The Prolyl Hydroxylase PHD3 Identifies Proinflammatory Macrophages and Its Expression Is Regulated by Activin A

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Modulation of macrophage polarization underlies the onset and resolution of inflammatory processes, with polarization-specific molecules being actively sought as potential diagnostic and therapeutic tools. Based on their cytokine profile upon exposure to pathogenic stimuli, human monocyte-derived macrophages generated in the presence of GM-CSF or M-CSF are considered as proinflammatory (M1) or anti-inflammatory (M2) macrophages, respectively. We report in this study that the prolyl hydroxylase PHD3-encoding EGLN3 gene is specifically expressed by in vitro-generated proinflammatory M1(GM-CSF) human macrophages at the mRNA and protein level. Immunohistochemical analysis revealed the expression of PHD3 in CD163+ lung macrophages under basal homeostatic conditions, whereas PHD3+ macrophages were abundantly found in tissues undergoing inflammatory responses (e.g., Crohn’s disease and ulcerative colitis) and in tumors. In the case of melanoma, PHD3 expression marked a subset of tumor-associated macrophages that exhibit a weak (e.g., CD163) or absent (e.g., FOLR2) expression of typical M2-polarization markers. EGLN3 gene expression in proinflammatory M1(GM-CSF) macrophages was found to be activin A dependent and could be prevented in the presence of an anti-activin A-blocking Ab or inhibitors of activin receptor-like kinase receptors. Moreover, EGLN3 gene expression was upregulated in response to hypoxia only in M2(M-CSF) macrophages, and the hypoxia-mediated upregulation of EGLN3 expression was significantly impaired by activin A neutralization. These results indicate that EGLN3 gene expression in macrophages is dependent on activin A both under basal and hypoxic conditions and that the expression of the EGLN3-encoded PHD3 prolyl hydroxylase identifies proinflammatory macrophages in vivo and in vitro. The Journal of Immunology, 2012, 189: 1946–1954.

The extreme sensitivity of macrophages to endogenous (e.g., cytokines) and exogenous (e.g., pathogens) stimuli explains their phenotypic and functional heterogeneity under homeostatic and pathological conditions (1). In fact, the existence of a plethora of macrophage polarization states is critical for the adequate onset, regulation, and resolution of immune and inflammatory responses (2). As a representative example, although GM-CSF and M-CSF contribute to macrophage survival and proliferation, they exert distinct actions on macrophage polarization. GM-CSF gives rise to monocyte-derived macrophages that exhibit high Ag-presenting capacity and produce proinflammatory cytokines in response to LPS (3, 4). Conversely, M-CSF generates macrophages with high phagocytic activity and IL-10–producing ability in response to pathogens (3, 4). Based on their respective cytokine profiles, human macrophages generated in the presence of GM-CSF or M-CSF are representative of the classical or alternative macrophage polarization states, respectively, and are considered as proinflammatory or anti-inflammatory macrophages (3, 5).

Variation in the levels of oxygen is a parameter that also determines the state of macrophage polarization (6). In healthy tissues, oxygen levels span from 150 mmHg in the lung to 40–100 mmHg in the circulation and 4–20 mmHg in the tissues (7). Pathologic hypoxia, commonly found in malignant solid tumors, inflammatory lesions, and healing wounds (8–10), also influences macrophage polarization and leads to transcriptional and metabolic changes (6) and the acquisition of effector functions to promote angiogenesis and restore tissue homeostasis (7, 11, 12). Macrophages accumulate in large numbers in damaged hypoxic tissues and respond to hypoxia by altering their gene expression program via upregulation of the hypoxia-inducible factor (HIF) 1 (HIF1α/HIF1β) and HIF2 (HIF2α/HIF2β) transcription factors (13, 14). In the presence of oxygen, both α subunits are prolyl-hydroxylated, recognized by the von Hippel-Lindau tumor suppressor protein (15–17), and degraded by the ubiquitin-
proteasome pathway (17). The three HIF prolyl hydroxylases (PHD1–3) (16, 18) require molecular oxygen as a cosubstrate and, therefore, constitute the link between oxygen availability and HIF-dependent transcription (19).

The prolyl hydroxylase PHD3 is encoded by the EGLN3 gene, for which expression is upregulated in platelet-derived growth factor-stimulated rat fibroblasts (20). PHD3 is involved in neurogrowth-factor-dependent survival of neurons (21), stimulates pyruvate kinase M2 coactivation for HIF1 (22), and also participates in nerve-stimulating rat fibroblasts (20). PHD3 is involved in nerve-dependent transcription (19). In the current study, we demonstrate that proinflammatory macrophages constitutively express the EGLN3-encoded PHD3 prolyl hydroxylase in vitro and in vivo and that activin A regulates EGLN3 gene expression both under normoxic and hypoxic conditions.

Materials and Methods

Generation of human monocyte-derived macrophages

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 PBMC by magnetic cell sorting using CD14+ microbeads or the CD16+ Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (0.5 × 10^6 cells/ml, >95% CD14+ cells) were cultured in RPMI 1640 supplemented with 10% FCS (completed medium) for 7 d at 37°C in a humidified atmosphere with 5% CO₂. For hypoxic conditions, cells were placed in a Lymphoprep (Nyomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14+ microbeads or the CD16+ Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (0.5 × 10^6 cells/ml, >95% CD14+ cells) were cultured in RPMI 1640 supplemented with 10% FCS (completed medium) for 7 d at 37°C in a humidified atmosphere with 5% CO₂ and containing 1000 U/ml GM-CSF or M-CSF (10 ng/ml; ImmunoTools, Friesoythe, Germany) to generate M1(GM-CSF) or M2(M-CSF)-monocyte-derived macrophages, respectively. Cytokines were added every 2 d. Where indicated, M1(GM-CSF) or M2(M-CSF) macrophages were treated with either IFN-γ (500 U/ml; R&D Systems, Minneapolis, MN) or IL-4 (1000 U/ml; R&D Systems) to generate M2(M-CSF) macrophages. GAPDH protein levels were determined as a loading control.

Expression of the EGLN3-encoded PHD3 prolyl hydroxylase is restricted to proinflammatory M1(GM-CSF) macrophages. (A) EGLN3 gene expression in M1(GM-CSF) and M2(M-CSF) macrophages derived from either CD14+CD16+ or CD14+CD16- monocytes, as determined by microarray analysis (GSE27792) of three independent samples. (B) EGLN3 gene expression in M1(GM-CSF) and M2(M-CSF) macrophages derived from CD14+CD16+ monocytes determined by qRT-PCR on three independent samples. Mean and SD of triplicate determinations are shown. Results are expressed as relative expression (relative to GAPDH RNA levels) and referred to the EGLN3 mRNA levels in M2(M-CSF) cells (p < 0.001). (C) Western blot analysis of PHD3 in lysates of M1(GM-CSF) and M2(M-CSF) macrophages derived from CD14+CD16- monocytes. Cells were cultured in complete medium for 48 h in the absence of GM-CSF, after which the conditioned medium was collected. Cells were routinely cultured in 21% O₂ and 5% CO₂ (normoxic conditions). For hypoxic conditions, cells were placed in an in vivo 400 hypoxia Work Station (Ruskinn Technology) that was infused with a mixture of 1% O₂, 5% CO₂, and 94% N₂ (Sociedad Española de Carbouros Metálicos) for 24 h and treated or not with the activin receptor-like kinase (ALK)4/5/7 inhibitor SB431542 (Sigma-Aldrich) or an anti-activin A-blocking Ab (R&D Systems).

Quantitative real-time RT-PCR

Oligonucleotides for selected genes were designed according to the Roche software for quantitative real-time PCR (Roche Diagnostics). Total RNA was extracted using the RNeasy kit (Qiagen), retrotranscribed, and individually amplified cDNA were quantified using the Universal Human Probe Roche library (Roche Diagnostics). Assays were made in triplicate and results were normalized according to the expression levels of GAPDH RNA. Results were expressed using the ΔΔ threshold cycle method for quantification.

ELISA

Macrophage supernatants from M1(GM-CSF) or M2(M-CSF) macrophages were assayed for the presence of activin A using an ELISA from R&D Systems and according to the protocol supplied by the manufacturer.

Western blot

Cell lysates were obtained in 10 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40 lysis buffer containing 2 mM Pefabloc, 2 µg/ml aprotinin/antipain/leupeptin/pepstatin, 10 mM NaF, and 1 mM Na 3VO4. Ten micrograms cell lysate was subjected to SDS-PAGE and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore). Protein detection was carried out using an anti-human PHD3 polyclonal Ab (Abcam, Cambridge, U.K.) or an mAb against GAPDH (sc-32233; Santa Cruz Biotechnology, Santa Cruz, CA).

Reporter gene assays

Mv1Lu cells were plated in 24-well plates at 1 × 10^6 cells/ml in RPMI 1640 with 10% FCS. The luciferase-based plasmid pGL3-EGLN3PEnhA, which is driven by the EGLN3 promoter, was cotransfected into these cells with either a constitutively active level of the activin receptor-like kinase (ALK)4/5/7 inhibitor SB431542 (Sigma-Aldrich) or an anti-activin A-blocking Ab (R&D Systems).
which contains the firefly luciferase gene under the control of the EGLN3 gene promoter and enhancer, has been previously described (25). Cells were transiently transfected with 2 μg DNA of the reporter construct using Superfect (Qiagen). Twenty to 24 h after transfection, cells were treated with 25 ng/ml human recombinant activin A (PeproTech) for 3 h. To normalize transfection efficiency, cells were cotransfected with an SV40 promoter-based β-galactosidase expression plasmid (RSV-βgal). Measurement of relative luciferase units and β-galactosidase activity was performed using the Luciferase Assay System (Promega) and the Galacto-Sensor measurement of relative luciferase units and β-galactosidase activity was performed using the Luciferase Assay System (Promega) and the Galacto-Sensor.

Statistical analysis

Statistical analysis was performed using Student t test, and a p value <0.05 was considered significant. A two-way ANOVA test was performed for analysis of immunohistochemistry results.

Results

**PHD3 is expressed by in vitro-generated proinflammatory M1 (GM-CSF) macrophages**

After 7 d in culture with GM-CSF, monocytes differentiate into M1 macrophages that, unlike those generated in the presence of M-CSF (M2), exhibit immunogenic, proinflammatory, and antitumor functions (3). Gene expression profiling (GSE27792) (27, 28) revealed that EGLN3 gene expression, which codes for PHD3, is considerably higher in M1(GM-CSF) than in M2(M-CSF) macrophages (data not shown). PHD3 expression in macrophages under homeostatic and inflammatory conditions

To determine whether EGLN3 gene expression could be detected in macrophages in vivo, normal human tissues were tested for PHD3-specific reactivity by immunohistochemistry. An extremely low percentage of skin and tonsil CD163+ macrophages were found to express PHD3 (Fig. 2A). Conversely, a moderate percentage of alveolar CD163+ macrophages exhibited PHD3 reactivity (Fig. 2B), indicating that macrophages can also express PHD3 under homeostatic conditions in vivo. In the case of the gut, a similar picture emerged under homeostatic conditions, as a very low percentage of PHD3+CD163+ macrophages were detected in the colon, regardless of the region analyzed (Q7, Q8, ascending; Q15, sigmoid) (Fig. 3A). However, a higher number of PHD3+CD163+ macrophages were detected in CD163+ macrophages from the ileum of patients with Crohn’s disease (Fig. 3B) and from the colon of patients with ulcerative colitis, independently of colon area analyzed (Q5, transverse; Q14, Q16, rectum) (Fig. 3C). To quantitate the level of PHD3 in macrophages in noninflamed and inflamed colon, individual CD163+ macrophages were identified.
and their PHD3 staining measured. As shown in Fig. 3D, CD163+ macrophages from inflamed tissues exhibit significantly higher intensity of PHD3 staining, thus demonstrating that PHD3+ CD163+ macrophages are found in inflamed gut and that PHD3 levels are higher in macrophages within an inflammatory environment.

**PHD3 is expressed by tumor-associated macrophages with low levels of M2-specific markers**

The pattern of PHD3 expression was also evaluated in tumor-associated macrophages (TAM), for which polarization critically influences tumor progression and metastasis (30). Immunohistochemical analysis revealed PHD3 expression in macrophages from tumors of three different origins (kidney, breast, and colon) (Fig. 3E). In CD163+ TAM, quantitative analysis indicated a heterogeneous expression of PHD3, which ranged from negative to strongly positive (Fig. 3F). A similar result was observed upon analysis of the PHD3 expression in melanoma TAM. A high PHD3 expression was detected in CD68+ macrophages primarily located within melanoma tumor cell clusters and exhibiting a weak expression of CD163 (Fig. 4A). Conversely, high expression of CD163 was detected in macrophages excluded from the tumor cell nests and that also exhibited a high level of expression of the FRβ (Fig. 4B). Like CD163, FRβ expression was primarily restricted to CD68+ macrophages surrounding melanoma cell nests (Fig. 4B). In fact, when coexpression of PHD3 and FRβ was compared, their respective pattern of staining was found to be almost opposite, as PHD3 expression was limited to CD68+ FRβ− macrophages that reside within the tumor cell clusters (Fig. 4B). Quantitative analysis of the expression of FRβ and PHD3 in four independent melanoma samples confirmed the negative correlation of their respective expression levels in CD68+ macrophages (Fig. 4C). Therefore, the expression of PHD3 in tumor-associated human macrophages is limited to a macrophage subset that is devoid of anti-inflammatory M2 markers.

**PHD3 expression in macrophages is regulated by activin A**

To determine the molecular basis for the restricted expression of PHD3 in proinflammatory macrophages, we first analyzed whether M1(GM-CSF) macrophage-derived soluble factors contribute to EGLN3 expression. As shown in Fig. 5A, M1(GM-CSF)-conditioned medium triggered a dramatic induction of EGLN3 expression.
expression in monocytes after 72 h, implying that the differential expression of \textit{EGLN3} in M1(GM-CSF) and M2(M-CSF) macrophages might be caused by soluble factors released along M1 (GM-CSF) polarization. Because activin A is actively produced by M1(GM-CSF) macrophages, where it constitutes a polarization marker (29), we next evaluated whether activin A had an influence on the M1(GM-CSF)-specific expression of \textit{EGLN3}. The presence of a blocking Ab against activin A reduced by 50% the acquisition of \textit{EGLN3} expression in monocytes exposed to GM-CSF–free M1(GM-CSF)-conditioned medium (Fig. 5B). Furthermore, \textit{EGLN3} expression levels were significantly reduced when M1(GM-CSF) macrophages were generated in the presence of inhibitors of TGF-\beta/activin/nodal type I receptors ALK5/ALK4/ALK7 SB431542 (Fig. 5C) or A-83 (Fig. 5D) or in the presence of a neutralizing anti-activin A Ab (Fig. 5E). Moreover, activin A (25 ng/ml) significantly increased the expression of \textit{EGLN3} in M2(M-CSF) macrophages after a 48-h treatment (Fig. 5F) and also enhanced the activity of \textit{EGLN3} gene regulatory regions in a heterologous cellular system (Fig. 5G). Taken together, these results indicate that activin A promotes \textit{EGLN3} gene expression in M1(GM-CSF) macrophages and contributes to the differential expression of \textit{EGLN3} in proinflammatory and anti-inflammatory monocyte-derived human macrophages.

\textit{Activin A mediates the hypoxia-induced expression of \textit{EGLN3} in M2(M-CSF) macrophages}

\textit{EGLN3} codes for the PHD3 prolyl hydroxylase oxygen sensor (16), and its expression is induced under hypoxic conditions in an HIF-dependent manner (31). The differential expression of \textit{EGLN3} in M1(GM-CSF) and M2(M-CSF) macrophages under normoxic

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\caption{EGLN3-encoded PHD3 is expressed by tumor-associated macrophages lacking M2 markers (CD163, FR\textbeta). (A) Confocal sections of a metastatic melanoma sample after triple immunofluorescence analysis of the melanoma marker high m.w. melanoma-associated Ag (HMW-MAA) (white), the macrophage-specific marker CD68 (white), CD163 (green), and PHD3 (red). Magnification of a CD163/CD68 and PHD3 colocalizing area appear enlarged in the bottom panel. In all cases, “T” and “S” indicate the melanoma nests and stroma devoid of tumor cells, respectively. When indicated, nuclei were counterstained with DAPI (blue). (B) Magnifications of an area at the tumor nests/stroma boundary stained with anti-CD163 (white), anti-FR\textbeta (green), and anti-PHD3 (red) Abs and with nuclei counterstained with DAPI (blue). (C) Correlation between FR\textbeta and PHD3 expression levels in CD163+ macrophages from four independent samples of s.c. melanoma metastases. In each case, five fields (750 \times 750 \mu m^2) were analyzed, and the background-subtracted mean fluorescence intensity (in arbitrary units [a.u.]) from 360 cells was plotted and statistically processed.
\end{figure}
conditions prompted us to analyze its expression in both macrophage types under low oxygen tensions. Surprisingly, hypoxia (1% O₂, 24 h) did not alter EGLN3 expression in M1(GM-CSF) macrophages (Fig. 6A). However, hypoxia significantly increased the EGLN3 gene expression level in M2(M-CSF) macrophages, whereas it led to a slight downregulation of EGLN1 and EGLN2 gene expression (Fig. 6A). This result implies that the expression of the EGLN genes is distinctly regulated by hypoxia between M1 (GM-CSF) and M2(M-CSF) and, more importantly, that both macrophage subtypes exhibit a distinct ability to respond to hypoxia.

Because activin A directs the expression of EGLN3 in M1(GM-CSF) macrophages and induces EGLN3 gene expression in M2 (M-CSF) macrophages (Fig. 5), we hypothesized that activin A might also mediate the hypoxia-dependent induction of EGLN3 expression in M2(M-CSF) macrophages. Determination of activin A levels in both macrophage subtypes after exposure to 1% O₂ for 24 h revealed that hypoxia significantly induced the expression of the INHBA gene (Fig. 6B) as well as the secretion of activin A in M2(M-CSF) macrophages (Fig. 6C). By contrast, hypoxia had no relevant effect on INHBA expression or activin A release from M1 (GM-CSF) macrophages (Fig. 6B, 6C). To fully test the above hypothesis, we evaluated the influence of a blocking anti-activin A Ab or the SB431542 inhibitor on the hypoxia-mediated upregulation of EGLN3 gene expression in M2(M-CSF) markers. The hypoxia-driven EGLN3 upregulation was significantly reduced in the presence of either anti-activin A or SB431542 (Fig. 6D). Therefore, activin A critically regulates EGLN3 expression in macrophages, mediating both its constitutive expression in proinflammatory M1(GM-CSF) macrophages and its hypoxia-driven upregulation in M2(M-CSF) macrophages.

**Discussion**

In the present report, we describe the preferential expression of the PHD3 prolyl hydroxylase-encoding EGLN3 gene by human proinflammatory macrophages in vivo and in vitro and that PHD3 expression is restricted to TAM that exhibit weak or absent levels of typical anti-inflammatory/M2 markers. Besides, we demonstrate that activin A regulates the constitutive expression of PHD3 in M1(GM-CSF) macrophages and its hypoxia-inducible expression in M2(M-CSF) macrophages. Considering that activin A production is an exclusive property of proinflammatory M1(GM-CSF) macrophages (29), the activin A–PHD3 axis appears to constitute a specific signature of proinflammatory macrophage polarization. Moreover, because activin A expression is upregulated by hypoxia in M2(M-CSF) macrophages (Fig. 6), the activin A–PHD3 axis might also influence macrophage effector functions under low oxygen pressures, a condition that is common to solid tumors and tissues undergoing active inflammatory responses (32).

The hypoxia-inducible expression of EGLN3 in M2(M-CSF) macrophages is in agreement with results in most cell types (33)
and with the presence of a functional hypoxia-responsive element within the first intron of the *EGLN3* gene (25). However, the presence of PHD3 in M1(GM-CSF) macrophages in vitro must reflect the existence of hypoxia-independent regulatory mechanisms controlling *EGLN3* gene expression, as HIF factors are not detectable in M1(GM-CSF) macrophages under normoxic conditions (data not shown). The presence of PHD3-positive macrophages in lung (Fig. 2), where partial pressure of O2 ranges between 150 and 100 mmHg, also supports the hypothesis of such a hypoxia-independent mechanism for regulation of PHD3 expression. Our results indicate that activin A complies with this requirement because it: 1) potentiates the activity of the *EGLN3* gene regulatory region; 2) positively regulates *EGLN3* expression in M1(GM-CSF) macrophages independently of low oxygen pressure; and 3) mediates the hypoxia-inducible *EGLN3* gene expression in M2(M-CSF) macrophages. Therefore, unlike promoter hypermethylation or miR-20a, which inhibit *EGLN3* gene expression in tumor cells (34) and cardiomyocytes (35), activin A represents a novel mechanism for positive regulation of *EGLN3* expression. Along this line, soluble growth factors may turn out to exert an influence on the expression of the hypoxia-dependent enzymes in myeloid cells, as *EGLN1* and *EGLN2* gene expression is up-regulated by hypoxic conditions (37).

Activin A mediates the hypoxia-triggered upregulation of *EGLN3* gene expression in M2(M-CSF) macrophages. (A) Relative mRNA expression of *EGLN3*, *EGLN2*, and *EGLN1* in M2(M-CSF) and M1(GM-CSF) macrophages maintained for 24 h under normoxia (Nx; 95% O2) or hypoxia (Hpx; 1% O2), as measured by qRT-PCR. In the case of *EGLN3*, mean and SD of three independent experiments are shown (**p < 0.001). For *EGLN1* and *EGLN2*, one representative experiment (out of two performed) is shown. Results are expressed as relative expression (relative to GAPDH RNA levels) and referred to the INHBA mRNA level in M2(M-CSF) macrophages. Mean and SD of three independent experiments are shown (*p < 0.05). (B) Relative mRNA expression of INHBA in M2(M-CSF) and M1(GM-CSF) macrophages maintained for 24 h under normoxia (Nx; 95% O2) or hypoxia (Hpx; 1% O2), as measured by qRT-PCR. Results are expressed as relative expression (relative to GAPDH RNA levels, log scale) and referred to the INHBA mRNA level in M2(M-CSF) macrophages maintained in normoxia. Mean and SD of three independent experiments are shown (***p < 0.001). (C) Activin A protein levels secreted by M2(M-CSF) (left panel) and M1(GM-CSF) (right panel) macrophages exposed to either normoxia or hypoxia for 24 h, as measured by ELISA. Shown are the mean and SD of five independent experiments (**p < 0.05). (D) Relative mRNA expression of *EGLN3* in M2(M-CSF) macrophages exposed to either normoxia or hypoxia for 24 h and in the absence or in the presence of DMSO, SB431542 (10 nM), isotype-matched Ab (IgG), or a blocking anti-activin A Ab (0.1 μg/ml). Results are expressed as relative expression (relative to GAPDH RNA levels) and referred to *EGLN3* mRNA levels in M2(M-CSF) macrophages exposed to DMSO under normoxia. Mean and SD of three independent experiments are shown (**p < 0.001).

Huge differences exist between the expression profiles of polarized macrophages of human or mouse origin (40). A recent report has conclusively demonstrated such a difference by comparing the transcriptome of bone marrow-derived murine and monocyte-derived human macrophages polarized by either GM-CSF or M-CSF (41). Interestingly, the expression of the *Egln3* gene has also been found to differentiate between M1(GM-CSF) and M2(M-CSF) bone marrow-derived macrophages, with a significantly higher *Egln3* expression in macrophages polarized by GM-CSF (ArrayExpress Archive, http://www.ebi.ac.uk/arrayexpress, accession number E-MTAB-791). Therefore, the lack of expression of the PHD3-encoding *EGLN3* gene appears to correlate with an anti-inflammatory macrophage phenotype. In addition to its identification as a marker for in vitro-generated M1 (GM-CSF) macrophages, the relevance of PHD3 expression in macrophages is underscored by the presence of PHD3-positive macrophages under homeostatic conditions (e.g., lung), in inflammatory conditions (Crohn’s disease and ulcerative colitis), and within tumor areas. A low percentage of PHD3+ intestinal macrophages was detected in normal noninflamed gut, where macrophages exhibit constitutive IL-10 production and promotion of regulatory T cell responses (42), whereas a high proportion of CD163+ PHD3+ macrophages was observed in inflamed gut (Fig. 3). PHD3 expression in macrophages from inflamed tissues can potentially be driven by hypoxia and/or activin A. However, based on the role
of activin A in inflammatory responses (43, 44) and its ability to support proinflammatory macrophage polarization and limit the acquisition of anti-inflammatory markers like IL-10 (29), it is tempting to speculate that activin A contributes to PHD3 expression in macrophages within inflamed gut and that macrophage EGLN3 gene expression identifies macrophages exhibiting an M1-skewed proinflammatory polarization. If so, and despite the difficulties in extrapolating polarization marker expression between the human and murine systems (40), our results would be in agreement with the proposed role of PHDs as positive regulators of the LPS-induced inflammatory process (45) and the link between HIF1α induction and M1 polarization (6). The correlation between PHD3 and macrophage M1 polarization is further supported by the restriction of PHD3 expression to a TAM subset that exhibits a low level of M2 polarization-associated markers and for which location differed from that of M2-polarized macrophages (highly positive for CD163 and FRβ). Based on the pro-M2 polarization ability of tumor-conditioned media (46), it seems reasonable to assume that PHD3+ macrophages would give rise to FRβ+ TAM under the influence of tumor-derived factors like M-CSF. Regardless of the ontogenic relationships between both TAM subsets, the appearance of two subsets of CD68+CD163+ TAM macrophages differing in their expression of M2 (FRβ) and M1 (PHD3) polarization markers is compatible with the heterogeneity and the M1/M2 mixed polarization of TAM in murine models of cancer (47, 48), in which M2-like TAMs are enriched within hypoxic areas (48).

As a defining hallmark of the myeloid cell lineage, plasticity allows macrophages to adjust their effector functions to their surrounding environment (49). In response to hypoxic conditions, macrophages modulate their metabolism (50, 51), effector functions in inflammatory responses (52), and ability to handle tumor progression or promote T cell stimulation (53). Because PHD3 regulates HIF protein stability (54) and is itself an HIF target gene (25), it can be hypothesized that the differential EGLN3 gene expression in M1 (GM-CSF) and M2 (M-CSF) macrophages may underlie a distinct response of both macrophage subtypes to hypoxia. In this regard, it is worth noting that, unlike their M2 (M-CSF) counterparts, PHD3-expressing M1 (GM-CSF) macrophages exhibited a defective response to hypoxia, at least in terms of EGLN3 and INHBA/activin A upregulation. Therefore, constitutive PHD3 expression appears associated to a certain state of hypoxia resistance that resembles the effects of chronic hypoxia (PHD’s overactivation and desensitization of HIFα) (55). As in the case of exposure to chronic hypoxia, constitutively elevated PHD3 expression might allow macrophages to exert their effector functions in a more efficient manner, protecting them from necrosis (55) or, as in the case of neutrophils (56), enhancing their survival during hypoxia. In this case, the activin A-regulated expression of PHD3 in macrophages would constitute an additional weapon for macrophages to efficiently fight against pathogenic stimuli.

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