which codes for FRβ, was preferentially expressed in M2 macrophages ($P = 1.3 \times 10^{-7}$; Fig. 1*A*). The *JDP2* gene, which encodes an activator protein-1 repressor, also showed higher expression in M2 macrophages ($P = 0.02$), whereas *SLC11A1*, which encodes the NRAMP1 protein associated with classic macrophage activation, was expressed at higher levels in M1 macrophages ($P = 0.029$; Fig. 1*A*). Interestingly, and in agreement with their anti-inflammatory activity, the expression of *IL10* was considerably higher in M-CSF–primed macrophages ($P = 1.2 \times 10^{-4}$). The differential expression of *FOLR2, JDP2, SLC11A1*, and *IL10* in both types of macrophages was confirmed by real-time RT-PCR on mRNA from independent donors (Fig. 1*A*). Besides, FRβ expression was exclusively detected in membrane lysates and on the cell surface of M2 macrophages (Fig. 1*B*), thus validating the transcriptome data.

Because FRβ binds folic acid and folate conjugates (32), the ability of FRβ to mediate folate-FITC uptake by M2 macrophages was assessed. Whereas both macrophage types endocytosed transferrin-FITC, M-CSF–polarized macrophages displayed folate binding and internalization ability, and GM-CSF–induced macrophages showed no folate uptake capacity, in agreement with their lack of FRβ expression (Fig. 1*C*). Folate binding and uptake by M-CSF macrophages were specific, as both were inhibited by a 100 mol/L excess of folic acid (Fig. 1*D*). Moreover, folate conjugates entered cells by endocytosis because most of the folate-FITC fluorescence could not be stripped from the cell surface by an acid wash step (Supplementary Fig. S1). Considering that neither *FOLR1* nor *FOLR3* was expressed by M-CSF macrophages (Supplementary Fig. S2), *FOLR2*-encoded FRβ protein must be responsible for the folate binding ability of M2 macrophages. Kinetic studies revealed that *FOLR2* mRNA and FRβ protein are initially detected 48 to 72 hours after M-CSF addition, and that their levels dramatically increase at later incubation times (Fig. 2*A* and *B*). Acquisition of folate uptake ability correlated with protein expression at all time points and showed its highest level at the end of the culture period (Fig. 2*C*). Therefore, M-CSF promotes the expression of a functional FRβ protein, which constitutes a marker of M-CSF–polarized M2 macrophages.

Expression of FRβ in TAM. TAM are an M2-skewed macrophage population that exhibits immunosuppressive activity within the tumor microenvironment, and whose recruitment and differentiation is influenced by M-CSF (9). Given the preferential expression of FRβ in M-CSF–polarized M2 macrophages, its presence was evaluated in TAM. Immunohistochemistry revealed that FRβ is frequently coexpressed with CD163 in TAM from primary and metastatic melanoma (Figs. 3*A* and 4*A*) but is absent from melanoma HMB-45$^+$ cells (Figs. 3*A* and 4*A*). In fact, *FOLR2* mRNA could be detected in three melanoma samples (Fig. 3*B*). *Ex vivo* isolated CD14$^+$ TAM from the pleural fluid of a metastatic melanoma

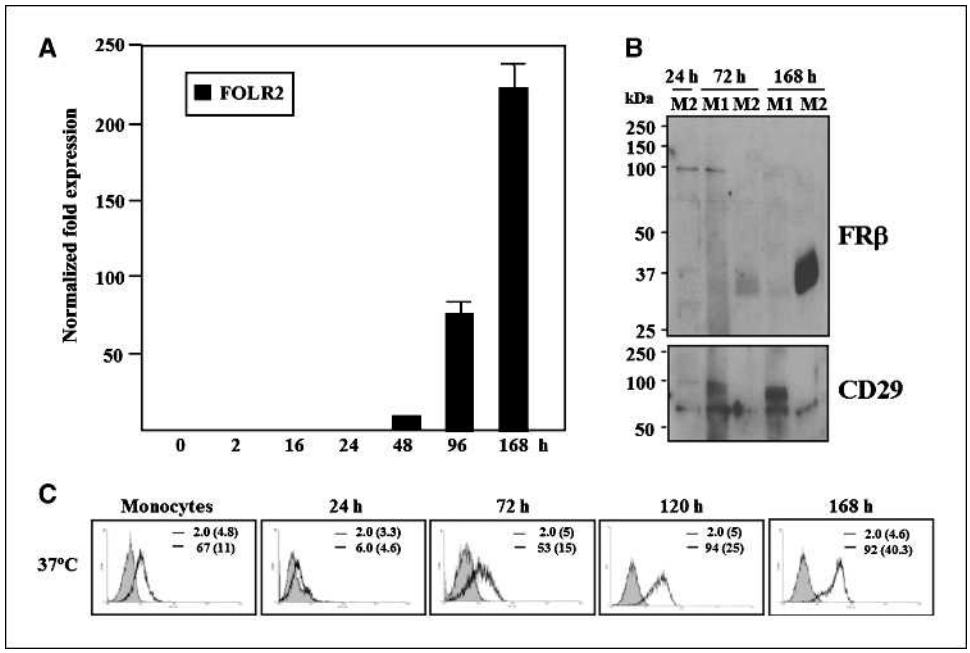

Figure 2. Acquisition of FRβ expression on monocyte treatment with M-CSF. *A, FOLR2* mRNA expression levels along M-CSF–induced polarization of macrophages, as determined by quantitative RT-PCR. *Columns,* mean normalized fold expression (relative to 18S rRNA levels) from triplicate determinations; *bars,* SD. *B,* FRβ expression along M1 and M2 macrophage polarization, as determined by Western blot at the indicated time points. As a control, CD29 expression levels were also determined. *C,* internalization of folate-FITC during M-CSF–induced macrophage polarization (*empty histograms*), as determined by flow cytometry at the indicated time points. *Filled histograms,* cell autofluorescence. The percentage of marker-positive cells and the mean fluorescence intensity (in parentheses) are indicated in each case.

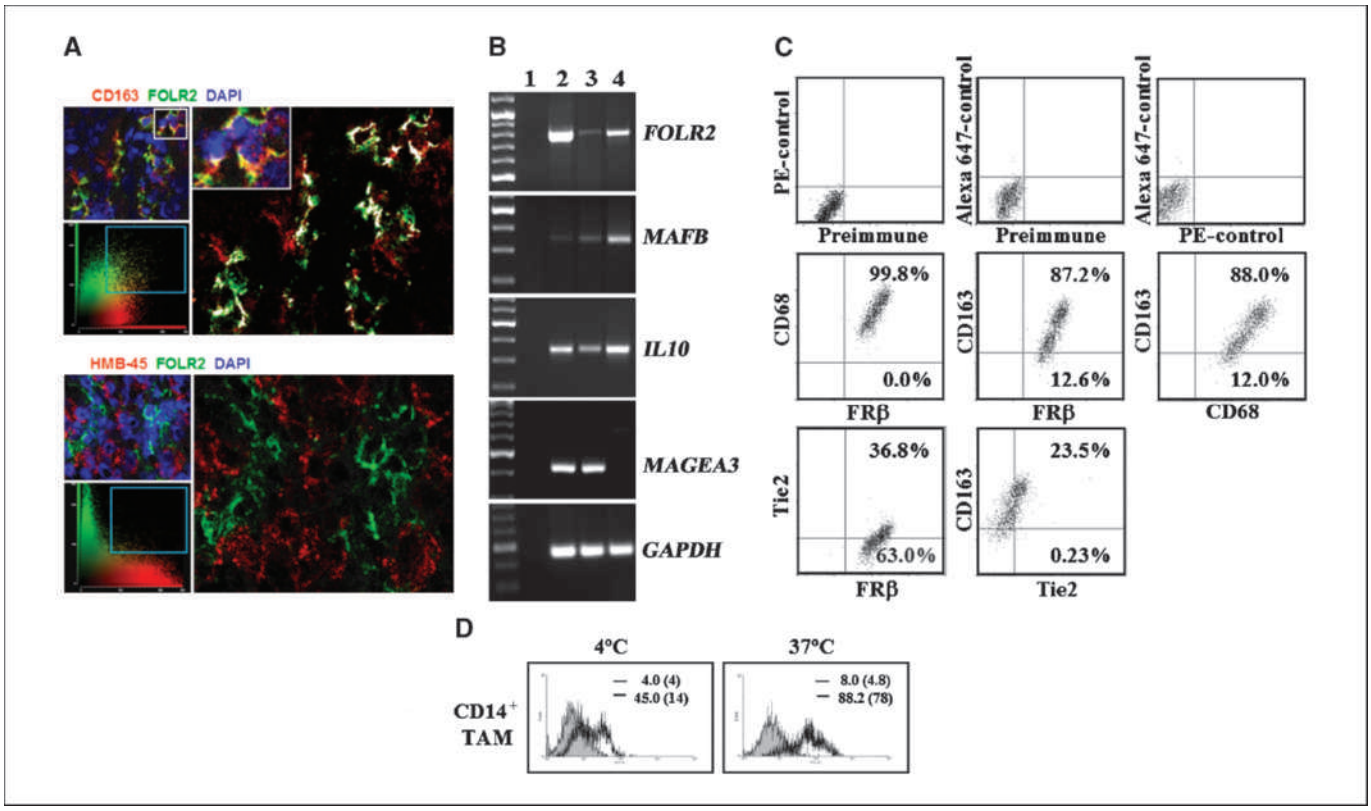

Figure 3. Expression and function of FRβ in TAM isolated from melanoma. *A,* confocal sections of infiltrating macrophages on a subcutaneous primary melanoma tissue sample, as determined by double immunofluorescence analysis of FRβ (*green*) and the macrophage marker CD163 (*red*; *top*), or FRβ and the melanoma marker HMB-45 (*red*; *bottom*). The corresponding scatter plots are shown, and colocalizing pixels (*blue rectangles*) are displayed on the merge images as white masks. Magnification of a FRβ/CD163 colocalizing area appears enlarged in the top image, and the enlarged area is depicted in white. In the top image, note the coexpression of FRβ by tumor-infiltrating macrophages ($CD163^+$), whereas nonstained areas correspond to tumor cells ($CD163^-$). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*). *B,* detection of *FOLR2, MAFB, IL10, MAGEA3,* and *GAPDH* mRNA by RT-PCR on RNA from two different primary melanoma tissues (*lanes 2* and *3*) and from $CD14^+$ cells isolated from the pleural fluid of a metastatic melanoma (*lane 4*). Control RT-PCR reactions were loaded in lane 1, next to the lane containing the molecular size markers. *C,* expression of CD68, CD163, Tie2, and FRβ in $CD14^+$ TAM isolated from a melanoma pleural fluid, as determined by three-color flow cytometry analysis on permeabilized cells. Isotype-matched monoclonal antibodies and a preimmune rabbit antiserum were used as negative controls. The percentages of single- and double-positive cells are indicated. *D,* binding (4°C) and internalization (37°C) of folate-FITC by $CD14^+$ TAM isolated from a metastatic melanoma (*empty histograms*). *Filled histograms,* cell autofluorescence. The percentage of marker-positive cells and the mean fluorescence intensity (in parentheses) are indicated.

expressed mRNA for *FOLR2, IL10*, and the macrophage-specific *MAFB* (31), whereas they lacked expression of the melanoma-specific marker *MAGEA3* mRNA (Fig. 3*B, lanes 4*) and were devoid of *FOLR1* and *FOLR3* mRNA (Supplementary Fig. S3). Three-color analysis on isolated melanoma TAM indicated that all $FR\beta^+$ macrophages are $CD68^+$, and that the percentage of $FR\beta^+$ $CD163^+$ macrophages (87%) is similar to that of $CD163^+$ $CD68^+$ cells (88%; Fig. 3*C*). Thus, most melanoma TAM from the analyzed sample coexpress CD163, CD68, and FRβ and exhibit folate-FITC internalization ability (Fig. 3*D*). It is also worth noting that a percentage of $FR\beta^+$ macrophages coexpress Tie2 (36%; Fig. 3*C*). Altogether, these results indicate that FRβ is functionally expressed on *IL10* mRNA–expressing $CD14^+$ $CD68^+$ $CD163^+$ melanoma TAM.

Evaluation of FRβ expression on other tumor tissues indicated that FRβ is detected in the stroma of lung, ovary, colon, gastric, and breast cancers, where numerous $CD68^+$ TAM were also present (Fig. 4*B*). Analysis of *ex vivo* isolated $CD14^+$ TAM from a metastatic breast adenocarcinoma also revealed the coexpression of CD68 and FRβ, and that 80% of the cells exhibited a $CD163^+$ $FR\beta^+$ phenotype (Fig. 5*A*). Importantly, primary and metastatic breast adenocarcinoma tissues were found to contain both *FOLR2* and *MAFB* mRNA (Fig. 5*B*), and *ex vivo* isolated $CD14^+$ metastatic breast adenocarcinoma TAM expressed *FOLR2, IL10*, and *MAFB* mRNA (Fig. 5*B, lane 5*). $CD14^+$ $CD163^+$ TAM also exhibited specific folate-FITC binding and uptake (Fig. 5*C*) and produced IL-10 in response to LPS stimulation (Fig. 5*D*). Because *FOLR2* and *IL10* mRNA are coexpressed in M2 macrophages *in vitro* (Fig. 1), and TAM from metastatic breast adenocarcinoma express functional FRβ and produce IL-10, these results indicate that FRβ activity marks anti-inflammatory M2-like TAM.

Parameters affecting FRβ expression on human macrophages. GM-CSF and M-CSF are tumor-derived factors that modulate myeloid cell differentiation (33). Unlike M-CSF, GM-CSF abrogated the aquisition of *FOLR2* mRNA during *in vitro* monocyte-to-macrophage differentiation, even in the presence of M-CSF (Fig. 6*A*). This result explains the differential expression of FRβ on both types of macrophages, and suggests that the relative levels of tissue GM-CSF and M-CSF determine macrophage FRβ expression. Other cytokines commonly released by tumors (33) also affected *FOLR2* mRNA; IL-6 alone and IL-10 in combination with M-CSF upregulated *FOLR2* mRNA expression (Fig. 6*B*). Therefore, tumor-derived cytokines (M-CSF, GM-CSF, IL-6, and IL-10) modulate FRβ expression in human macrophages.

Expression of FRβ on TAM led us to analyze the nature of the stimuli that might control its presence in the tumor microenvironment. *FOLR2* mRNA was variably upregulated by supernatants from tumor cell lines, with placenta choriocarcinoma JAR and JEG-3 cells and ovary carcinoma NIH-OVCAR-3 cells promoting the highest level of upregulation (Fig. 6*C*). In contrast, conditioned media from colon carcinoma Colo320 cells had no effect (Fig. 6*C*). More importantly, ascitic fluid from the breast carcinoma analyzed in Fig. 5 promoted a strong upregulation of *FOLR2* mRNA (Fig. 6*C*), confirming that tumor cells release factors that upregulate human macrophage FRβ expression. The addition of a blocking anti–M-CSF monoclonal antibody greatly reduced the upregulation of *FOLR2* mRNA promoted by ascitic fluid from breast carcinoma (Fig. 6*D*) or by conditioned medium from tumor-associated fibroblasts or JEG-3 tumor cells (Fig. 6*D*). Therefore, M-CSF is a major determinant for FRβ expression on human macrophages and contributes, alone or in combination with other cytokines, to FRβ cell surface expression on TAM.

Discussion

GM-CSF and M-CSF contribute to the generation of different macrophage subsets and enhance myeloid cell survival and proliferation (9). However, GM-CSF promotes the generation of myeloid cells with potent antigen presentation activity, whereas M-CSF leads to the generation of macrophage cells with regulatory properties (9). Gene expression profiling allowed us to identify FRβ as preferentially expressed by macrophages generated under the influence of M-CSF, which display FRβ-dependent folate binding ability. FRβ expression on *in vitro* differentiating macrophages was enhanced by M-CSF and by tumor cell-conditioned medium in an M-CSF–dependent manner. Conversely, GM-CSF prevented the acquisition of FRβ expression. Importantly, FRβ was detected in TAM, where FRβ-mediated folate binding activity correlates with the presence of *IL10* mRNA. Therefore, FRβ constitutes a marker for M-CSF–primed IL-10–expressing M2-polarized macrophages, providing a molecular basis for the value of folate-conjugated drugs in cancer therapy approaches.

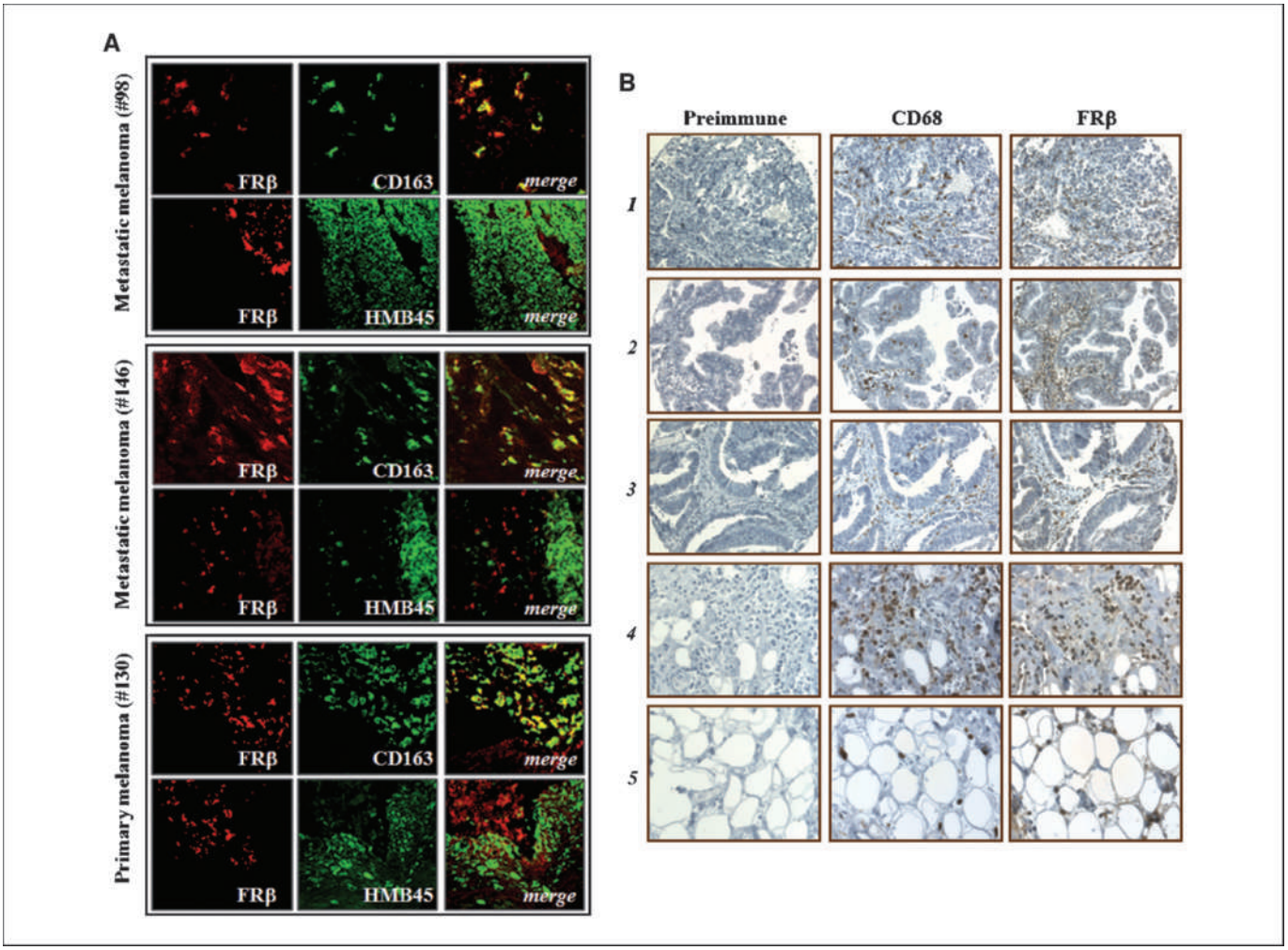

Figure 4. Expression of FRβ in TAM from primary and metastatic melanoma. *A,* expression of FRβ in melanoma-infiltrating macrophages on a primary melanoma (#130, *bottom*) or two metastatic melanomas (#98 and #146, *top* and *middle*), as determined by double immunofluorescence analysis of FRβ and the macrophage marker CD163 (*top rows*) or FRβ and the melanoma tumor marker HMB-45 (*bottom rows*). *B,* expression of FRβ in tumors of distinct tissue origins. Light microscopy images of the macrophage marker CD68 (*middle*) and FRβ (*right*) staining of tumor tissue from lung squamous cancer (*1*; magnification, ×20), ovarian cystadenoma-mucous (*2*; magnification, ×20), rectal colon adenocarcinoma (*3*; magnification, ×20), gastric adenocarcinoma (*4*; magnification, ×40), and breast invasive ductal cancer (*5*; magnification, ×40). *Left,* staining yielded by normal rabbit serum, used as a control.

Because macrophage polarization is stimulus dependent (1), alternatively activated M2 macrophages have been further classified as M2a, M2b, or M2c in an effort to link genetic markers to specific macrophage-activating stimuli (34). The expression of FRβ in M-CSF–generated macrophages indicates that it is preferentially expressed by IL-10–producing M2 macrophages and, therefore, identifies a population of macrophages with anti-inflammatory/regulatory properties. The presence of FRβ in M-CSF–primed *in vitro* macrophages is in agreement with its upregulation in human decidual macrophages, which exhibit an immunosuppressive phenotype and whose gene expression profile closely corresponds to that of M2-polarized macrophages (35). Further supporting its presence on M2 macrophages, FRβ has been detected on F4/80$^+$ CD68$^+$ murine peritoneal macrophages (36), where its mRNA levels can be further upregulated by IL-4 (37). Therefore, FRβ expression seems not to be restricted to anti-inflammatory/regulatory IL-10–producing M2 macrophages and marks a wider range of alternatively activated macrophages in the human and murine systems. However, the functional state of FRβ on murine peritoneal macrophages is still not clear because folate binding ability is only detected after stimulation with inflammatory stimuli (26).

The expression of FRβ on TAM from primary and metastatic melanoma and breast carcinoma (Figs. 3–5) is also in agreement with a previous report describing the presence of FRβ in CD68$^+$ CD163$^+$ cells within human and rat glioblastoma (36). In an apparent contradiction, gene expression profiling has revealed downregulated FRβ mRNA levels in murine fibrosarcoma TAM relative to the levels detected in thioglycollate-elicited peritoneal macrophages (12). However, because the latter exhibit functional characteristics of M-CSF–driven M2 macrophages (38), these results do not rule out the presence of detectable levels of FRβ in murine TAM. Besides, it is also possible that FRβ is expressed by TAM in a tumor-dependent manner, a phenomenon which would be in agreement with its differential upregulation by distinct tumor-conditioned media (Fig. 6) and the variable levels of FRβ in TAM from a variety of human tumors (Fig. 4). Finally, it is also possible that differences might exist between murine and human TAM, as it is already evident that paradigmatic M2 murine macrophage markers (Arginase and Ym1) are not useful to identify human alternatively activated macrophages (39). Whether the acquisition of FRβ expression by tumor-infiltrating macrophages is detrimental for the tumor (e.g., by removing folate) or favors tumor cell growth is a matter that deserves further investigation. Regardless of the precise role of FRβ on TAM, the presence of FRβ on their cell surface provides an opportunity for depletion of TAM through the use of folate-conjugated drugs. As an example, and while this article was being completed, Nagai and coworkers have shown the feasibility of reducing tumor growth by

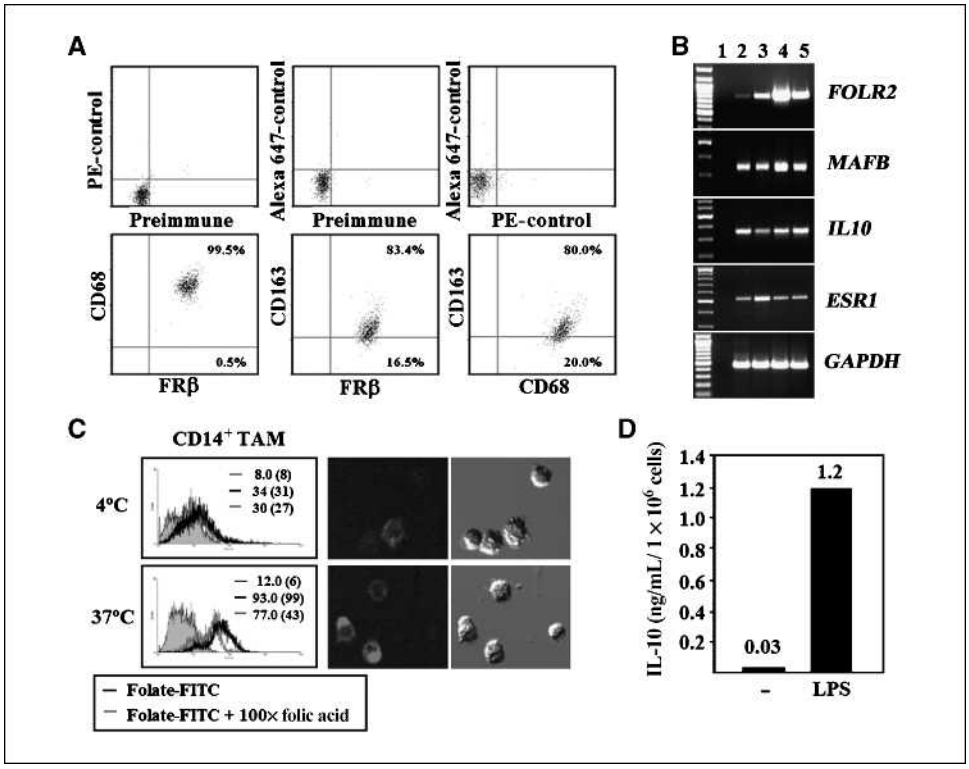

Figure 5. Expression and function of FRβ in TAM isolated from breast adenocarcinoma. *A,* expression of FRβ, CD68, and CD163 in permeabilized CD14$^+$ TAM from a metastatic breast adenocarcinoma, as determined by flow cytometry using PE-labeled anti-CD68, Alexa Fluor 647–labeled anti-CD163, and a polyclonal antiserum against human FRβ (18), followed by FITC-labeled goat anti-rabbit antibodies. Isotype-matched monoclonal antibodies and a preimmune rabbit antiserum (29) were used as negative controls (*top*). The percentages of single-positive and double-positive cells are indicated. *B,* detection of the indicated mRNA by RT-PCR on RNA from three different primary breast adenocarcinoma tissues (*lanes 2–4*) and from CD14$^+$ cells isolated from ascitic fluid from a metastatic breast adenocarcinoma (*lane 5*). Control RT-PCR reactions were loaded in lane 1, next to the lane containing the molecular size markers. *C,* binding (4°C; *top*) and internalization (37°C; *bottom*) of folate-FITC by CD14$^+$ TAM isolated from a metastatic breast adenocarcinoma, in the absence (*empty histograms, black line*) or the presence (*empty histograms, gray line*) of a 100 mol/L excess of folic acid. *Filled histograms,* cell autofluorescence. The percentage of marker-positive cells and the mean fluorescence intensity (in parentheses) are indicated. The experiment was done two times, and one of the experiments is shown. Representative confocal sections and differential interference contrast microscopy images of macrophages incubated with folate-FITC. *D,* CD14$^+$ TAM isolated from a metastatic breast adenocarcinoma were either untreated or stimulated with LPS (50 ng/mL) for 24 h, and IL-10 release was determined by ELISA. *Columns,* mean of triplicate determinations; *bars,* SD.

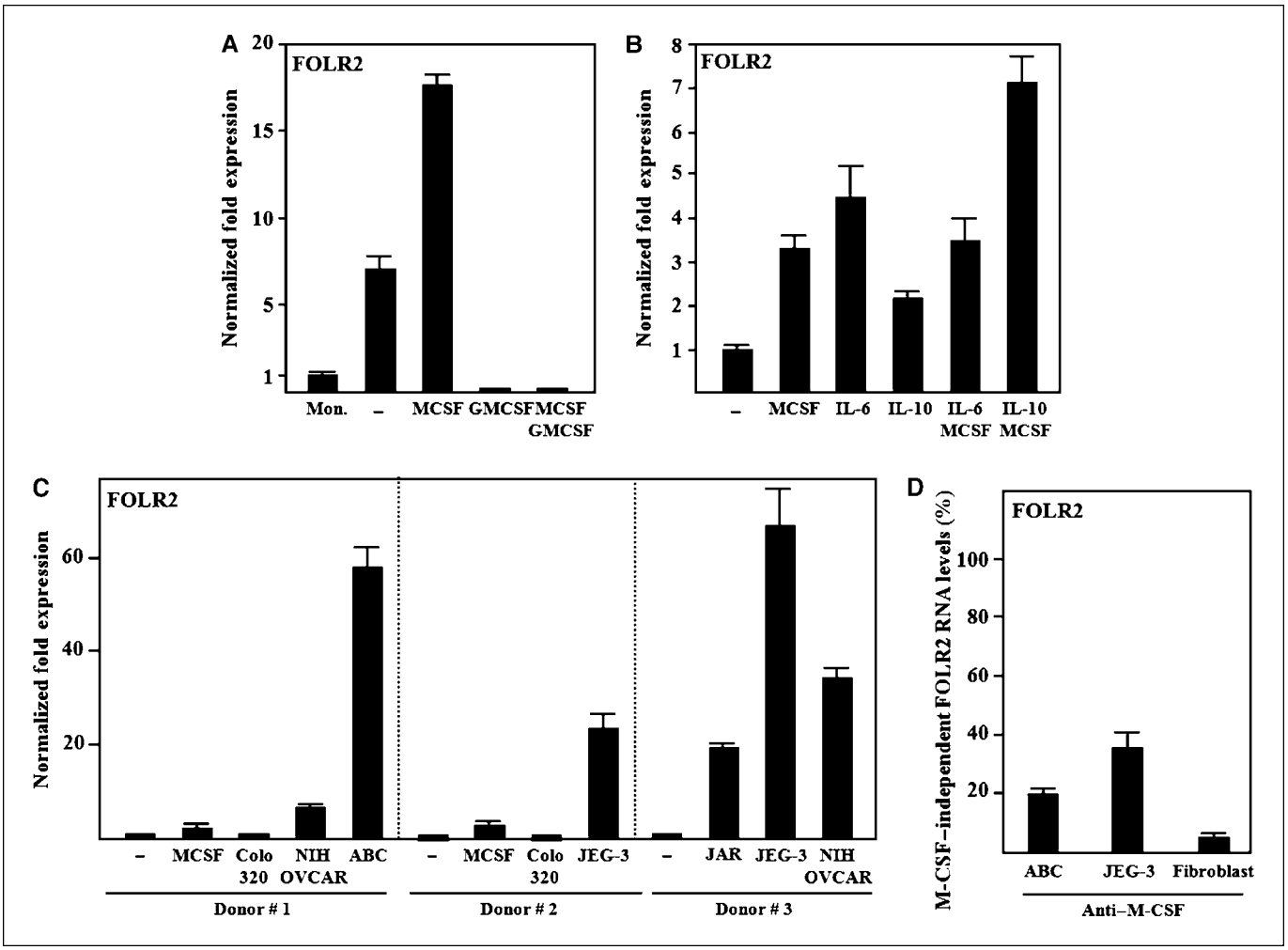

Figure 6. Parameters affecting FRβ expression on human macrophages. *A* and *B, FOLR2* mRNA expression in macrophages exposed for 72 h to the indicated cytokines, as determined by quantitative RT-PCR. Results are expressed as normalized fold expression relative to 18S rRNA levels and the *FOLR2* RNA levels in peripheral blood monocytes (*Mon.*). *Columns,* mean of triplicate determinations; *bars,* SD. *C, FOLR2* mRNA expression in macrophages exposed for 72 h to conditioned media from the ascitic fluid of a breast carcinoma (*ABC*) or tumor cell lines, as determined by quantitative RT-PCR. Results are expressed as normalized fold expression (relative to 18S rRNA levels). *Columns,* mean of triplicate determinations from three independent macrophage preparations; *bars,* SD. *D,* inhibitory effect of anti–M-CSF on *FOLR2* mRNA levels induced by ascitic fluid from metastatic breast carcinoma (*ABC*) or conditioned-medium from JEG-3 placenta choriocarcinoma (*JEG-3*) or tumor-associated fibroblasts (*Fibroblast*). The results are depicted as the *FOLR2* mRNA levels detected in the presence of the anti–M-CSF antibody relative to the levels seen in untreated cells (set to 100 in the three cases).

targeting an immunotoxin to TAM using an antimouse FRβ monoclonal antibody (36).

Given the tumor influence on macrophage functions (40), FRβ might specifically mark tumor-infiltrating human macrophages whose effector functions have been already skewed by tumor-derived factors. In this regard, our data also suggest that tumor-derived M-CSF, which recruits and shapes macrophage functions (33), would be the primary determinant for FRβ expression. However, FRβ expression is also detected in resident macrophages within nontumor tissue (data not shown). This fact, together with the increase in FRβ expression during the *in vitro* macrophage differentiation that takes place in the absence of exogenous cytokines, might indicate that FRβ could be a macrophage differentiation marker under homeostatic conditions, and whose levels could be maintained or upregulated by anti-inflammatory cytokines and downregulated by pro-inflammatory stimuli. In this regard, cytokines such as IL-4, IL-13, and IL-10, which promote macrophage alternative activation, trigger a transient increase of *FOLR2* mRNA levels in M2 macrophages (Supplementary Fig. S4*A* and *B*). By contrast, LPS greatly downregulates *FOLR2* mRNA levels (Supplementary Fig. S4*A* and *B*) although it does not lead to a great decrease in cell surface FRβ (Supplementary Fig. S4*C*). This divergence might be explained by the fact that *FOLR2* is an endocytic receptor whose protein levels are higher than those present on the cell surface. In fact, flow cytometry on permeabilized cells showed that a large proportion of FRβ is located intracellularly in both *in vitro* M2 macrophages and TAM (Supplementary Fig. S5).

The presence of functional FRβ on M-CSF–primed macrophages and the detection of FRβ mRNA in other types of M2-polarized macrophages (12, 35, 37) are difficult to reconcile with its expression (25) and function (26) in synovial macrophages from rheumatoid arthritis patients, which are embedded in a inflammatory pro-M1 environment. It could be speculated that synovial macrophages might exhibit a mixed M1/M2 phenotype, similar to what occurs with myeloid populations within tumors (41), an explanation that would be compatible with the high levels of M-CSF

present in rheumatoid arthritis synovia (42). M-CSF is produced by synovial fibroblasts, and administration of M-CSF is known to exacerbate arthritis in some settings (9, 43). Therefore, the levels of M-CSF within the synovia of rheumatoid arthritis might suffice to promote FRβ expression on surrounding macrophages, although the concomitant presence of extremely high levels of tumor necrosis factor α might override its immunosuppressive actions. Alternatively, because M-CSF contributes to macrophage recruitment, FRβ expression might mark macrophages newly recruited into the arthritic synovia, whose later levels of FRβ expression would be determined by the pro-inflammatory environment. In this regard, it is worth noting that (*a*) FRβ$^+$ macrophages are more prominently detected at early stages during development of animal models of atherosclerosis and muscle injury and in rheumatoid arthritis in humans;[6] (*b*) *in vitro* acute (48 hours) exposure of FRβ-expressing M2 macrophages to M1-polarizing stimuli (e.g., LPS, GM-CSF, and IFNγ) does not result in loss of FRβ expression, which is only moderately downregulated by IFNγ (Supplementary Fig. S4*C*). Therefore, folate-targeted killing of FRβ$^+$ macrophages in inflammatory disease murine models might contribute to inflammation resolution by preferentially eliminating newly recruited macrophages.

Although further studies are needed to correlate FRβ expression and function in macrophages within inflamed tissues, our results indicate that cytokines favoring the generation of anti-inflammatory/regulatory macrophages, and known to shape TAM effector functions (M-CSF and IL-10), promote and are permissive for FRβ expression, whereas factors skewing macrophage polarization toward the proinflammatory branch either inhibit (IFNγ) or abrogate FRβ expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[6] P. Low, personal communication.

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