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Use of Folate-Conjugated Imaging Agents To Target Alternatively Activated Macrophages in a Murine Model of Asthma

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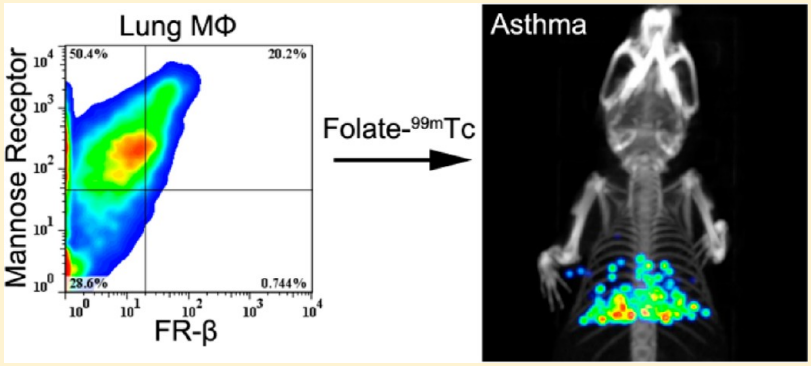
Supporting Information



ABSTRACT: Pro-inflammatory macrophages play a prominent role in such autoimmune diseases as rheumatoid arthritis, Crohn's disease, psoriasis, sarcoidosis, and atherosclerosis. Because pro-inflammatory macrophages have also been shown to overexpress a receptor for the vitamin folic acid (i.e., folate receptor beta; FR-β), folate-linked drugs have been explored for use in imaging and treatment of these same diseases. To determine whether allergic inflammatory disorders might be similarly targeted with folate-linked drugs, we have examined the characteristics of macrophages that are prominent in the pathogenesis of asthma. We report here that macrophages from the lungs of mice with experimental allergic asthma express FR-β. We further document that these FR-β^+ macrophages coexpress markers of alternatively activated (M2-type) macrophages, including the mannose receptor and arginase-1. Finally, we demonstrate that folate-conjugated fluorescent dyes and radioimaging agents can be specifically targeted to these asthmatic lung macrophages, with little uptake by macrophages present in healthy lung tissue. These data suggest strategies for the development of novel diagnostic agents for the imaging of asthma and other diseases involving alternatively activated macrophages.

KEYWORDS: *folate receptor-β, alternatively activated macrophages, radioimaging of asthma, EC20, technetium 99m imaging, folate targeting*

INTRODUCTION

Allergic asthma, the most common form of asthma, is a chronic inflammatory disease characterized by the development of a type 2 helper T cell (T_H2) response toward inhaled allergens. Allergen-specific T_H2 cells secrete cytokines that regulate the synthesis of allergen-specific immunoglobulin E (e.g., IL-4), cause airway hyper-reactivity (e.g., IL-13), stimulate mast cell infiltration (e.g., IL-9), promote pulmonary eosinophilia (e.g., IL-5), and induce accumulation of alternatively activated macrophages in the lungs (e.g., IL-4 and IL-13).[1]

Evidence for the prominent involvement of alternatively activated macrophages (AAMs) in allergic asthma arises from several observations. First, two molecular markers of AAMs, arginase-1 (Arg1) and acidic mammalian Chitinase (AMCase), are simultaneously markers for asthma,[2,3] suggesting that AAMs may constitute an abundant cell type in the asthmatic lung. In fact, treatment with either an inhibitor of Arg1 or a neutralizing antibody to AMCase has been found to ameliorate the symptoms of asthmatic mice.[3,4] Second, T_H2 cells, eosinophils, and basophils that aggravate the allergic inflammation are attracted into asthmatic lung tissue by chemokines specifically released by AAMs (i.e., CCL17, CCL18, CCL22, and CCL24).[5] Third, adoptive transfer of AAMs into the lungs of sensitized mice increases the influx of myeloid dendritic cells and thereby exacerbates airway inflammation.[6] Thus, in aggregate, these findings suggest that AAMs participate centrally in the pathogenesis of allergic asthma.

Folate receptor-beta (FR-β), a homologue of FR-α,[7] is a glycosylphosphatidylinositol (GPI)-anchored membrane protein that is normally expressed in human placenta and on a subset of cells of myelomonocytic lineage.[8,9] Although FR-β has

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been detected on both CD34$^+$ bone marrow cells and normal human neutrophils, the receptor on these cells is functionally inactive and unable to bind folate or folate-linked drugs.[10,11] In contrast, a functional FR-β with nanomolar affinity for the vitamin has been identified on activated macrophages that accumulate in inflammatory diseases such as rheumatoid arthritis,[12,13] atherosclerosis,[14] systemic lupus erythrematosus,[15] Crohn's disease,[16] and osteoarthritis.[17] A functional FR-β has also been reported on macrophages induced by repeated treatment of human monocytes with M-CSF,[18] but whether macrophages recently detected by anti-FR-β staining of pancreatic cancers[19] or fibrotic lung tissues[20] express a functionally active FR-β has not yet been determined.

Upregulation of a functional FR-α or FR-β on a pathologic cell type has been recently exploited for imaging and/or therapy of the associated disease. Thus, attachment of the vitamin, folic acid, to optical or radioimaging agents has allowed the selective imaging of both cancers and inflammatory diseases in humans and appropriate animal models,[12−14,16,17] and similar folate-targeted therapeutic agents have been exploited to treat several of the same diseases.[12,15] To determine whether folate-linked drugs might be analogously applied in the diagnosis of asthma, we have isolated and characterized AAMs from the lungs of mice with allergic asthma. We demonstrate here that AAMs from the lungs of asthmatic mice express FR-β and that this folate receptor is functionally active and useful for the selective delivery of folate-linked drugs to asthmatic tissues.

■ EXPERIMENTAL SECTION

Animal Model. All experimental procedures were approved by the Purdue Animal Care and Use Committee. Eight-week-old virgin female BALB/c mice purchased from Harlan Laboratories were sensitized by intraperitoneal (i.p.) injection of 0.3 mL solution containing 100 μg of ovalbumin (OVA) (Sigma-Aldrich) bound to Imject Alum (Pierce) on days 0, 7, and 14, followed by 50 μg OVA intranasal challenge on days 17, 18, and 19, as previously described.[2,21] Mice were either injected with folate-linked drugs as described below or anesthetized or euthanized for further analysis 24 h after the last OVA challenge.

Lung Histological Studies. For histopathologic assessment, lungs of mice were excised, fixed in IHC zinc fixative solution (BD Pharmingen), and embedded in paraffin. Sections of fixed tissues (4 μm thick) were prepared and stained with hematoxylin and eosin (H&E) for light microscopic examinations.

Isolation of Immune Cells from the Lungs of Asthmatic Mice. Euthanized mice were subjected to bronchoalveolar lavage prior resection and mincing of their lung tissue into small cubes. The minced tissue was then digested for 60 min in 5 mL of folate-deficient RPMI 1640 media (Life Technologies) containing 0.1% collagenase (Type IV; Sigma-Aldrich), 0.01% hyaluronidase (Sigma-Aldrich), and 0.002% DNase (Sigma-Aldrich). Digested tissue was further disaggregated into single cells by gentle pipetting through a 1 mL pipet tip and then passed through a 40 μm cell strainer (BD Biosciences). After lysis of residual erythrocytes using red blood cell lysis buffer (Sigma-Aldrich), dissociated cells were layered onto 5 mL of Ficoll-Paque PLUS (GE Healthcare) lymphocyte separation solution and centrifuged at 500*g* for 30 min. The mononuclear cells in the middle layer and the granulocytes at the bottom of the tube were collected for flow cytometric analysis.

Flow Cytometry Analysis and Cell Sorting. Single cell suspensions obtained as described above from asthmatic lungs were preincubated with anti-CD16/32 antibodies (eBioscience) to block F_c receptors, then treated for 1 h with a 1:100 dilution of polyclonal rabbit anti-FR antibody (FL-257; Santa Cruz Biotechnologies) followed by staining for 1 h with a 1:100 dilution of FITC-conjugated antirabbit second antibody (Sigma-Aldrich). Evaluation of the isolated cells for functional FR was performed by incubation with folate-Oregon Green (FOG) or folate-AlexaFluor 647 (prepared in our lab) in the presence and absence of 1000-fold excess folic acid to block unoccupied FR, as reported previously.[13] For characterization of coexpression of FR with inducible nitric oxide synthase (iNOS) or mannose receptor (MR), folate-fluorescent probe (200 nM) and PE-conjugated anti-F4/80 (eBioscience) labeled cells were fixed, permeabilized, and incubated with FITC-labeled anti-iNOS (BD Biosciences) or AlexaFluor 647-labeled anti-MR (Biolegend). To distinguish FR negative from FR positive cells, the fluorescence gate for FR expression was set such that <1% of the macrophages were counted as FR$^+$ in the competition control. Similarly, the fluorescence gate for other cell markers was set such that <1% of the gated cell population appeared positive when examined with a nonspecific antibody isotype control. Experiments from each group were repeated at least three times, and representative data from each group are shown. Flow cytometry and cell sorting were performed on a BD FACSCalibur flow cytometer (BD Biosciences) and iCyt Reflection Sorter (iCyt), respectively. Data were analyzed using CellQuest (BD Biosciences).

RT-PCR and Quantitative Real-Time PCR. Total RNA was prepared from sorted cells using the RNAeasy kit (Qiagen). cDNA was synthesized using qscript flex cDNA synthesis kit (Quanta Biosciences) or Superscript III reverse-transcriptase and oligo$(dT)_{20}$ primer (Invitrogen). PCR consisted of a denaturation step at 94 °C for 2.5 min, followed by 45 cycles for FR-β (sense primer, gctggaagactgaactaagacagaa; antisense primer, ggagtcttggatgaagtgactctta) and 30 cycles for hypoxanthine phosphoribosyltransferase (HPRT, sense primer, tgaagagctactgtaatgatcagtcaac; antisense primer, agcaagcttgcaacctta-acca).[22] Each cycle consisted of 30 s denaturation at 94 °C, 30 s annealing at 60 °C, and 45 s extension at 72 °C. Quantitative real-time PCR was carried out using TaqMan primer and probe sets for mouse FR-α, FR-β, FR-δ, arginase-1 (Arg1), iNOS, and 18 s rRNA (Applied Biosystems). Relative mRNA expression was calculated from the formula, RmE = 2 − (Ct of gene − Ct of 18 s rRNA), where Ct is the threshold cycle time and RmE is the relative mRNA expression. Data were normalized to 18 s RNA and are representative of three independent experiments.

Arginase Activity Assay. For analysis of Arg1 enzyme function, sorted cells from freshly isolated lungs were resuspended at a concentration of 1×10^7 cells/mL in lysis buffer. Arg1 activity was measured in supernatants of cell lysates using the QuantiChrom Arginase Assay Kit (DARG-200, BioAssay Systems). Protein concentration was determined using BCA Protein Assay Kit (Pierce Biotechnology Inc.).

Preparation of ^{99m}Tc-EC20. Vials containing formulated EC20, a folate-targeted technetium (^{99m}Tc) radioimaging agent, were a generous gift from Endocyte (West Lafayette, IN). EC20 was reacted with ^{99m}Tc by submersing in a boiling water bath, allowing to heat for 5 min, and then injecting with 2 mL of a 925 MBq/mL solution of ^{99m}Tc (Cardinal Health). After heating for an additional 15 min, the folate-targeted radioimaging agent was diluted with the desired volume of saline,

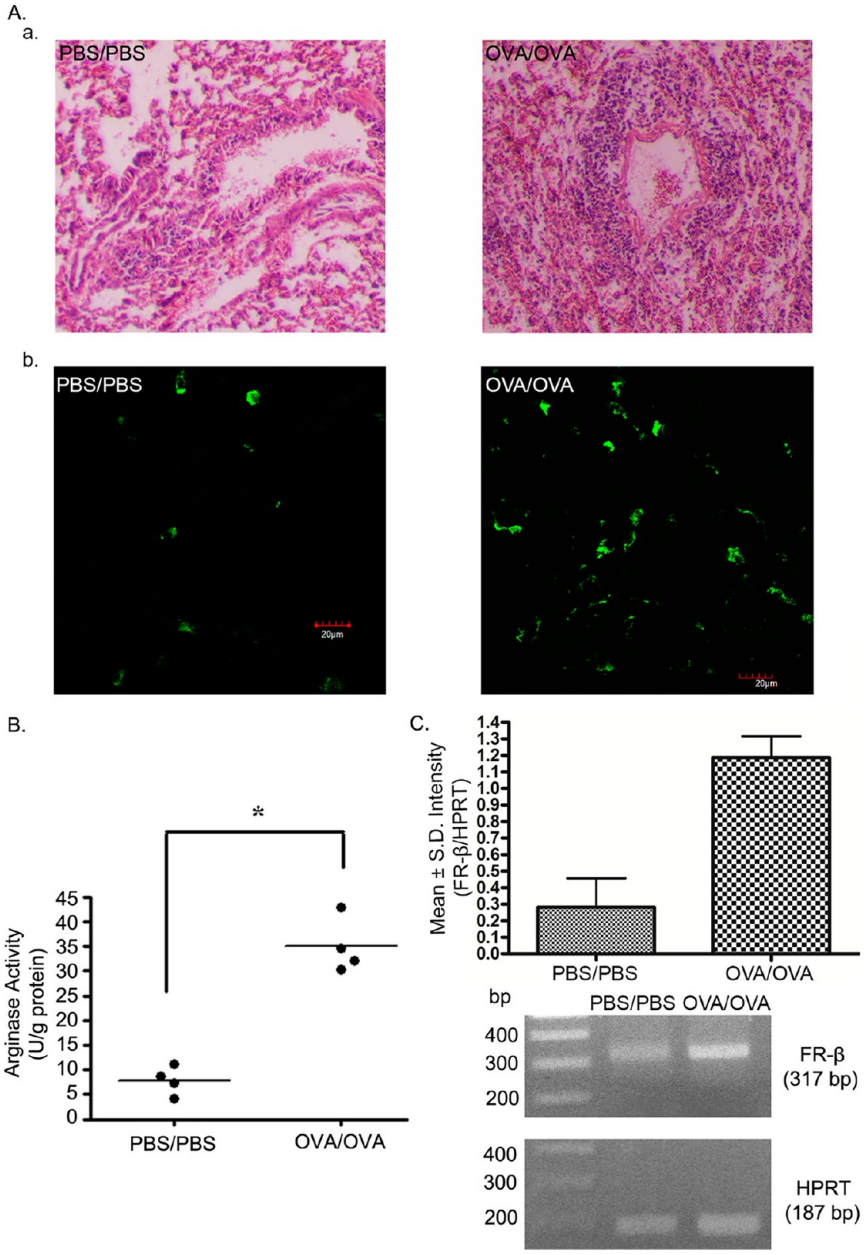

Figure 1. FR-β transcripts are significantly increased in murine lung tissue during OVA-induced acute allergic inflammation related to asthma. Balb/c mice (n = 4) were sensitized and challenged with OVA to induce experimental asthma or treated similarly with saline (n = 4) and used as controls. (A) Representative photographs of H&E (×100) stained lung tissue sections (a) and of immunofluorescence staining of CD68 in lung parenchyma sections (b) from control and OVA-induced asthmatic mice. (B) Measurement of arginase activity in the lungs of saline- and OVA-induced mice (P < 0.05). (C) Expression of FR-β transcripts in lung tissues from control and OVA-induced asthmatic mice as detected by RT-PCR. Band intensities were quantified by densitometry, and the relative FR-β mRNA expression normalized with HPRT is shown above the gel image. Results are expressed as mean ± SD of four mice.

and mice were injected i.p. with either 400 μL of ^{99m}Tc-EC20 (18.5 MBq, ~250 nmol of EC20 per kilogram) or the same volume of ^{99m}Tc-EC20 plus with a 100-fold molar excess of free folic acid (to compete for unoccupied FRs). Unbound ^{99m}Tc-EC20 was allowed to clear from the tissues for 4 h before imaging.

Radioimaging and Assessment of ^{99m}Tc-EC20 Accumulation. Mice injected with ^{99m}Tc-EC20 were anesthetized with 3% isoflurane and imaged using a Kodak Image Station (Carestream Molecular Imaging). Radioimage acquistion was performed for 2 min (whole body) and 15 min (resected lungs) using a radioisotopic phosphor screen (Carestream Molecular Imaging), no illumination source, a 4 × 4 binning setting, and an f-stop of 0. Radiographic images used to coregister anatomic structures with γ-scintigraphic images during overlays were acquired for 55 s using the same Kodak Imaging Station. Both radiographic and γ-scintigraphic images had a focus setting of 7 mm and a field of view of 200 × 200 mm. γ-emission from the abdomen was shielded using a 5-mm-thick lead shield. All data were analyzed using the Kodak molecular imaging software (version 4.5; Carestream Molecular Imaging). Single-photon-emission computed tomography/computed tomography (SPECT/CT) scans were performed using MILab's subhalf-mm resolution U-SPECT-II/CT scanner (Utrecht, The

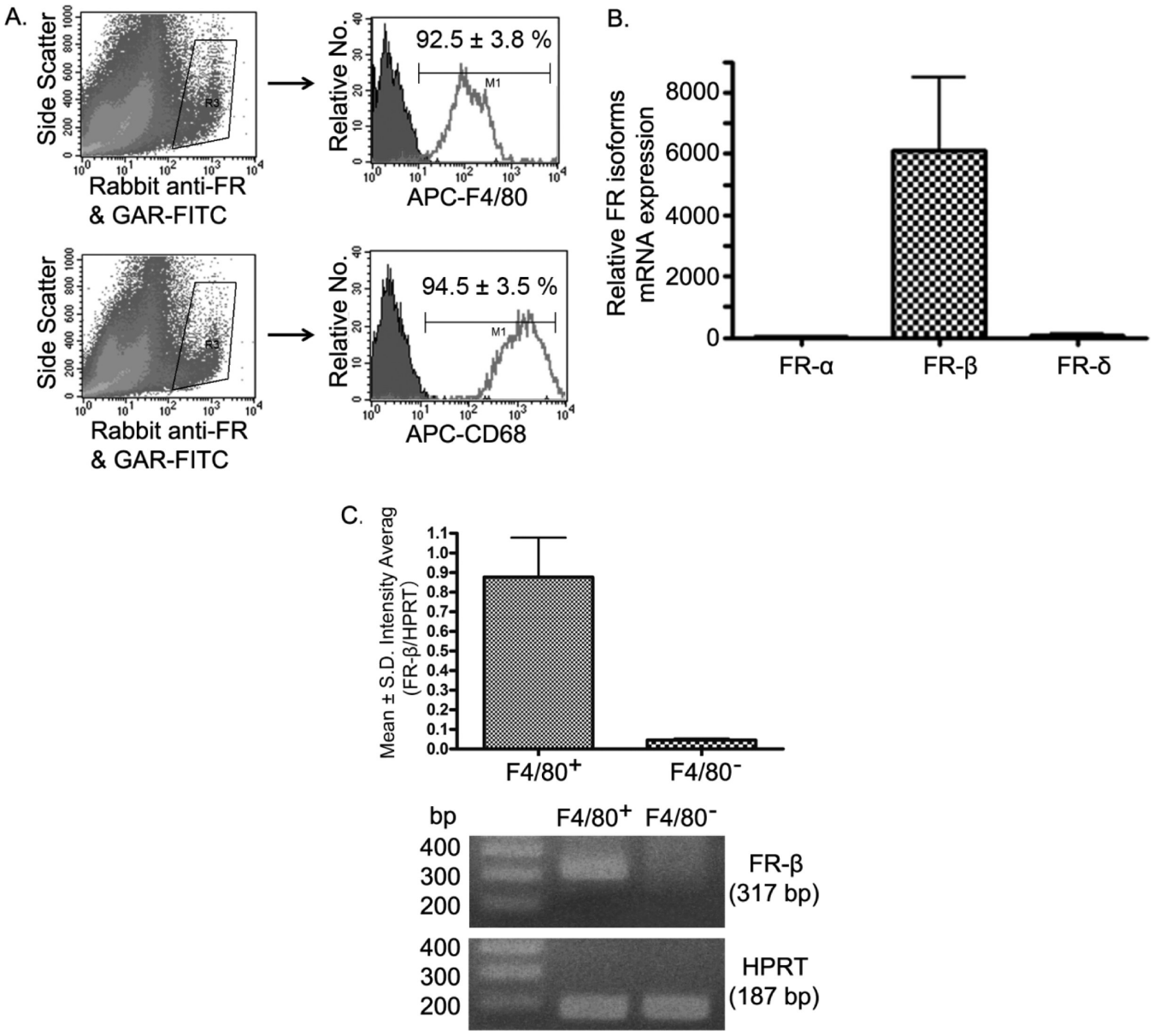

Figure 2. FR-β is expressed on F4/80$^+$ macrophages in lungs from mice during OVA-induced acute allergic inflammation related to asthma. Lungs from asthmatic and control mice were excised, sliced into small cubes, and processed for preparation of single cell suspensions. After staining with rabbit anti-FR primary antibody followed by fluorescein isothiocyanate (FITC)-conjugated antirabbit IgG secondary antibody to define the FR$^+$ cell population, the percentage of F4/80$^+$ and CD68$^+$ macrophages within this FR$^+$ cell population was analyzed by flow cytometry. Quantitative results listed in each panel represent the mean ± SD from four mice (A). FR$^+$F4/80$^+$ cells were then sorted and used for analysis of the expression of various FR isoforms by real-time PCR (B). Total RNA isolated from sorted F4/80$^+$ macrophages was converted to cDNA and used for PCR analysis of FR-β or HPRT (control). Densitometry analysis of amplified gene products is shown above the gel image, and results are expressed as mean ± SD of four mice (C).

Netherlands). Before scanning, kidneys and livers were removed from the mice to reduce noise from radioactivity accumulation in those organs in the field of view. The acquisition time was 60 min for SPECT (30 min/frame, 2 frames) and 30 min for CT ("normal" acquisition at 55 kV and 500 μA). SPECT and CT reconstruction was performed using a POSEM algorithm and a cone-beam filtered back-projection algorithm (NRecon v1.6.3, Skyscan), respectively. Co-registered images were visualized using the PMOD software (PMOD Technologies, Zurich, Switzerland). For the quantitation of accumulation of ^{99m}Tc-EC20 in the lungs, mice were euthanized by CO_2 asphyxiation, lungs were excised, and radioactivities were counted for 2 min using a γ-counter (Packard Bioscience). Results are reported as percentage injected dose per gram of tissue (%ID/g).

***In Vivo* Near-Infrared Fluorescent Imaging.** Mice were injected via the tail vein with 10 nmol of folate-DyLight680 (prepared in our lab) in 50 μL of saline. After a 2 h clearance period, mice were anesthetized with 3% isoflurane, and near-infrared fluorescence imaging was performed using the IVIS Lumina II Imaging System (Caliper Life Sciences). For fluorescence acquisition, a 692 nm excitation filter and a 712 nm emission filter were used. Abdomens were shielded with black construction paper to avoid interference from fluorescence emanating from the kidneys and bladder. After *in vivo*

imaging, animals were euthanized by CO_2 aphyxiation, and lungs were resected for further imaging.

Statistical Analysis. Statistical significance among experimental groups was calculated using *t*-tests. Values of *P* less than 0.05 were considered significant.

RESULTS

FR-β Identifies Lung Macrophages in a Murine Model of Allergic Asthma. To determine whether macrophages from the lungs of asthmatic mice might express a folate receptor, Balb/c mice were immunized against ovalbumin (OVA) and challenged with OVA by intranasal instillation (see Methods). After allowing 24 h for allergic inflammation to develop, reverse transcription PCR (RT-PCR) was performed on isolated lung tissue to look for expression of FR-β. As seen in Figure 1A, the OVA sensitization protocol induced the anticipated allergic inflammation, as demonstrated by a massive increase in eosinophil and macrophage content in the OVA-treated mice. The specific accumulation of alternatively activated macrophages (AAMs) could also be documented by the rise in arginase activity in the same sample (Figure 1B). Evidence that at least some of the accumulating macrophages express FR-β was then shown by demonstrating an enhancement in FR-β mRNA (Figure 1C).

Although other studies have previously reported that FR-β expression is restricted to activated macrophages in non-pregnant mice,[13,20,23] we wished to confirm this limited distribution pattern by characterizing the FR-β positive cells isolated by collagenase digestion of asthmatic lungs. As seen in Figure 2A, 92.5 ± 3.8% and 94.5 ± 3.5% of the FR positive asthmatic lung cells also stained for F4/80 and CD68, respectively, suggesting that FR expression is limited to monocyte/macrophage lineage cells in the lungs. Further, quantitative RT-PCR (qRT-PCR) analysis of sorted FR^+ $F4/80^+$ cells showed that FR-positive macrophages express mRNA for FR-β, but not for FR-α or FR-δ (Figure 2B). Moreover, FR-β mRNA could be detected in sorted $F4/80^+$ cells, but not in $F4/80^-$ cells (Figure 2C). Taken together, these data demonstrate that expression of FR-β is limited to the macrophage population of cells in asthmatic lungs.

Fraction of Macrophages That Are FR-β Positive Increases ∼15-fold upon OVA-Induced Allergic Inflammation. To obtain more quantitative information on the increase in FR-β^+ macrophages upon induction of allergic asthma, the change in FR-β^+ macrophage content was examined as a function of time after OVA challenge. As seen in Supplemental Figure S1, the proportion of FR-β^+ macrophages increases steadily from 0 to 24 h after OVA challenge and remains high for up to 72 h after stimulation. By the 24 h time point (Figure 3A), FR-β^+ macrophages (FR-β^+ $F4/80^+$ cells) have increased nearly 5-fold above baseline levels, from ∼8% to 39% of the total macrophage population. Moreover, the fraction of total lung cells that were macrophages ($F4/80^+$) rose from 6% to 19%. Taken together, the fraction of lung macrophages that express FR-β can be calculated to have increased ∼15-fold from PBS- to OVA-treated mice. Consistent with these observations, the total number of FR-β^+ macrophages in the lung was found to increase from 2.6×10^4 to 8.7×10^5 upon induction of allergic inflammation (Figure 3B). When adjusted for the ∼70% increase in lung weight in the asthmatic mice, an ∼20-fold rise in FR-β^+ macrophages/gram lung tissue was observed upon induction of allergic inflammation.

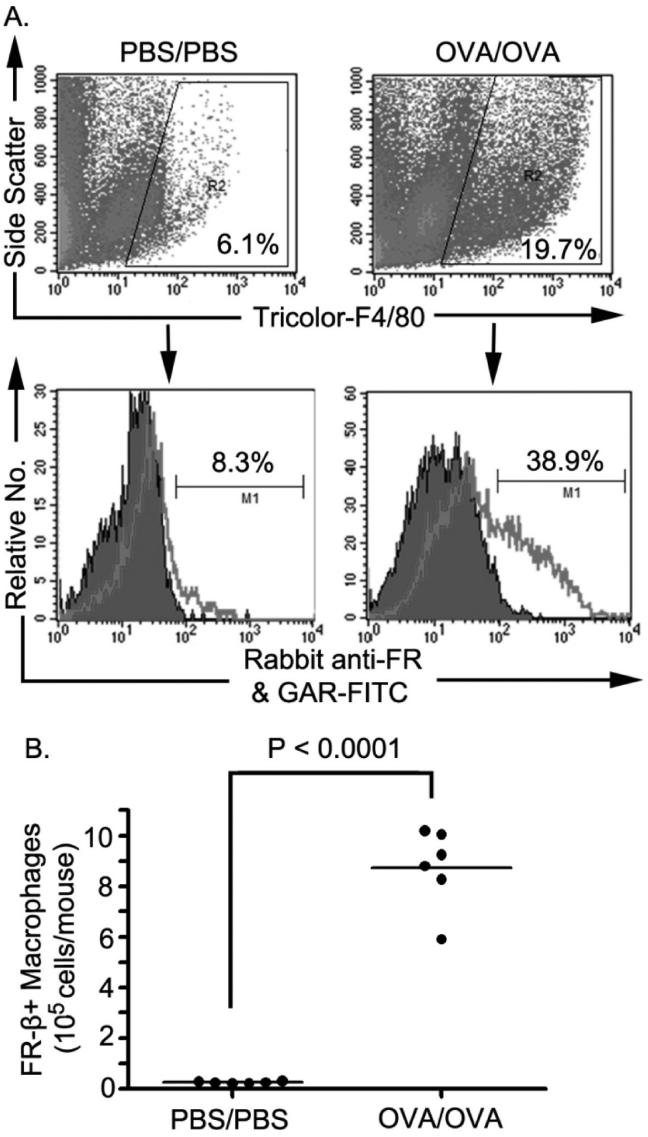

Figure 3. $F4/80^+$ cells expressing FR-β increase markedly in lung tissue during OVA-induced asthma in mice. Mononuclear cells isolated from lungs of mice during OVA-induced experimental asthma were analyzed by flow cytometry for F4/80 expression (a macrophage marker) and for FR-β expression. (A) The percentage of FR^+ cells within the $F4/80^+$ macrophage population. (B) Mice (n = 6) with ovalbumin (OVA)-induced airway inflammation have significantly more $FR^+F4/80^+$ cells in their lung tissue than PBS-treated control mice (n = 6). Pairwise comparison using t test with pooled SD were used for statistical analyses ($P < 0.0001$).

FR-β Expressed on Asthmatic Lung Macrophages Is Functional. The ability of FR-β to bind folic acid (FA) conjugates is essential for use of the receptor as a mediator of FA-conjugated drug delivery. To test whether the FR-β that is upregulated in asthmatic lungs is functional, as seen on thioglycolate-elicited macrophages[13] but not on some myeloid cells,[9−11] harvested lung mononuclear cells were incubated with folate-Oregon Green (FOG) and anti-F4/80 for flow cytometry. As illustrated in Figure 4A, a subpopulation of lung macrophages ($F4/80^+$ cells) from asthmatic mice bound high levels of FOG. Moreover, the binding of FOG could be inhibited by 1000-fold molar excess of free folic acid, demonstrating that cell binding of FOG was FR-β mediated.

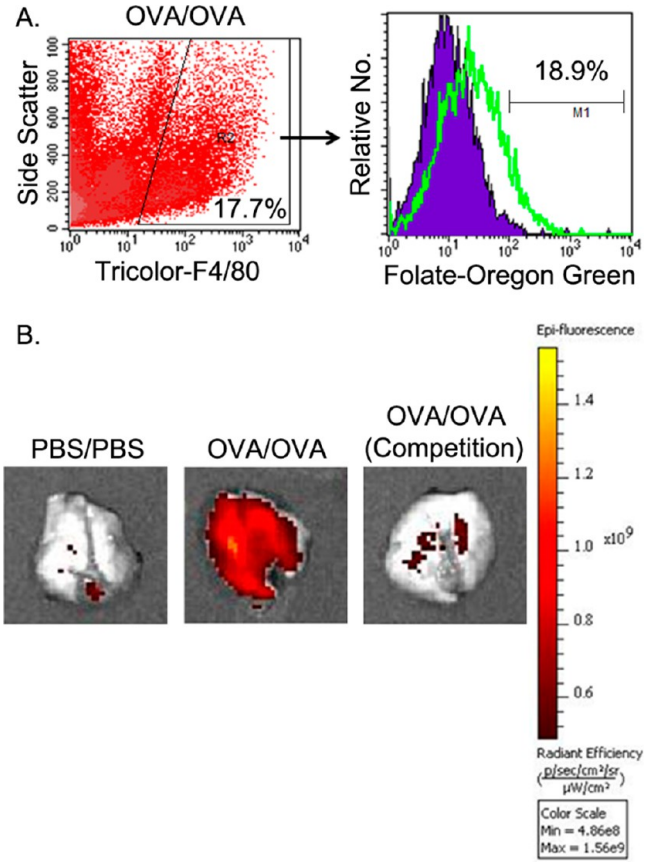

Figure 4. FR-β expressed on asthmatic lung macrophages can bind folate-conjugates. (A) Macrophages isolated from lungs of mice with OVA-induced experimental asthma (gated by side scatter and F4/80$^+$ fluorescence) were examined for binding of FOG. Flow cytometry showed strong surface binding of FOG after 1 h of incubation. Moreover, binding of FOG could be completely blocked by 1000-fold excess of free folic acid (filled histogram). (B) *Ex vivo* near-infrared fluorescent (NIRF) images of representative lungs from control (left panel) and asthmatic mice (middle and right panels) following intravenous (i.v.) injection of folate-Dylight 680 in the absence (left and central panels) or presence (right panel) of 100-fold molar excess of free folate-glucosamine to block all FR-β.

To further verify the functionality of FR-β on asthmatic macrophages *in vivo*, folate-DyLight 680 was injected intravenously (i.v.) into mice previously immunized and challenged with OVA. Two hours after folate-DyLight 680 injection, lungs were resected and imaged *ex vivo* for folate conjugate uptake. As demonstrated in the representative samples shown in Figure 4B, lungs from the OVA-treated mice were brightly fluorescent, whereas lungs from PBS-treated mice were essentially nonfluorescent. Furthermore, as indicated in the competition study, folate-DyLight680 binding to asthmatic lungs was FR-β specific, since uptake of the folate-targeted dye was readily inhibited by 100-fold excess of folate-gluocosamine, a ligand that associates with FR-β with high affinity (Figure 4B, right panel).

FR-β Constitutes a Marker for Alternatively Activated Macrophages in Lungs of Mice Induced to Develop Allergic Asthma. Puig-Kröger et al.[18] have previously shown that human peripheral blood monocytes can be differentiated *in vitro* into FR-β expressing AAMs by repeated treatment with M-CSF. Given the strong evidence that AAMs participate intrinsically in the development of asthma,[3,6,24−26] we elected to examine the characteristics of FR-β-positive macrophages present in the lungs of asthmatic mice. As shown in Figure 5B, virtually all FR-β^+ macrophages express the mannose receptor (MR), a hallmark of AAMs.[27] Moreover, AAMs from the same mice do not express iNOS (Figure 5A), a marker of classically activated macrophages (CAMs).[27] In agreement with the flow cytometry data, mRNA expression of Arg1, another AAMs marker, is also elevated in the FR-β-positive macrophage fraction (Figure 5C), and these qRT-PCR data were readily confirmed by analysis of arginase catalytic activity in the macrophage populations of naïve and asthmatic mice (Figure 5D).

A Folate-Targeted Radioimaging Agent Can Be Used to Assess the Activation Status of Lung Macrophages in Asthmatic Mice *in Vivo*. Because FR-β-positive macrophages are virtually absent from lungs of healthy mice (Figure 3), we decided to evaluate whether a radioimaging agent might be used to noninvasively assess the activation status of pulmonary macrophages in asthmatic mice *in vivo*. For this purpose, a folate-targeted ^{99m}Tc-based radioimaging agent, ^{99m}Tc-EC20,[28] was injected into mice with OVA-induced asthma, after which γ-scintigraphic and SPECT/CT images were acquired. As seen in the representative images of Figure 6A, healthy mice showed no pulmonary uptake of ^{99m}Tc-EC20, whereas asthmatic mice displayed prominent uptake in multiple loci throughout the affected lungs (Figure 6B). That the elevated lung accumulation derived, in fact, from FR-β expressing cells could be demonstrated by the absence of uptake in mice preinjected with 100-fold excess of folate-glucosamine, a high affinity FR-β ligand that can block empty folate receptors. Further confirmation of the *in vivo* imaging data was also obtained from *ex vivo* γ-scintigraphic images of lungs resected from mice treated analogously to those shown in panel A (see panel C), and these scintigraphic images could be readily confirmed by gamma counting of the same tissues (panel D). Taken together, these data demonstrate that folate conjugate uptake constitutes a property of asthmatic but not healthy lungs, suggesting the folate-targeted imaging agents can potentially be exploited to evaluate the inflammatory status of an asthma patient.

■ DISCUSSION

We demonstrate here that both the total macrophage content and the fraction of FR-β positive macrophages increases significantly upon OVA induction of allergic asthma in mice. We further show that the FR-β positive macrophages exhibit an alternatively activated phenotype, based both on their upregulation of the mannose receptor and arginase 1 and downregulation of iNOS. While not all FR on these macrophages may be functional, our data also show that at least a sufficient fraction can bind folate to enable delivery of folate-linked imaging agents selectively into the asthmatic lung macrophages. This conclusion was established using both folate-linked fluorescent dyes and folate-targeted ^{99m}Tc-based radioimaging agents.

Past results have documented the ability of folate to target attached drugs to pro-inflammatory (classically activated) macrophages *in vivo*.[12] Thus, not only have inflammatory diseases such as rheumatoid arthritis,[29] atherosclerosis,[14] osteoarthritis,[30] and bacterial infections[31] been imaged in humans and/or mice with folate-linked imaging agents, but tissue biopsies of inflamed lesions have also revealed uptake of folate-conjugated drugs by these proinflammatory macrophages.[12] In these previous cases, the responsible macrophages have been found to express CD80, CD86, TNF-α, and Ly6C/G

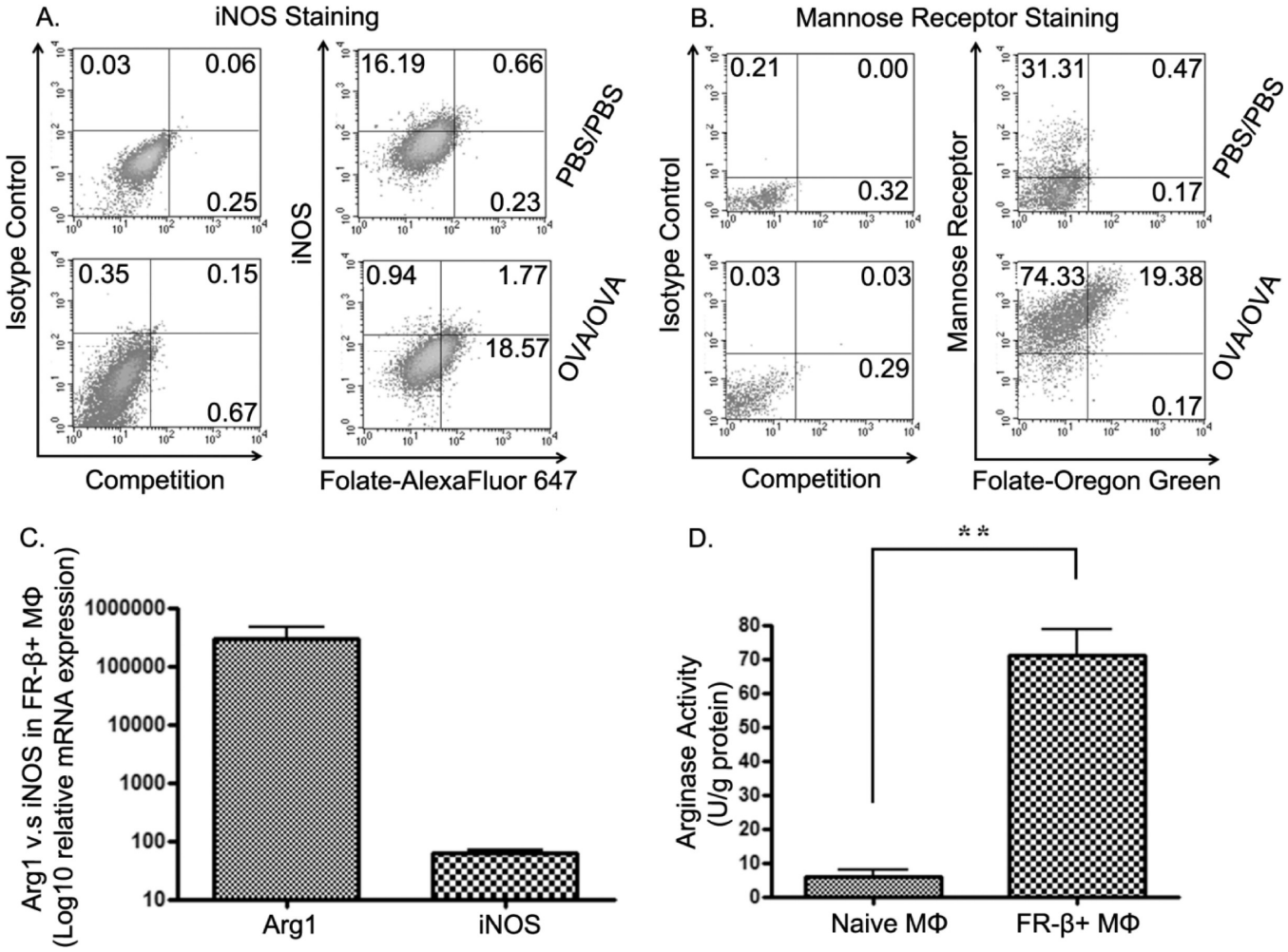

Figure 5. FR$^+$F4/80$^+$ cells express markers consistent with an alternatively activated macrophage phenotype. (A, B) Expression of FR-β and either iNOS (A) or mannose receptor (B) in macrophages harvested from lungs of OVA-challenged mice or saline-treated control mice. (C) Quantitative PCR showing expression of iNOS and Arg 1 mRNAs (relative to 18S rRNA). Total RNA was prepared from sorted FR$^+$F4/80$^+$ cells collected from lungs of OVA-challenged mice (n = 3). (D) Arginase activity in the sorted FR$^+$ macrophages from lungs of OVA-treated mice or in the sorted naïve macrophages from lungs of saline-treated mice (n = 3 mice in each group).

and also to produce reactive oxygen species, that is, markers of classically activated macrophages.[13] Moreover, treatment of rodent models of such inflammatory diseases as rheumatoid arthritis and systemic lupus erythematosus with folate-targeted drugs has been shown to both ameliorate symptoms and prevent disease progression.[15,32] Unfortunately, because alternatively activated macrophages are phenotypically very different from classically activated macrophages,[27] it was not possible to assume that folate could be similarly exploited to target attached compounds to these alternatively activated cells.

Demonstration of selective targeting of folate conjugates (e.g., folate-Dylight 680 and folate-^{99m}Tc) to alternatively activated macrophages *in vivo* now raises the question regarding how folate-targeted compounds might be used to diagnose and treat AAM-mediated diseases. Although the AAM content of most autoimmune/inflammatory diseases has not been examined, available data to date suggest that asthma, idiopathic pulmonary fibrosis, and cirrhosis of the liver and other fibrotic diseases are characterized by strong AAM involvement.[33,34] Moreover, many human cancers have been found to accumulate large numbers of FR-β positive anti-inflammatory macrophages[18,19] (personal observations), and these macrophages have been reported to contribute measurably to the survival and growth of the tumor.[35−37] Taken together, these observations raise the possibility that folate conjugates might eventually be exploited to diagnose, image, and treat important anti-inflammatory and malignant diseases if an appropriate warhead can be identified and delivered. However, because folate conjugate uptake is also frequently observed in the liver as a consequence of FR-β expression on activated macrophages/Kuppfer cells, care will have to be taken to ensure that any targeted therapeutic agent does not cause liver toxicity. Similar uptake of folate conjugates in the kidneys should not prove problematic, since capture of folate-targeted cytotoxic drugs by FR in the kidneys has not been observed to promote any kidney toxicity, probably because the conjugates are not permanently retained in the kidneys.[38]

Objective measures of disease severity can be important for evaluating: (i) how well a patient responds to a therapy, (ii) when a different treatment option is mandated, or (iii) if discontinuation of a drug is warranted due to disappearance of symptoms. Currently, no such assay exists for assessment of asthma. Because folate-^{99m}Tc accumulation in any section of the lungs likely correlates with disease activity, the possibility now arises for spatially quantitating the severity of inflammation in asthmatic lungs. However, whether such an assay will prove

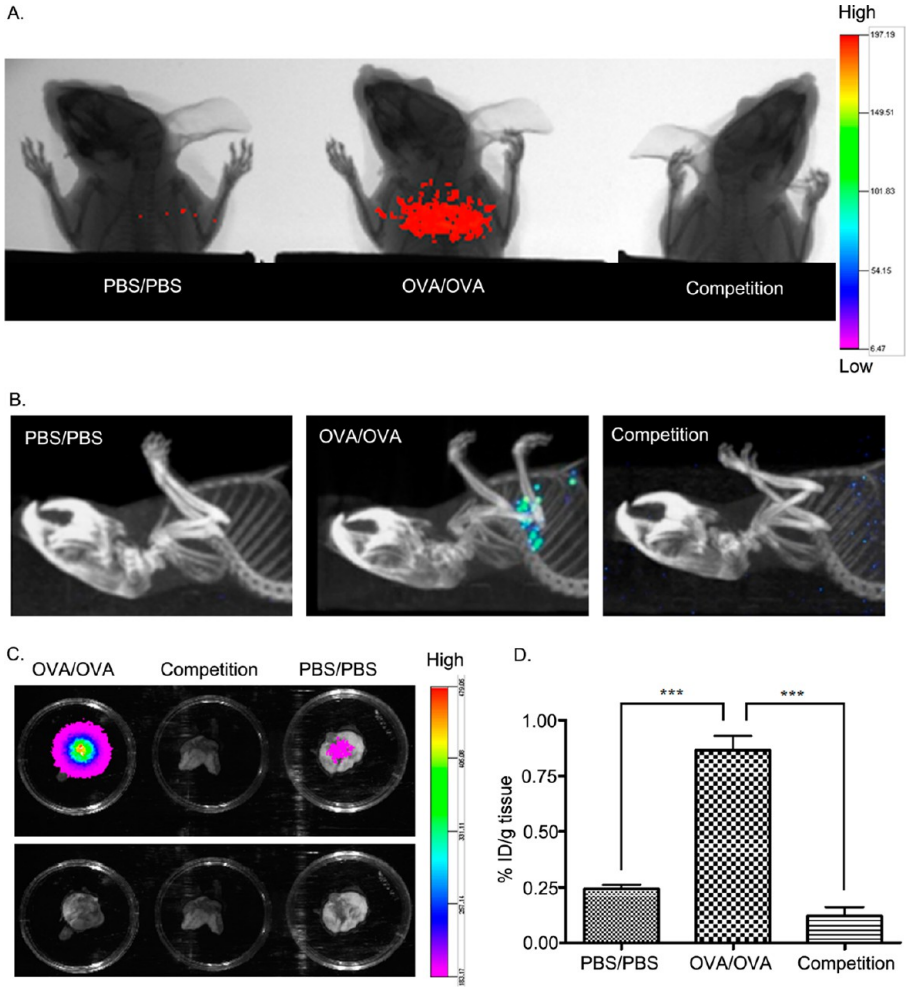

Figure 6. Folate-linked radioimaging agents can be used to assess the activation status of macrophages in asthmatic lungs *in vivo*. (A) Representative standard γ-scintigraphic images demonstrating ^{99m}Tc-EC20 uptake in the lung of mice with OVA-induced experimental asthma. Balb/c mice sensitized and challenged with OVA. Twenty-four hours later, mice were injected i.p. with either ^{99m}Tc-EC20 or ^{99m}Tc-EC20 plus 100-fold excess free folic acid. Radioimages were obtained on Kodak Imaging Station 4 h after injection. (B) Representative SPECT/CT images of mice with OVA-induced experimental asthma. Balb/c mice sensitized and challenged with OVA. Twenty-four hours later, mice were injected i.v. with either ^{99m}Tc-EC20 or ^{99m}Tc-EC20 plus 100-fold excess free folate-glucosamine. SPECT/CT scans were performed 2 h after injection. (C) *Ex vivo* γ-scintigraphic images of lungs excised from euthanized mice from the indicated groups. (D) Uptake of ^{99m}Tc-EC20 in lungs from healthy mice (n = 6) and mice with OVA-induced experimental asthma (n = 6). Lungs from different groups were harvested, and the uptake of ^{99m}Tc-EC20 was determined by γ-counting. ***P < 0.0001.

useful in disease management will obviously require clinical testing.

Finally, as noted in the Introduction, there is abundant evidence that AAMs are involved in the pathogenesis of asthma.[2−6] Thus, AAMs not only invite other immune cells into inflamed lungs via their release of specific cytokines and chemokines, but they also generate proallergic factors such as chitinase-like molecules, arginase, and resistin-like molecule alpha (Relma/FIZZ1) that contribute to airway inflammation and tissue remodeling during asthma.[27] Based on these unwanted activities, it can be anticipated that blockade of the inflammatory properties of AAMs might constitute a strategy for treatment of asthma and other AAM-caused diseases. Although an initial examination of the ability of a folate-targeted anti-inflammatory drug (folate-aminopterin) to suppress symptoms in a murine model of asthma failed to demonstrate any benefit (unpublished observations), future studies are still planned to look for other possible therapeutic cargos that might prove effective in treatment of AAM-mediated pathologies.

■ CONCLUSIONS

FR-β is shown to be substantially upregulated on AAMs, which are thought to be intrinsically involved in the development of asthma, idiopathic pulmonary fibrosis, cirrhosis of the liver, and other fibrotic diseases. Because these folate receptors can be selectively targeted with folate-linked imaging agents, such folate conjugates could prove useful in assessing disease severity and response to therapy in affected patients.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional quantitative time course evaluation of FR-β+ macrophage infiltration by flow cytometry, dual-color immunofluorescence analysis of the distribution of FR-β-expressing macrophages in asthmatic lung tissues, and three-dimensional SPECT/CT movie of folate-^{99m}Tc uptake in asthmatic mice.

This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written by J.S. and P.S.L. C.V. synthesized folate-DyLight 680 and folate-glucosamine. G.C. helped with real time PCR analyses.

Notes

The authors declare the following competing financial interest(s): P.S.L. has received fees and stock from Endocyte Inc. (West Lafayette, IN, USA), a company that he cofounded in 1995 to develop treatments for cancer and inflammation. All other authors declare no competing interests.

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SUPPORTING INFORMATION

Use of Folate-conjugated Imaging Agents to Target Alternatively Activated Macrophages in a Murine Model of Asthma

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Immunofluorescent Staining

After *in-situ* lung perfusion with PBS and fixation with 3% paraformaldehyde (PFA) in PBS, lungs were carefully removed, embedded in OCT Compound (Tissue-Tek), frozen in liquid nitrogen and maintained at -80 ^{o}C until use. The embedded lung tissues were then cut into 5 µm cyosections using a cryostat (Thermo Scientific). The cryosections were fixed and permeabilized using BD Cytofix/Cytoperm^{TM} Plus Fixation and Permeabilization Kit (BD Biosciences), followed by incubating cells with 1% BSA in PBST (0.25% Triton X-100) for 30 min at room temperature to block nonspecific binding of the antibodies. Then, the cryosections were stained overnight at 4°C with the following primary antibodies at 1:100 dilution: rabbit anti-FR polyclonal Ab (FL-257, Santa Cruz Biotechnologies) and rat anti-mouse CD68 mAb (137001, Biolegend). The cryosections were incubated with the appropriate mixture of secondary antibodies which were raised in different species with different fluorochromes: AlexaFluor 647

conjugated donkey-anti-rabbit IgG (Life Technologies), and AlexaFluor488 labeled goat-anti-rat IgG (Life Technologies) in 1% BSA for 1 hr at room temperature in the dark, washed extensively in PBS, and mounted with a drop of mounting medium. The stained sections were imaged using a V1000 confocal unit and a 60×/1.2 NA oil objective (Olympus). Images were processed using FLUOVIEW software (Olympus).

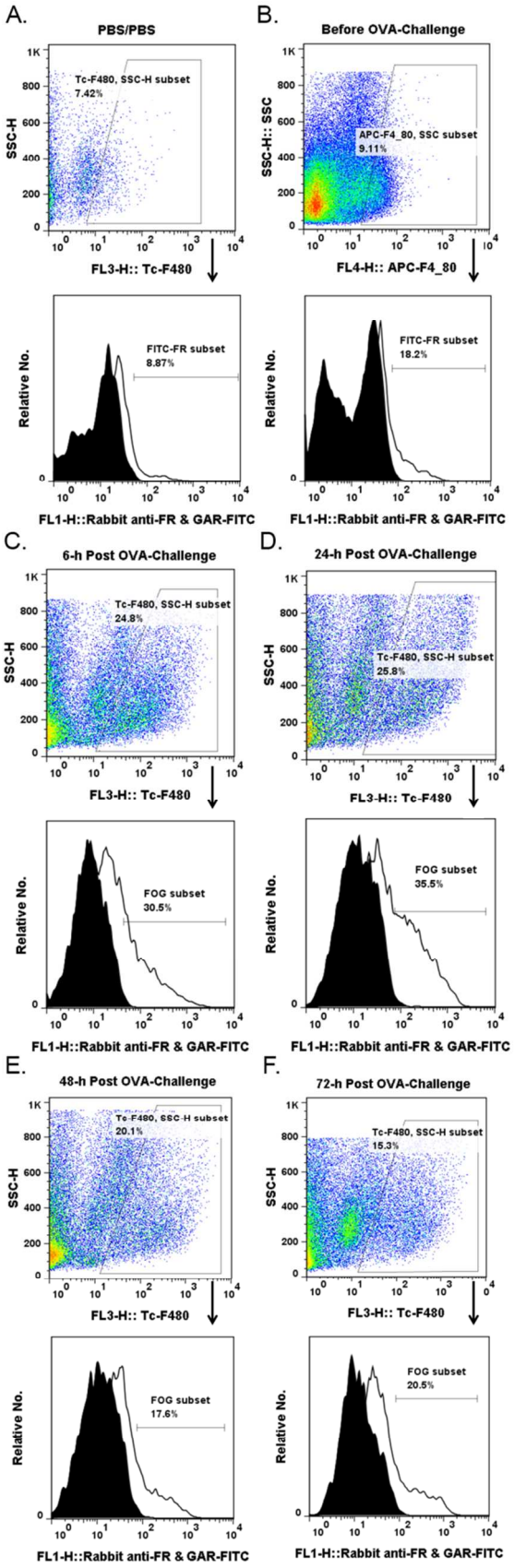
A.
PBS/PBS
Tc-F480, SSC-H subset
7.42%
SSC-H
FL3-H:: Tc-F480
FITC-FR subset
8.87%
Relative No.
FL1-H::Rabbit anti-FR & GAR-FITC
B.
Before OVA-Challenge
APC-F4_80, SSC subset
9.11%
SSC-H:: SSC
FL4-H:: APC-F4_80
FITC-FR subset
18.2%
Relative No.
FL1-H::Rabbit anti-FR & GAR-FITC
C.
6-h Post OVA-Challenge
Tc-F480, SSC-H subset
24.8%
SSC-H
FL3-H:: Tc-F480
FOG subset
30.5%
Relative No.
FL1-H::Rabbit anti-FR & GAR-FITC
D.
24-h Post OVA-Challenge
Tc-F480, SSC-H subset
25.8%
SSC-H
FL3-H:: Tc-F480
FOG subset
35.5%
Relative No.
FL1-H::Rabbit anti-FR & GAR-FITC
E.
48-h Post OVA-Challenge
Tc-F480, SSC-H subset
20.1%
SSC-H
FL3-H:: Tc-F480
FOG subset
17.6%
Relative No.
FL1-H::Rabbit anti-FR & GAR-FITC
F.
72-h Post OVA-Challenge
Tc-F480, SSC-H subset
15.3%
SSC-H
FL3-H:: Tc-F480
FOG subset
20.5%
Relative No.
FL1-H::Rabbit anti-FR & GAR-FITC

Figure S1. Quantitative time course evaluation of FR-β$^+$ macrophage infiltration by flow cytometry. Lung tissue from PBS-treated control mice (A), and from OVA-sensitized mice both before (B), and 6 hours (C), 24 hours (D), 48 hours (E), and 72 hours (F) after OVA challenge were digested in 5 mL folate-deficient RPMI 1640 media containing 0.1% collagenase IV, 0.01% hyaluronidase and 0.002% DNase. Mononuclear cells isolated from lungs of mice during OVA-induced experimental asthma were analyzed by flow cytometry for F4/80 and FR-β expression.

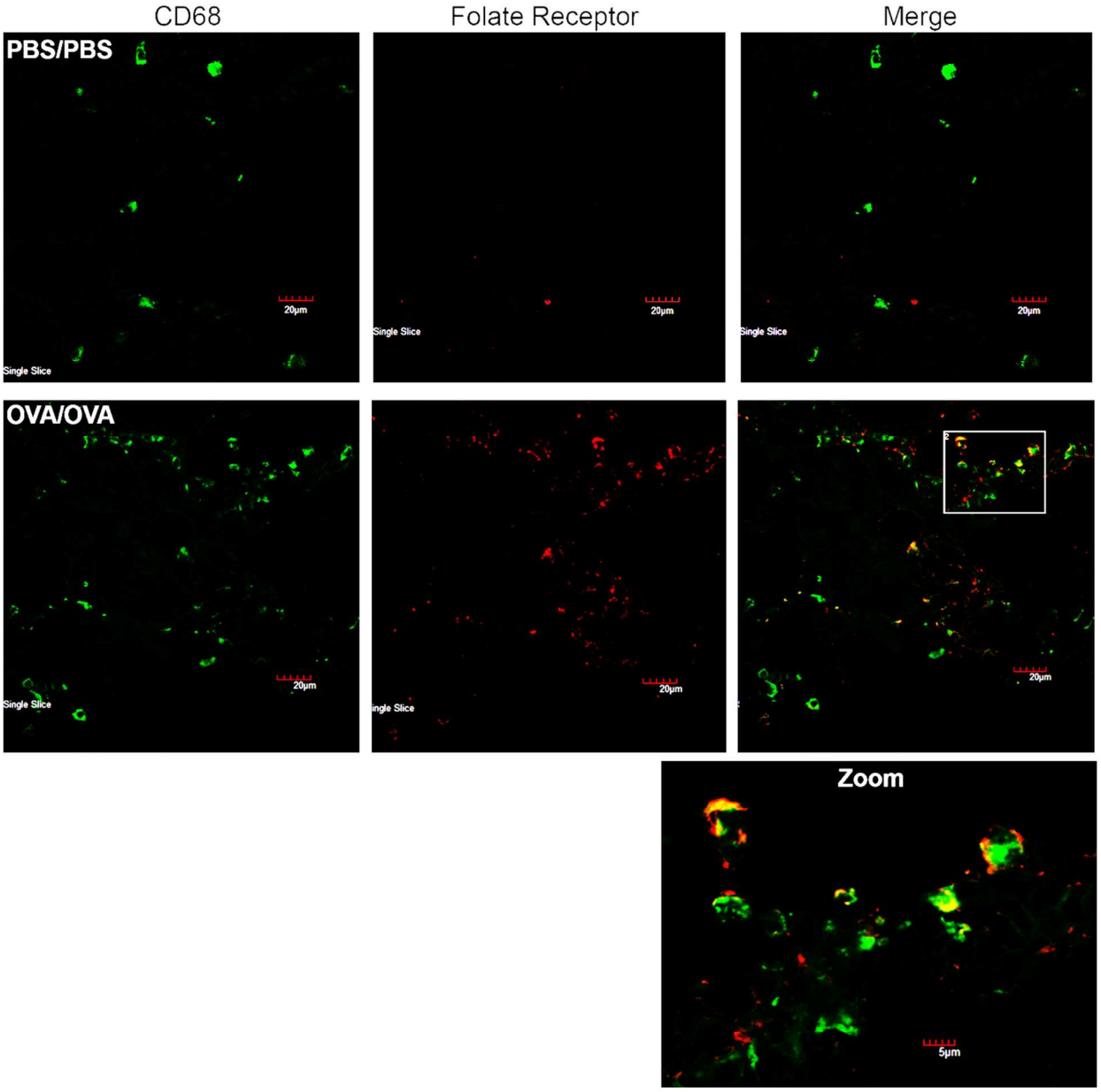

Figure S2. Distribution of folate receptor β (FR-β)-expressing macrophages (CD68$^+$) in the lungs of mice with allergic asthma. Cryosectioned lung tissues from PBS-treated control mice

(upper panel) and OVA-induced asthmatic mice (middle panel) were stained with rat anti-mouse CD68 mAb and rabbit anti-FR polyclonal antibody, followed by AlexaFluor488 labeled goat-anti-rat IgG and AlexaFluor 647 conjugated donkey-anti-rabbit IgG. Immunofluorescence staining for macrophages is revealed in the FITC channel (left, green). FR-positive cells are revealed in the AlexaFluor647 channel (middle, red). Superimposed images show the colocalization of CD68-positive cells and FR-expressing cells (far right). The image in the lower panel presents a magnification of the boxed area.

Video S1. Three-dimensional SPECT/CT movie of folate-^{99m}Tc uptake in an asthmatic mouse 2 hours post injection.