Macrophage uptake and accumulation of folates are polarization-dependent in vitro and in vivo and are regulated by activin A

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ABSTRACT

Vitamin B9, commonly known as folate, is an essential cofactor for one-carbon metabolism that enters cells through three major specialized transporter molecules (RFC, FR, and PCFT), which differ in expression pattern, affinity for substrate, and ligand-binding pH dependency. We now report that the expression of the folate transporters differs between macrophage subtypes and explains the higher accumulation of 5-MTHF—the major folate form found in serum—in M2 macrophages in vitro and in vivo. M1 macrophages display a higher expression of RFC, whereas FRβ and PCFT are preferentially expressed by anti-inflammatory and homeostatic M2 macrophages. These differences are also seen in macrophages from normal tissues involved in folate trans- (placenta, liver, colon) and inflamed tissues (ulcerative colitis, rheumatoid arthritis, breast cancer resistance protein, FSGS glioma-derived nutrient transporter, qRT-PCR quantitative RT-PCR, RA rheumatoid arthritis, RFC reduced folate carrier, ROI regions of interest, siRNA small interfering RNA, SLC solute-carrier gene, SPT spleen focus forming virus, TBP TATA box binding protein, THF tetrahydrofolate)

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Macrophages are phagocytic cells present in all tissues and show a remarkable plasticity in response to environmental signals [1, 2]. The development of macrophages from monocytes is regulated by the CSFs M-CSF and GM-CSF [3], with a prominent role for the transcription factor PU.1, together with C/EBPα and runt-related transcription factor 1 [4, 5]. In vitro, GM-CSF and M-CSF generate phenotypically and functionally different macrophages [6]; whereas GM-CSF-driven macrophages produce proinflammatory TNF-α, IL-23, and IL-12 in response to TLR ligands and promote type 1 immunity, M-CSF-derived macrophages produce IL-10 in response to pathogens, inhibit Th1 responses, and exhibit regulatory properties [3, 7, 8]. Therefore, GM-CSF- and M-CSF-induced macrophages are considered proinflammatory (M1 macrophages) and anti-inflammatory (M2 macrophages), respectively.

Folic acid is a member of the B-complex family of vitamins produced by most bacteria and plants by de novo biosynthesis. Mammalian cells do not have the metabolic machinery to synthesize folates, and folate requirements depend mainly on dietary sources [9]. The reduced form of the vitamin, 5-MTHF, functions as a cofactor in one-carbon transfer reactions and plays a key role in metabolic pathways involved in DNA synthesis, epigenetic processes, and proliferation [9, 10]. The regulation of total cellular folate concentrations depends on uptake, polyglutamylation, export, and catabolism [10]. Folate uptake is mediated by three distinct systems [9]: (1) the RFC (gene symbol SLC19A1), a bidirectional transporter ubiquitously expressed in normal adult tissues and the major route of folate transport to tissues at physiological pH; (2) the high-affinity

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Abbreviations: 5-MTHF 5-methyltetrahydrofolate, ABC ATP-binding cassette, ALK activin receptor-like kinase, BCRP breast cancer resistance protein, FOLR2 folate receptor 2, FPGS glutamate synthetase, FR folate receptor, GGH γ-glutamyl hydrolase, HCP1 home carrier protein 1, MRP multidrug resistance protein, PCFT proton-coupled folate transporter, qRT-PCR quantitative RT-PCR, RA rheumatoid arthritis, RFC reduced folate carrier, ROI regions of interest, siRNA small interfering RNA, SLC solute-carrier gene, SPT spleen focus forming virus, TBP TATA box binding protein, THF tetrahydrofolate
PCFT (HCP-1, gene symbol SLC46A1), involved in intestinal absorption of folate [11]; (3) and the high-affinity GPl-linked FRs, FRα, FRβ, and FRβ [12]. Whereas FRα is expressed in the apical surface of epithelial cells and tumors of epithelial origin [12], and FRβ marks regulatory T cells [13], FRβ (gene symbol FOLR2) expression appears to be myeloid-restricted [14], but the molecular basis for its tissue-restricted expression remains unknown. Within the myeloid lineage, FRβ is expressed in a nonfunctional state in CD34+ cells and neutrophils [15], whereas it mediates folate binding in activated synovial macrophages from RA and osteoarthritis [16, 17], M-CSF-driven M2 macrophages and TAMs [18]. The restricted expression of FRα, FRβ, and FRβ, together with the high-affinity for folate binding and endocytosis, has prompted the evaluation of the potential therapeutic value of folate drug conjugates in cancer and in inflammatory pathologies [19, 20].

5-MTHF is trapped within the cells by polyglutamylation of THF. The enzyme FPGS catalyzes the polyglutamylation of folates, whereas the enzyme GGH catalyzes the hydrolysis of the glutamate residues from polyglutamylated folates [21]. Long-chain polyglutamylated folate derivatives are no substrate of efflux systems that include MRP s (ABC exporters), BCRP, as well as the bidirectional folate transporter RFC [21]. Polyglutamylation is a determinant of intracellular retention and increases the efficiency of THF cofactor use.

We have reported previously that FRβ is preferentially expressed on human anti-inflammatory M2 (M-CSF) macrophages and in M2-polarized TAM [18, 22]. We have now analyzed the expression of RFC, PCFT, and FRβ in macrophages under homeostatic and inflammatory conditions and found that the pattern of expression of folate transporters explains the different 5-MTHF transport ability of M1 and M2 macrophages, allowing the discrimination of macrophage polarization states in vitro and in vivo. Besides, we found that activin A controls the expression of folate transporters in macrophages and modulates 5-MTHF uptake.

MATERIALS AND METHODS

Cell culture

Human PBMCs were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient, according to standard procedures. Monocytes were purified from PBMCs by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Auburn, CA, USA). Monocytes were cultured at 0.5 × 10⁶ cells/ml for 7 days in RPMI 1640 (standard RPMI, which contains 1 mg/L folic acid), supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO₂, containing standard RPMI, which contains 1 mg/L folic acid), supplemented with 10% FCS at 37°C in a humidified atmosphere with 5% CO₂, containing 1% human Ig, and incubated with primary (1–5 μg/ml) and matched control antibodies and fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For CD68 and CD209 staining, cells were permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). CD206 staining was carried out with PE-labeled anti-CD206 (BioLegend, San Diego, CA, USA); and PE-labeled anti-CD68 (BioLegend, San Diego, CA, USA). For CD68 and CD209 staining, cells were permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). CD206 staining was carried out with PE-labeled anti-CD68 (BioLegend, San Diego, CA, USA) and the high-affinity GPl-linked FRs, FRα, FRβ, and FRβ [12]. Whereas FRα is expressed in the apical surface of epithelial cells and tumors of epithelial origin [12], and FRβ marks regulatory T cells [13], FRβ (gene symbol FOLR2) expression appears to be myeloid-restricted [14], but the molecular basis for its tissue-restricted expression remains unknown. Within the myeloid lineage, FRβ is expressed in a nonfunctional state in CD34+ cells and neutrophils [15], whereas it mediates folate binding in activated synovial macrophages from RA and osteoarthritis [16, 17], M-CSF-driven M2 macrophages and TAMs [18]. The restricted expression of FRα, FRβ, and FRβ, together with the high-affinity for folate binding and endocytosis, has prompted the evaluation of the potential therapeutic value of folate drug conjugates in cancer and in inflammatory pathologies [19, 20].

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Flow cytometry

Phenotypic analysis was carried out by immunofluorescence using standard procedures. Mouse mAb used for cell-surface staining included FITC-labeled anti-CD14, FITC-labeled anti-CD209, PE-labeled anti-CD40, PE-labeled anti-CD86, PE-labeled anti-CD83, PE-labeled anti-CD80, and PerCP-labeled anti-HLA-DR (BD Biosciences, San Jose, CA, USA); FITC-labeled anti-FRβ and FITC-labeled anti-CD163 (MBL International, Woburn, MA, USA); and PE-labeled anti-CD68 (BioLegend, San Diego, CA, USA). For CD68 and CD209 staining, cells were permeabilized using the Cytofix/Cytoperm kit (BD Biosciences). CD209 staining was carried out by indirect immunofluorescence, followed by incubation with FITC-labeled goat anti-rabbit affinity-purified antibody, FITC/PE/PerCP-labeled, isotype-matched irrelevant antibodies were included as negative controls.

ELISA

 Supernatants from M1 and M2 macrophages were tested for the presence of cytokines using commercially available ELISA for TNF-α and IL-10 (BioLegend), IL-6 (ImmuNoTools, Friesoythe, Germany), and IL-23 (eBioscience, San Diego, CA, USA), following the protocols supplied by the manufacturers.

Multicolor confocal microscopy

Human tissue samples were obtained from patients undergoing surgical treatment following the Medical Ethics Committee procedures (Hospital General Universitario Gregorio Marañon and Hospital Clinic de Barcelona). Histopathology diagnosis was confirmed for each specimen. The following antibodies were used: mouse mAb against FRβ [23], FITC-labeled anti-CD163 (Ber-Mac3; MBL International), anti-CD68 (PG-M1; Dako, Denmark), anti-CD209 (MR1; kindly provided by A. L. Corbi, Centro de Investigaciones Biológicas, Madrid, Spain), anti-PCFT (ab25134, Abcam, Cambridge, UK; and SAB2104833, Sigma, St. Louis, MO, USA), anti-RFC (ab62302; Abcam), anti-TNF-α (ab66671; Abcam), anti-CD31 (Lis2/5.1) and anti-THF (ab20475; Abcam), and isotype-matched control antibodies and fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Sections of cryopreserved tissues were fixed with acetone, blocked with 1% human Ig, and incubated with primary (1–5 μg/ml) and proper secondary antibodies. Imaging was performed with an inverted confocal microscope (SP2; Leica Microsystems, Buffalo Grove, IL, USA), using the ×20 PL-APO NA 0.7 and the ×63 PL-APO NA 1.3 glycerol immersion objectives. Quantification of protein expression was performed using similar acquisition settings in all tissues and a control colon sample as a reference. Although CD68 and CD163 fully colocalized in all tissues analyzed, we selected CD163 as a more suitable pan marker to depict ROIs around macrophages (Supplemental Fig. 1A). Mean fluorescence intensities (arbitrary units) within ROIs were assessed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). At least three independent samples were evaluated for each type of tissue, quantifying all of the macrophages present in three random fields (×20)/sample. After background subtraction, data were plotted using GraphPad software (GraphPad Software, La Jolla, CA, USA). For in vitro imaging, cells were plated on Poly-L-lysine (Sigma)-coated coverslips, fixed with 4% formaldehyde, and when indicated, permeabilized with 0.1% saponin for 10 min.

Real-time qRT-PCR

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) and retrotranscribed, and cDNA was quantified using the Universal Probe Library (Roche Diagnostics, Indianapolis, IN, USA). Oligonucleotides specific for FOLR2, SLC46A1, SLC19A1, TNFA, SPI, CCL2, CSF1, IL1α, IL6,
Characterization of M1 (GM-CSF) and M2 (M-CSF) macrophages

As a preliminary step to determine their 5-MTHF uptake capacity, GM-CSF and M-CSF macrophages were analyzed phenotypically and functionally. Flow cytometry on GM-CSF and M-CSF macrophages, isolated from five independent donors, revealed that M-CSF macrophages expressed higher levels of CD163, CD14, CD86, HLA-DR, and CD68 than GM-CSF macrophages (Fig. 1A) [24]. Importantly, the expression of CD209 and FRβ identified M-CSF macrophages but not GM-CSF macrophages [18, 22]. By contrast, the expression of CD40 and CD206 was higher in GM-CSF than in M-CSF macrophages, whereas both macrophage subsets lacked CD80 and CD83 expression (Fig. 1A). Regarding cytokine release and in agreement with previous reports [7, 8, 24], LPS stimulation of GM-CSF macrophages led to the production of the proinflammatory cytokines TNF-α, IL-23, and IL-6 (Fig. 1B), whereas LPS-stimulated M-CSF macrophages produced high levels of IL-10 (Fig. 1B). Therefore, M-CSF-differentiated macrophages are FRβ⁺, CD209⁺, CD163low, CD14high, IL-10low, TNF-αlow, IL-6low, IL-23⁻, whereas GM-CSF-differentiated macrophages are FRβ⁻, CD209⁻, CD163low, CD14high, IL-10high, TNF-αlow, IL-6low, IL-23⁺, a phenotype that correlates with M2 and M1 polarization, respectively [7, 8, 22, 25], and will be referred to as M1 (GM-CSF) and M2 (M-CSF) macrophages.

In vitro and in vivo M2-like macrophages accumulate THF

We have reported previously that M2 (M-CSF) macrophages exhibit a higher folate-FITC capture ability than M1 (GM-CSF) macrophages [18]. As folate-FITC conjugates are not naturally occurring folates, we initially tested the capacity of both macrophage subtypes for uptake of 5-MTHF, the major folate form found in mammalian serum [9, 10]. When exposed to physiological concentrations of [³H]-5MTHF [26] and in agreement with their higher expression of FRβ (Fig. 1A), M2 (M-CSF) macrophages showed a significantly higher 5-MTHF uptake capacity (Fig. 2A), an activity that reached a plateau after 60 min (Fig. 2A). A 20-fold molar excess of unlabeled folic acid (1 μM) almost abolished 5-MTHF uptake by M2 (M-CSF) macrophages but did not affect the lower 5-MTHF uptake ability of M1 (GM-CSF) macrophages (Fig. 2B), thus indicating that 5-MTHF transport in M2 (M-CSF), but not M1 (GM-CSF) macrophages, is mediated by FRβ. Furthermore, 5-MTHF uptake was inhibited by 50% in the presence of an antibody against FRβ in M2 (M-CSF) macrophages (Fig. 2C). 5-MTHF uptake was also increased significantly in IL-4-exposed M1 (GM-CSF) and M2 (M-CSF) macrophages compared with IFN-γ-treated cells (Fig. 2D). THF staining confirmed that M2 (M-CSF) macrophages exhibit a potent intracellular THF accumulation when exposed to physiological concentrations of folic acid (50 nM; Fig. 2E). Moreover, evaluation of THF content in tissues involved in folate absorption [27] revealed that CD163⁺ macrophages from healthy colon and placenta exhibit detectable levels of intracellular THF, whereas no THF staining was observed in CD163⁻ macrophages from ulcerative colitis (Fig. 2F). In normal placenta and colon, CD163⁺/THF⁺ expression was limited to CD209⁺/TNF-α⁻ macrophages, a phenotype that correlates with a M2-like polarization (Fig. 2G) [28, 29]. CD163⁺/THF⁻ tissue macrophages from ulcerative colitis were devoid of the M2 marker CD209 (Supplemental Fig. 1C) [22] but expressed the M1 proinflammatory marker TNF-α (see Fig. 6B), indicating that THF⁺ macrophages from ulcerative colitis are M1-like [30]. Altogether, the above results indicate that
THF uptake and accumulation characterize M2 macrophage polarization in vitro and in vivo.

The expression of folate influx transporters distinguishes proinflammatory/M1 and anti-inflammatory/M2 human macrophage in vitro

To elucidate the molecular basis for the differential handling of folates by M1 and M2 macrophages, the expression of genes encoding molecules involved in THF uptake (SLC19A1, FOLR2, SLC46A1), retention (FPGS, GGH), and export (MRPs or ABCC) and BCRP (ABCG2) was determined [21]. Gene-expression profiling on proinflammatory M1 (GM-CSF) and anti-inflammatory M2 (M-CSF) macrophages (GSE27792) revealed that FOLR2 and SLC46A1 genes are expressed at significantly higher levels in M2 (M-CSF) macrophages, whereas SLC19A1 gene expression was much higher in M1 (GM-CSF) macrophages (Fig. 3A and B). Regarding THF efflux transporters, ABCC3 (MRP3) and ABCC2 (MRP2) gene expression was significantly higher in M1 (GM-CSF), whereas ABCC5 (MRP5) expression was higher in M2 (M-CSF) macrophages (Fig. 3A and B). Therefore, M2 macrophages exhibit a higher expression of genes (FOLR2, SLC46A1) that encode molecules mediating an influx of folates (FRβ and PCFT, respectively), whereas M1 macrophages display a higher expression of genes coding for folate efflux transporters and the bidirectional folate transporter RFC (SLC19A1) [31].

At the protein level and in agreement with RNA results, FRβ and PCFT proteins were highly expressed in M2 (M-CSF) macrophages, located on the surface (Fig. 4A) and a perinuclear endosomal compartment (Fig. 4B) enriched in GPI-linked molecules [32] but were absent (PCFT) or very weakly expressed (FRβ) by peripheral blood monocytes (Fig. 4A). Conversely, expression of RFC protein was only detected on the cell surface of monocytes and M1 (GM-CSF) macrophages, which lack FRβ protein and exhibit lower levels of PCFT (Fig. 4A). In line with these findings, the uptake of folic acid and 5-MTHF at pH 5.5 [11, 33] was much higher in M2 than in M1 macrophages (Fig. 4C). Kinetics analysis showed that SLC19A1, FOLR2, and SLC46A1 RNA levels are essentially kept constant along GM-CSF-driven M1 polarization, whereas SLC19A1 RNA levels decreased, and FOLR2 and SLC46A1 RNA levels increased during M-CSF-driven M2 macrophage polarization (Fig. 4D and E). Thus, monocytes and M1 and M2 macrophages display a different and specific complement of folate transporters: monocytes and M1 macrophages exhibit a higher expression of the
Bidirectional folate transporter RFC, whereas FRβ and PCFT are preferentially expressed by M2-polarized macrophages. As a consequence, M1 and M2 macrophages differ in folate-harvesting ability, with M2 macrophages exhibiting higher 5-MTHF and folic acid uptake at neutral (FRβ/H9252-dependent) and acidic (PCFT-dependent) pH.

FRβ and PCFT are coexpressed in tissue-resident macrophages involved in folate transit and absorption

Given that macrophage accumulation of THF differs between normal and inflamed tissues (Fig. 2F) and that folate transporter expression is polarization-dependent (Figs. 3 and 4), we next evaluated FRβ, PCFT, and RFC expression in macrophages from normal and inflamed tissues. In the case of tissues with a predominance of M2-like macrophages (colon, placenta, and liver), immunohistochemistry analysis revealed that CD163+/CD209+ crypt macrophages coexpressed FRβ and PCFT, whereas enterocytes only expressed PCFT (Fig. 5A and Supplemental Figs. 1C and D and 2A and B). In human term placenta and liver, FRβ/H9252 and PCFT were also detected in CD163+/CD209+ macrophages, either below the PCFT/H9252 syncytiotrophoblast [34] or in Kupffer cells within the lumen of the liver sinusoids, respectively (Fig. 5B and C and Supplemental Figs. 1B and D and 2A). In fact, analysis of the distinct liver cell types (hepatocytes, sinusoidal endothelial cells, Ito cells, and Kupffer cells) confirmed that FOLR2 and SLC46A1 mRNA are found exclusively in PU.1/H11001 (SPI1) human and F4/80 (EGF-like module-containing mucin-like hormone receptor-like 1) murine Kupffer cells (Fig. 5D, and not shown). Therefore, FRβ and PCFT are highly expressed by CD163+/CD209+ resident mac-
rophen from tissues involved in folate absorption and transit and with a predominance of M2-like macrophages.

FRβ and RFC are coexpressed in tissue macrophages in inflammatory conditions

To determine the pattern of folate transporters in macrophages within inflamed tissues in vivo, immunofluorescence analysis was done on tissue samples from ulcerative colitis and RA. Unlike in healthy colon tissue, where macrophages were PCFT⁺/FRβ⁺/RFC⁻, CD163⁺ macrophages from ulcerative colitis were RFC⁺ (Fig. 6A). Similarly and in the case of the synovium, CD163⁺ macrophages from homeostatic synovium expressed FRβ but lacked RFC, whereas macrophages from RA samples were FRβ⁺ and RFC⁺ (Fig. 6D). The proinflammatory M1 marker TNF-α was expressed in CD163⁺ macrophages from ulcerative colitis and RA tissues but not in the normal counterpart (Fig. 6B and E and Supplemental Fig. 1D). Quantitative analysis of the expression of FRβ and RFC in macrophages from homeostatic and pathological tissues confirmed that RFC is expressed predominantly by macrophages within inflamed tissues and represents a valuable M1-like marker (Fig. 6C and F and Supplemental Fig. 2A). Furthermore, analysis of synovial fluid from RA patients, which are also embedded in a proinflammatory milieu [35], revealed that ex vivo-isolated RA synovial fluid CD163⁺ macrophages (not shown) contained mRNA for SLC19A1 and TNFA, lacked FOLR2 mRNA (Fig. 7A), stained positive for RFC and TNFα, and were almost devoid of FRβ (Fig. 7B). Therefore, and in agreement with the expression profile of folate transporters observed in in vitro-generated macrophages, RFC expression marks inflammatory macrophages in vivo, whereas PCFT and FRβ are expressed primarily by macrophages under homeostatic conditions, thus implying that the expression profile of folate transporters reflects the tissue-inflammatory state and can be used to discriminate between macrophage-polarization states in vitro and in vivo.

Activin A modulates 5-MTHF uptake

To determine the molecular basis for the enhanced RFC expression in proinflammatory macrophages, we first performed repolarization assays on M2 (M-CSF) macrophages. GM-CSF increased SLC19A1 mRNA levels significantly in M2 (M-CSF) macrophages (Fig. 8A). As we have reported previously that GM-CSF-induced activin A contributes to M1 (GM-CSF) macrophage polarization and inhibits FRβ expression [8], we determined whether activin A also regulates the expression of the RFC-coding SLC19A1 gene in M1 (GM-CSF) macrophages. As shown in Fig. 8B, activin A increased the expression of SLC19A1 during M2 (M-CSF) macrophage differentiation, whereas it concomitantly reduced FOLR2 mRNA levels. Along the same line, the presence of a blocking antibody against activin A reduced by 80% the acquisition of SLC19A1 during M1 (GM-CSF) differentiation (Fig. 8C), and SLC19A1 expression was also reduced by SB431542, an inhibitor of activin receptor-like kinase 4/5/7 signaling [8] (Fig. 8D). Taken together, these results indicate that activin A mediates the up-regulation of SLC19A1 and the down-regulation of FOLR2 during GM-CSF-driven M1 macrophage polarization and therefore, critically controls the profile of folate transporters in human macrophages. Finally, 5-MTHF uptake was enhanced significantly on the blockade of activin A activity during M1 (GM-CSF) polarization process, thus indicating that activin A modulates 5-MTHF uptake in macrophages (Fig. 8E).

Folic acid diminished the proinflammatory cytokine profile of M1 (GM-CSF) macrophages

As M1 and M2 macrophages display different uptake of 5-MTHF, we analyzed whether a link exists between folate metabolism and macrophage polarization. To that end, we cultured monocytes in folic acid-free RPMI medium containing 10% dialyzed FCS in the absence or in the presence of folic acid (2.3 μM) and differentiated them to M1 (GM-CSF) and M2 (M-CSF) macrophages. The presence of folic acid did not modify the GM-CSF- or M-CSF-driven polarization significantly, as determined by the acquisition of polarization-specific markers (not shown). However, the presence of folic acid diminished the mRNA levels significantly for TNF-α, IL-6, CSF-1, and CCL2 after LPS stimulation, exclusively in M1 macrophages (Fig. 9). These results indicate that folic acid conditions M1
DISCUSSION

The intrinsic metabolism is an important parameter that determines macrophage polarization and the acquisition of specific macrophage effector functions [36]. 5-MTHF, the reduced form of folic acid (vitamin B9), functions as a cofactor in a plethora of metabolic reactions, including nucleotide biosynthesis, homocysteine remethylation, methionine biosynthesis, and methylation processes [10]. We now report that 5-MTHF can be internalized by macrophages through three different transporters (RFC, FRβ, and PCFT) that differ in binding affinity and pH dependency; that RFC is mainly expressed by human proinflammatory macrophages in vivo and in vitro; that PCFT and FRβ are coexpressed by M2-skewed, tissue-resident macrophages from tissues involved in folate transit; and that activin A is a critical factor for determining the set of folate transporters expressed by macrophages. The fact that macrophages with different polarizing phenotypes display a distinct complement of folate transport proteins, which will ultimately determine their THF uptake capacity, is especially relevant considering the ability of folate conjugates or pharmacological antifolates (methotrexate) to target macrophages in therapeutic protocols for RA and cancer [20, 37].

The polarization-dependent expression of folate transporters by macrophages suggests an important role for this cell type in determining folate availability under physiological and pathological situations, an activity that reminisces the macrophage contribution to iron recycling [38]. As professional scavenging cells, macrophages are critically involved in systemic iron homeostasis through ingestion and degradation of senescent/damaged erythrocytes for iron recovery from hemoglobin and iron-containing enzymes [39]. From this point of view, folate handling by macrophages may exhibit similarities with iron handling, as both are essential nutrients, highly demanded during pregnancy, whose deficiency leads to anemia [40]. In fact, the folate transporter PCFT, which we now report to be

(GM-CSF) macrophages for a diminished, LPS-induced expression of the mRNA of proinflammatory cytokines (TNF-α, IL-6) and chemokines (CCL2).

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preferentially expressed by M2-polarized macrophages, also functions as a weak hemin transporter [41, 42], hence, its alternative name, HCP-1. However, whereas M2 macrophages enhance extracellular iron availability through reduced intracellular retention and enhanced release (possibly as a mean to facilitate proliferation of surrounding cells), it appears that folate retention is higher in M2-polarized macrophages because (1) placental and colon macrophages (FR/H9252/PCFT/H11001/RFC/H11002) exhibit a detectable and previously unnoticed accumulation of THF; and (2) no THF staining can be detected in M1-like macrophages from inflammatory conditions, such as ulcerative colitis (Fig. 2F). This explanation is compatible with the fact that M1 macrophages exhibit a much lower uptake ability and a higher expression of the folate exporters ABCC3, ABCG2, and RFC, which also mediate folate efflux [31], and suggests the hypothesis that M1 macrophages might maintain a higher extracellular folate concentration to ensure availability of the vitamin for lymphocyte proliferation during immune responses.

Regarding the differential complement of folate transporters in M1 and M2 macrophages, we have described previously the preferential expression of FRβ in CD163+ TAM and its regulation by M-CSF and M2-driving cytokines (IL-10, IL-6) in monocyte-derived macrophages [18]. Further extending those results, we now report the expression of FRβ in CD163+ tissue-resident macrophages in noninflamed tissues and its absence in macrophages isolated from the synovial fluid of RA patients that exhibit a folate transporter profile compatible with an M1 polarization at the mRNA and protein level (FRβ+/PCFT+/RFC−). However, and unlike macrophages within RA synovial fluid, we have detected FRβ expression in tissue macrophages from ulcerative colitis and RA synovium (Fig. 6). This finding is compatible with the presence of FRβ+ macrophages that had been seen previously in other inflammatory conditions, where M1-polarized macrophages predominate [16, 37, 43]. A possible explanation for this apparent discrepancy is the fact that FRβ expression levels in macrophages are critically dependent on M-CSF, whose expression is constitutive under homeostatic conditions but also enhanced during inflammation or in response to infection [3]. Therefore, whereas FRβ expression might mark macrophages newly recruited to an inflammatory focus, it might also identify macrophages exposed to an M-CSF-rich inflammatory milieu.

FRβ and PCFT bind folate with high-affinity but with a different pH [9]. The role of PCFT in FR-expressing cells has been studied in HeLa subline cells during FR-mediated endocytosis, and these studies have concluded that PCFT is expressed with FRh in endosomes and that PCFT exports folates from acidified endosomes to the cytoplasm [44]. FRβ and PCFT colocalized in a characteristic perinuclear compartment

Figure 5. Expression of folate transporters in macrophages from normal tissues. (A) Normal colon stained for FRβ, CD163, and DAPI (left) and PCFT and CD163 (right). (B) Normal term placenta stained for FRβ, CD163, and DAPI (left) and PCFT, CD163, and CD31 (right). (C) Normal liver stained for FRβ, CD163, and DAPI (left) and PCFT and CD163 (right). Original scale bars as indicated. (D) Relative levels of FOLR2, PU.1, and SLC46A1 mRNA in human hepatocytes, liver sinusoidal endothelial cells (LSEC), Kupffer cells, and Ito cells, as determined by qRT-PCR. Results were referred to FOLR2 RNA levels in Kupffer cells.
Figure 6. Expression of folate transporters in macrophages from normal and inflammatory tissue samples. (A) Human normal colon (left) and ulcerative colitis (right) tissue sections stained for CD163, FRβ, RFC, and DAPI, as indicated. (B) Human normal colon (left) and ulcerative colitis (right) tissue sections stained for CD163 and TNF-α, as indicated. (C) Quantification of FRβ and RFC expression in crypt CD163+ macrophages from normal colon (left) and ulcerative colitis (right) tissues. For each protein, background-subtracted mean intensities (arbitrary units) from 200 to 400 macrophages/tissue were plotted. (D) Human normal (synovium, left) and RA (right) synovial membrane tissue sections stained for CD163, FRβ, RFC, and DAPI, as indicated. (E) Human normal (synovium, left) and RA (right) synovial membrane tissue sections stained for CD163 and TNF-α, as indicated. (F) Quantification of FRβ and RFC expression in CD163+ macrophages from normal synovial membrane (synovium, left) and RA synovial membrane (right) tissues. For each protein, background-subtracted mean intensities (arbitrary units) from nearly 350 macrophages/tissue were plotted.
similar to that observed for folate/PCFT in HeLa cells [44] and FRA/PCFT in retinal Müller cells [45], so this mechanism might also operate in FRβ+ PCFT+ M2 macrophages. If so, it would be interesting to determine if macrophage effector functions are altered in patients with hereditary folate malabsorption that have loss-of-function mutations in the PCFT gene [11]. In any event, the physiologic role of FRβ remains unsolved, as unlike the lethal phenotypes of the other folate transporter knockout mice [27], FRβ null mice is associated with an apparently normal phenotype, except for the increased susceptibility to arsenic-induced neural tube defects [46]. Nevertheless, FRβ is able to bind and transport a wide variety of chemical conjugates of folic acid and antifolate drugs, thus providing an opportunity for targeting FRβ+ macrophages [47].

Macrophages are involved in the initiation and progression of numerous inflammation-related diseases, including cancer, obesity, and RA [48]. Tumors are infiltrated with TAM that promotes malignancy by stimulating angiogenesis and tumor-cell migration, whereas the number of macrophages in the synovial sublining of patients with RA is a biomarker for disease severity and for response to therapy [49]. For this reason, the development of antimacrophage therapies is an active area of research (e.g., antibodies against CCL2, M-CSF, or GM-CSF). Although TAMs and RA macrophages exhibit functional FRβ [18, 37], our results suggest that caution should be taken with FRβ-targeted and anti-folate- or folate-conjugated-based therapies, as they might also affect other tissue-resident macrophages with strong FRβ expression (i.e., Kupffer cells).

**AUTHORSHIP**

R.S., B.S.P., and Á.D-S. performed research and analyzed data. C.V., A.S., T.M., I.T., and M.E.M-C. contributed vital reagents and materials. C.S-T., M.E.M-C., and P.S-M. analyzed data.
A.P-K. and R.S. conceived the study. A.P-K. designed research, analyzed data, and wrote the paper.

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DISCLOSURES

The authors declare no financial or commercial conflict of interest.

REFERENCES


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