Monocytic cells derived from human embryonic stem cells and fetal liver share common differentiation pathways and homeostatic functions

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ABSTRACT

The early emergence of macrophages and their large pattern of tissue distribution during development suggest that they may play a critical role in initial steps of embryogenesis. Here, we show that monocytic cells derived from human ES cells (hESC) and fetal liver follow a differentiation pathway different to that of adult cells, leading to specific functions. Embryonic and fetal monocytic cells differentiated from a CD14lowCD16– precursor to form CD14highCD16+ cells without producing the CD14highCD16– cell population that predominates in adult peripheral blood. Both demonstrated an enhanced expression of genes encoding tissue degrading enzymes, chemokines and scavenger receptors, previously reported for M2 macrophages. In comparison to adult blood monocytes, they secreted high amounts of proteins acting on tissue remodeling and angiogenesis and most of them expressed the Tie2 receptor. Furthermore, they promoted the vascular remodeling in xenotransplanted human tumors. These findings suggest that the regulation of human fetal and embryonic monocytic cell differentiation leads to the generation of cells endowed mainly with anti-inflammatory and remodeling functions. Trophic and immunosuppressive functions of M2-polarized macrophages link fetus and tumor development and human ES cells offer a valuable experimental model for in vitro studies of mechanisms sustaining these processes.
INTRODUCTION

The mononuclear phagocyte system (MPS) is a network of highly versatile and multifunctional cells that include peripheral blood monocytes, dendritic cells and tissue macrophages and plays major roles in development, scavenging, inflammation and antipathogen defenses. These cells originate from hematopoietic stem cells in the bone marrow through differentiation steps generating common myeloid progenitors shared with neutrophils, then enter the blood as monocytes to be recruited into normal healthy tissues or at sites of injury where they differentiate into dendritic cells or macrophages. Macrophages demonstrate a remarkable heterogeneity related to their phenotype, localization and function.

It was proposed to distinguish M1 macrophages attributed with pro-inflammatory functions and M2 macrophages attributed with wound healing or tissue remodeling functions, which is an over-simplistic but useful categorization of this complex cell lineage. It remains unsolved whether these heterogeneous functions are determined only by the local tissue environment or reflect also specific developmental maturation pathways.

In mice and birds, macrophages are part of primitive hematopoiesis and first emerge in the yolk sac at day 7.5 post-coitum (dpc). A second wave of macrophages is formed in the yolk sac between 8-8.5 dpc through maturation of a myeloid-erythroid progenitor. These macrophages resemble those appearing later from the differentiation of a lympho-myeloid hematopoietic stem cell present in the fetal liver. Macrophages generated in the fetal liver are rapidly dispersed in all embryonic tissues. The early
emergence of macrophages and their large pattern of tissue expression during development suggest a critical role of these cells in initial steps of embryogenesis 13,14.

As in adult mice, macrophages play a key role in innate response to pathogens and constitute the primary host defense in the mouse embryo. Several lines of evidence suggest also trophic roles for mouse embryonic macrophages, including bone morphogenesis, ductal branching, neuronal networking and angiogenesis 14-15. Gene signature of mouse embryonic macrophages is shared with a subset of adult peripheral blood monocytes expressing the angiopoietin receptor Tie2, known as TEMs, which may be part of Gr1+Ly-6c+/CX3CR1high/CCR2-/CD62L- cells 16-17. Their human counterparts belong to the less frequent subpopulation of ‘non classical” circulating monocytes with a CD14+CD16+ phenotype 3,18-19. Part of the embryonic mouse macrophage signature is shared also with the so-called tumor-associated macrophages (TAM) that have tumor remodeling and immunosuppressive functions 13,17,20-21. Collectively, these data suggest that embryonic mouse macrophages are specialized in tissue remodeling and that similar cells are recruited or reprogrammed during tumor development 14. Common remodeling and immunosuppressive functions of M2-polarized macrophages may link normal developmental and pathological processes.

Our knowledge on the development and functional properties of human MPS during ontogeny is much lower, due to the limited availability of human embryos at the earliest stages of development 22. In recent years, human embryonic stem cells (hESC) have been used as an in vitro model of the early stages of tissue maturation. In vitro hESC hematopoietic differentiation recapitulates the main stages of early in vivo embryogenesis 23-27. This suitable model was used here for studying embryonic monopoiesis. More specifically, we examined monocytes/macrophages obtained from
differentiation of hESC and fetal liver CD34+ cells issued from first trimester fetuses. We show that, in early stages of human development, most of the generated fetal and embryonic monocytic cells share a specific pathway of differentiation which correlated with transcriptional and functional patterns that characterize anti-inflammatory and tissue remodeling cells.
MATERIALS AND METHODS

hESC maintenance and differentiation.

H1 (NIH code WA01) and H9 hES (NIH code WA09) cell lines were obtained from WiCell Research Institute (Madison, WI). Most experiments were performed with the H9 cell line. Undifferentiated hESC were grown as previously described. Differentiation was achieved from embryoid bodies (EB) and the differentiation medium consisted of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 15% FBS. Mesoderm and hematopoietic specifications were induced by adding bone morphogenetic protein 4 (BMP-4, 10 ng/ml), vascular endothelial growth factor (VEGF, 5 ng/ml), interleukin-3 (IL-3, 100 U/ml), fetal liver tyrosine kinase 3 ligand (FLT3-ligand, 10 ng/ml) (all from Peprotech, Neuilly-sur-Seine, France), stem cell factor (SCF, 50 ng/ml) (a gift from Amgen, Thousand Oaks, CA) and thrombopoietin (TPO, 10 ng/ml) (a gift from Kirin laboratories, Tokyo, Japan). CD14+ cells were sorted and their terminal differentiation towards the monocyte/macrophage lineage was achieved in presence of monocyte-colony stimulating factor (M-CSF, 50 ng/ml), granulocyte-macrophage CSF (GM-CSF, 20 ng/ml), IL-3 (100 U/ml) and FLT3-L (10 ng/ml) (Peprotech). Cultures were maintained in a 5% CO2/5% O2 environment and differentiation medium was changed every four days.

Generation of monocyte/macrophage from fetal and adult CD34+ cells

Adult CD34+ cells were recovered from cytapheresis performed in healthy donors submitted to hematopoietic stem cell (HSC) harvest. Fetal CD34+ cells were recovered
from fetal liver ranging from 10–17 weeks estimated gestational age (EGA), obtained after legal abortion. CD34+ cells from fetal liver or from adult peripheral blood mononuclear cells (PBMCs) were purified using CD34 immunomagnetic beads (MACS; Miltenyi Biotec). Proliferation and differentiation of CD34+ cells towards the monocyte/macrophage lineage was achieved in conditions similar to those described for hESC, in the presence of M-CSF, GM-CSF, IL-3, and FLT3-L.

Human peripheral blood monocyte sorting

PBMCs were sorted from cytaphereses performed in healthy donors. PBMCs were isolated using a Ficoll gradient and monocytes were enriched by adherence in IMDM supplemented with 15% FBS.

Flow cytometry and cell sorting

To obtain a single-cell suspension, EB cultures were treated with trypsin-EDTA (0.25%) (Sigma-Aldrich, St. Louis) for 5 min at 37°C. Adherent monocytes from hESC, fetal liver and blood CD34+ cells, or peripheral blood were detached from the plastic plates by incubation with cold EDTA/PBS (2 mM) for 10 min. Single-cell suspension was incubated for 30 min at 4°C with the different conjugated monoclonal antibodies (MoAbs) and washed twice. Flow cytometric characterization was performed using a panel of anti-human MoAbs: phycoerythrin (PE)–conjugated-anti-CD14, CD16, CD163, CD64, CCR5, CD34 (Becton Dickinson) and Tie2 (R&D); (FITC)–conjugated anti-CD16, CD15 (Becton Dickinson) and APC-conjugated anti-CD14, CD32, CD15, CD36, CD62L (Becton Dickinson), CD115 (R&D) and CD16 (Caltag). Cells were analyzed with a FacSort flow cytometer (Becton Dickinson, San Jose, CA). The percentage of positive
cells was determined as compared with isotypic controls establishing the background level of non-specific staining. CellQuest software (Becton Dickinson) was used for data acquisition and analysis. CD14^+ or CD14^+CD16^- and CD14^+ CD16^+ cells were sorted after labeling with PE-conjugated anti-CD14 and APC-conjugated anti-CD16 MoAbs using a FacsDiva cell sorter (Becton Dickinson).

**Cell morphology**

CD14^+ cells obtained from hESC were sorted after 21 days of differentiation from EB cultures and then cultured in the presence of FLT3-L, IL-3, GM-CSF and M-CSF. Differentiated cells were stained with May-Grünwald-Giemsa solution, myeloperoxidase (MPO) and double-specific (naphthol AS-D chloroacetate esterase/non-specific-naphthyl butyrate esterase) esterase (Sigma Aldrich) in accordance with manufacturer’s protocols.

**Quantification of hematopoietic progenitors in semi-solid cultures**

To study hematopoietic progenitors, dissociated cells from EB were plated in triplicate at a density of 3x10^4 cells/mL in human methycellulose medium H4434 containing cytokines (Stem Cell Technologies, Vancouver, BC, Canada). Progenitor-derived hematopoietic colonies were scored after 9 to 14 days.

**Total RNA isolation, RT-PCR and quantitative RT-PCR**

CD14^+CD16^+ cells were sorted from embryonic or fetal cells or from adult PBMN cells (>4 repeated experiments in each condition). When indicated, cells were activated with 100 ng/mL LPS (Sigma, St Louis, MO) and 1,000 U/mL IFN γ (Peprotech, France) for 24 hours. Total RNA was isolated using the RNeasy microkit (QIAGEN, Cortaboeuf,
France) and RNA concentration was adjusted at 100 ng/µL (NanoDrop Technologies, Wilmington, DE). Quality was assessed using the Lab-on-a-Chip 2100 bioanalyzer technology (Agilent Technologies, Palo Alto, CA). For each condition, RNA of 3 experiments was mixed and a total of 1 µg of RNA was reverse transcribed into cDNA with SuperScript III (Invitrogen), and qPCR analyses were performed with TaqMan low-density arrays. Immune panel and customized gene array (Applied Biosystems, Foster City, CA) on which 0.1 to 1 µg of cDNA was loaded. qPCR was run for 35 cycles in standard mode using an ABI7900HT apparatus (Applied Biosystems). Raw data were extracted using the SDS 2.2.1 software and the difference between the threshold cycle (CT) of each gene and that of the endogenous controls 18S and Gapdh was used to determine (ΔCT) gene expression.

Cytokine and chemokine secretion and expression

CD14⁺CD16⁺ sorted cells (300x10³) were washed and cultured for 24 hours in 0.2 ml IMDM with 10% FBS. Cells were either untreated or stimulated with LPS or IFNγ (n=4 repeated experiments in each condition). Levels of 20 cytokines released into the supernatants were determined using customized «Procarta™ Cytokine Assay Kit » (Ozyme, France) and the xMAP Luminex technology (Multi-analyse profiling). Quantification of cytokines was realized using the Bio-Plex 200 (Bio-Rad). Culture medium was used as a negative control. Data were analyzed with the Bio-Manager 5.0 software (BioRad) using 4-5 parameters values. The “Human Angiogenesis Array Kit” (R&D) was used to measure relative levels of tissue remodeling-related proteins (n=55) following the Proteome Profiler™ Array technology. All experiments were performed in duplicates.
**In vivo tumor angiogenesis assays**

All animal treatment and procedures were approved by the Institut Gustave Roussy animal care and use committee. Sorted CD14\(^+\)CD16\(^+\) from hESC, fetal CD34\(^+\) cultures and adult peripheral blood cells \((2.5\times10^5)\) were co-injected subcutaneously with U87 human glioma cells \((5\times10^6)\) in nude mice; control mice received only glioma cells \((n=6, \text{ for each group})\). Tumors were grown for 5-7 days. To quantify angiogenesis, serial sections spanning the whole tumor were immunostained for CD34 (anti–mouse CD34, HyCult biotechnology, Uden, the Netherlands), followed by incubation with a rabbit antibody (Southern Biotech., Birmingham, AL) for 1 hour. Quantification of CD34-stained micro-vessels was achieved using PIXCIT, a software package designed by the Groupe Régional d’Études sur le Cancer (Caen, France). Cell quantification was also performed using flow cytometry analysis with a (PE)–conjugated anti–human CD45 MoAb and a (FITC)–conjugated anti–mouse CD34 MoAb \((n=6, \text{ for each group})\).

**Phagocytosis assay**

Cells \((250,000)\) were suspended in 0.5 ml HBSS containing 10 uM luminol, incubated at 37°C in a thermostatic luminometer and stimulated (or not) with opsonized zymosan \((0.5 \text{ mg/ml})\)(Sigma Aldrich, St.Louis). Changes in chemiluminescence were measured over a 30 min period.
Statistical analysis.

Differences in qPCR and protein analysis and in tumor angiogenesis assays between groups were assessed by pairwise t tests (Software, Inc.). The pool SD switch calculated a common SD for all groups and used that for all comparisons. The reported $p$ value is a result of Bonferroni adjustment method. A $P$ value $< 5\%$ was considered statistically significant.
RESULTS

Embryonic monocytes and macrophages develop from hESCs through a process similar to bone marrow monopoiesis.

We induced human ESC differentiation into the monocyte/macrophage lineage through a 3 steps protocol (Fig 1A) that included: step 1) embryoid body (EB) formation by culturing SSEA4+ H9 cell line (Fig 1B) in low-attachment plates in the presence of BMP4 and VEGFA for 5 days; this led to mesoderm specification attested by the appearance of CD34+VEGFR2+ cells (Fig 1B); step 2) hematopoietic differentiation in the presence of SCF, FLT3-L, TPO and IL-3 from day 5 to day 21, which resulted in the emergence of CD34+CD43+ cells (Fig 1B), a population of embryonic hematopoietic progenitors. Hematopoietic cells were detected in EB and floating cells emerging from EB and included cells expressing monocytic lineage markers, CD45, CD14 (Fig 1B) and CD115 (M-CSFR, shown subsequently) as early as day 14; step 3) expansion and differentiation of the monocyte/macrophage lineage by sorting CD14+ cells between day 14 and day 21, then culturing these cells in the presence of M-CSF, GM-CSF, FLT3-L and IL-3, which efficiently supported the development of adherent cells with typical macrophage morphology (Fig 1A, B).

Using a methylcellulose-based colony-forming assay, we detected the emergence, at day 8, of primitive erythroid colonies comprising 6-20 cells (Ery-P) and primitive macrophage colonies (Macro-P) similar to those described in mouse hematopoiesis and ex vivo human hemangioblast culture (Fig 1C, left panel). At day 15, hematopoietic activity was mainly granulocyte and macrophage progenitors (CFU-G, CFU-GM, CFU-M) with only rare erythroid (BFU-E) and mixed (CFU-GEMM) progenitors (Fig 1C, left)
and middle panel). The production of macrophage progenitors peaked at day 21 (Fig 1C, right panel). Cytological examination revealed the presence of mature macrophages in CFU-M, CFU-GM and CFU-GEMM derived colonies (Fig 1C, left panel). These data suggested two waves in the in vitro development of embryonic monocyte/macrophage lineage. The second wave generated a progenitor hierarchy close to that observed during fetal or adult hematopoiesis. The proliferative potential of monocytic cells issued from these embryonic progenitors is shown in Fig 1D.

To dissect more accurately the maturation of embryonic monocytes and macrophages, CD14⁺ cells from step 2 were sorted, cultured, harvested at different times and stained with May-Grunwald Giemsa. After one week in culture, we observed a large spectrum of cells belonging to the monocyte/macrophage lineage (Fig 1D), ranging from monoblasts (top panel), to mature macrophages (bottom panel), with the presence of monocytes (middle panel). Later on, the cultures contained mainly macrophages, which were negative for myeloperoxydase (MPO) and positive for nonspecific esterase (NSE) (Supplemental Fig1A). Electron microscopy showed typical features of macrophages with cytoplasmic pseudopodia, highly developed vacuoles and rough dark granules of various densities (Supplemental Fig 1B). Altogether, this experimental approach enabled the efficient generation of hESC-derived monocytes and macrophages (subsequently named monocytic cells) through the main stages of adult monopoiesis.

**CD16 expression identifies two populations of CD14⁺ hESC-derived cells.**

CD14 and CD16 (FcγR-III) are used to distinguish 2 subsets of monocytes with distinct functional properties in human adult peripheral blood.¹ ³ ²⁸ The major subset called classical monocytes expresses CD14 but lacks CD16 (CD14<sup>high</sup>CD16⁻) whereas a minor
subset that expresses CD16 includes at least two populations with distinct functions, CD14^{high}CD16^{+} and CD14^{low}CD16^{+} cells. Macrophages usually express both CD14 and CD16, whatever their function. Analysis of CD14 and CD16 expression at the surface of hESC-derived cells identified a major population of CD14^{high}CD16^{+} cells and a minor population of CD14^{low}CD16^{−} cells (Fig 2A, top panel). These two subsets demonstrated distinct size and granularity, with CD14^{high}CD16^{+} cells showing scatter properties similar to those of macrophages (Fig 2A, top panel). This pattern of CD14 and CD16 expression was quite different from that reported for adult monocytes and macrophages. To determine whether this differential pattern was related to the ontogenic stage or to the experimental procedure, we cultured fetal liver and adult peripheral blood CD34^{+} cells according to the step 3 of the culture protocol used for hESC. Culture of fetal liver CD34^{+} cells for 10 days in the presence of M-CSF, GM-CSF, IL-3 and FLT3-L generated the same pattern of CD14 and CD16 expression than hESC-derived cells (Fig 2A, middle panel) whereas adult CD34^{+} cells gave rise to CD14^{+}CD16^{−} cells with a large spectrum of CD14 expression (Fig 2A, bottom panel), including a majority of CD14^{high}CD16^{−} cells with low scatter properties, similar to peripheral blood classical monocytes. These results suggested that mononuclear phagocytes generated in embryo and fetus might differ from those generated in adults.

We then sorted CD14^{low}CD16^{−} cells and cultured these cells in the presence of IL-3 or GM-CSF or M-CSF. CD14^{low}CD16^{−} cells derived from hESC and fetal liver CD34^{+} cells rapidly formed groups of 4–10 round proliferating cells that became larger clusters (10–50 cells) and semi-adherent colonies (more than 50 cells) after 7-10 days of culture (Supplemental Fig 2A). At day 9, cells with morphological characteristics of macrophages and a unique CD14^{high}CD16^{+} phenotype were observed, whatever the
cytokine conditions (Fig 2B, top and middle panel). In contrast, sorted CD14\textsuperscript{low}CD16\textsuperscript{-} cells derived from adult CD34\textsuperscript{+} cells gave rise to CD14\textsuperscript{high}CD16\textsuperscript{-} cells that subsequently differentiated towards CD14\textsuperscript{high}CD16\textsuperscript{+} cells (Fig 2B, bottom panel). We subsequently focused on the phenotypic, molecular and functional characterization of the embryonic and fetal CD14\textsuperscript{high}CD16\textsuperscript{+} subset.

**hESC- and fetal liver-derived monocytic cells demonstrate a phenotype of non-classical monocyte.**

Additional immunophenotyping of the embryonic and fetal CD14\textsuperscript{high} CD16\textsuperscript{+} subset revealed the expression of the scavenger receptors CD36 and CD163, Fc receptors CD32 (Fc\gamma RII) and CD64 (Fc\gamma RI). These cells also expressed CD15, CD33 and the cytokine receptors CD115 (M-CSFR) and CD116 (GM-CSFR). The majority of the cells expressed CCR5 and CX3CR1, but only marginal amounts of CD62L (L-selectin) and CCR2 (Fig 3, top panel). Monocytic cells generated from adult CD34\textsuperscript{+} cells in the same conditions expressed more CCR2 and CCR5 and less CX3CR1 as compared to embryonic and fetal CD14\textsuperscript{high} CD16\textsuperscript{+} (Fig 3, bottom panel). Expression of CX3CR1, CD115 and CD135 (FLT3) has been also described at the surface of the mouse macrophage-dendritic precursor \textsuperscript{31}; we confirmed that hESC- and fetal liver-derived monocytic cells gave rise to dendritic cells after 8-10 days in the presence of GM-CSF and IL-4 (Supplemental Fig 2 B, C). These results enforced the previous suggestion that embryonic and fetal monocytic cells were developmentally close and distinct from adult cells. We thus determined the functional state of this population and this was performed in reference with the subset of peripheral blood monocytes expressing the same CD14\textsuperscript{+}16\textsuperscript{+} phenotype.
Cytokine expression in hESC- and fetal liver-derived monocytic cells evokes anti-inflammatory cells.

Cytokine production is the hallmark of the M1 versus M2 polarization paradigm in macrophages. hESC- and fetal liver-derived CD14^{high}CD16^{+} cells demonstrated a similar pattern of immune and anti-inflammatory cytokine mRNA expression (Supplementary Table 1). In both cases, IL-10 mRNA expression was higher than IL12α and IL12β mRNA, which suggested a M2 anti-inflammatory state (Fig 4A). These data were confirmed at the level of secreted proteins (Fig 4B). In contrast, CD14^{+}CD16^{+} adult monocytes demonstrated a similar production of IL-10 and IL-12. Treatment of hESC and fetal-derived monocytic cells with IFNγ and LPS stimulated the production of pro-inflammatory cytokines. This production remained ~10-fold lower than their adult counterpart with a significant differences in IL-12, IL-6 and TNFα secretion (p<0.05) (Fig 4C). Overall, levels of secreted cytokines in embryonic and fetal monocytic cells remained low when compared to adult CD14^{+}CD16^{+} monocytes, except for TGFβ1 whose level was respectively 7- and 2.5-fold increased in the supernatants of embryonic and fetal cells (Fig 4D). These results suggested that hESC and fetal liver-derived monocytic cells might have anti-inflammatory and immunosuppressive properties as well as tissue remodeling activity.

Monocytic cells derived from hESC and fetal liver have a gene signature of tissue remodeling activity.

We used qRT-PCR to compare the expression of 48 genes whose product is involved in tissue remodeling, angiogenesis, immune response, scavenging and chemotaxis in
CD14<sup>+</sup>CD16<sup>+</sup> cells derived from hESC, those derived from fetal liver CD34<sup>+</sup> cells or those isolated from adult peripheral blood. The expression of these genes was similar in hESC and fetal liver-derived monocytic cells in comparison with adult CD14<sup>+</sup>CD16<sup>+</sup> peripheral blood monocytes (Fig 5A). By pair-wise comparisons using \( t \) test, we observed a significant increase in the expression of genes encoding chemokines (CCL22, CCL17, CCL13; \( P = 0.01 \)) related to a M2 polarization state and a decrease in the expression of the gene encoding the Th1 chemokine CXCL9 (\( P = 0.05 \)) in hESC or fetal liver-derived cells compared to sorted adult cells (Fig 5 A, B). hESC- and fetal liver-derived cells exhibited several fold up-regulation of genes encoding tissue degrading enzymes such as metalloelastases and metalloproteases, including MMP2, MMP12 and MMP1 (\( p = 0.01 \)) and MMP7 and MMP9, in comparison to adult monocytes (Fig 5 A, B). hESC- and fetal liver-derived cells also expressed high levels of scavenger receptor encoding genes MSR1 (macrophage scavenger receptor 1) and STABILIN-1, while the class A scavenger receptor gene SCARA5 was not detected. Compared to adult subset, they expressed high levels of the phagocytic mannose receptor gene MRC1, MARCO or the hyaluronan receptor-1 gene LYVE-1 that are characteristic markers of M2 macrophages (supplementary data, Table S2). This transcriptional pattern indicated that hESC and fetal liver-derived monocytic cells have characteristic of M2 polarized macrophages known to exert a wide range of homeostatic functions.

**Monocytic cells derived from hESC and fetal liver express tissue remodelling proteins.**

We used a protein array to explore the expression of 55 proteins involved in angiogenesis, vascular morphogenesis and tissue remodeling in hESC- and fetal liver-
derived monocytic cells compared to sorted adult CD14^+CD16^+ cells. Embryonic macrophages extensively secreted proteins acting on extracellular matrix and tissue remodeling such as TIMP-1, MMP-8, and Serpins (Fig 6A). Analysis of secreted protein using the Luminex technology corroborated these data and identified an impressive secretion of MMP-12 several folds higher than in adult cultures, in agreement with transcriptional studies ($P = 0.04$) (Fig 6B). Surprisingly, the secretion pattern of the fetal subset was closer to adult than embryonic macrophages (Fig 6A and Supplementary data; table S3). Proteins acting specifically on angiogenesis, including VEGF, angiopoietin, thrombospondin (TSP), urokinase plasminogen activator (uPA) and pentraxin 3, were secreted at a lower level (Fig 6B), but embryonic macrophages still secreted a larger amount of angiogenic proteins when compared to adult blood CD14^{low}CD16^+ monocytes that include ~70% of Tie-2-expressing monocytes (TEMs) known for their pro-angiogenic activity $^{19}$. Proteins significantly increased in hES-derived cells in comparison to fetal and adult cells are indicated in Fig 6B. We confirmed that the specific profile of embryonic and fetal monocytic cells was not related to culture conditions by comparison with adult CD34^+ cells derived-monocytic cell, obtained in the same culture conditions (n=3) (Supplementary data, Fig S3).

Embryonic and fetal CD14^{high} CD16^+ cells demonstrated phagocytic functions that can contribute to their remodeling activity. They showed ability to ingest apoptotic cells, including embryonic erythrocytes and granulocytes (Supplemental Fig 4A, top left panel) and *Escherichia coli* at 37 °C (Supplemental Fig 4A, top right panel). Phagocytosis of IgG-opsonized E. Coli induced actin polymerization and the formation of a phagocytic cup in hESC-derived macrophages (Fig 7A, bottom panel) and phagocytosis of
opsonized zymozan particles triggered an oxidative burst as measured by chemiluminescence (Supplemental Fig 4B).

Monocytes/macrophages derived from hESC and fetal liver promote tissue remodeling

Tie-2 angiopoietin receptor is expressed on a subset of CD14+CD16+ adult peripheral blood monocytes and Tie-2 expressing cells are required for the vascularization and growth of several tumor models. Tie-2 was detected at the surface of ~70% of embryonic and virtually all fetal monocytes/macrophages (Fig 7A). Sorted hESC- and fetal liver-derived CD14^{high}CD16+ cells were co-injected subcutaneously with human U87 glioma cells in nude mice, a xenogenic model used for the demonstration of the proangiogenic effect of adult TEMs. U87 cells alone or co-injected with adult CD14+CD16+ monocytes were used as controls. Developing CD34+ blood vessels were studied in tumors by immunochemistry at day 5-7. Computer-assisted image analysis showed that the overall vascular area was not significantly greater in tumors produced by co-injection of U87 cells with embryonic or fetal macrophages than in controls (Fig 7B). However, despite the early times of tumor growth (5-7 days), we observed a profuse vascular framework both in control and co-injected tumors (Supplemental Fig 4). In an independent set of experiments, we observed, using cytometric analysis, that embryonic, fetal and adult cells were still present in the tumors on days of analysis, but at a significant larger extent for adult CD14+CD16+ monocytes (p<0.05) (Fig 7C). Additional detailed morphological analysis showed that embryonic and fetal/macrophages promoted the development of large blood vessels with wide lumens, whereas CD14+CD16+ adult
monocytes induced a framework of small capillaries (Fig 7D). Thus, embryonic and fetal monocytes/macrophages promote vascular morphogenesis and tissue remodeling.

The specific pattern of CD14 and CD16 expression, inflammatory cytokines and remodelling proteins was confirmed from monocytic cells issued from the H1 ES cell line (WiCell Research Institute; Madison, WI). (Supplementary data, Fig S5 )
DISCUSSION

Ontogeny of monocyte/macrophage development has been investigated mainly in mice. In contrast, the establishment of MPS in human remains poorly understood. The present study used hESC and fetal liver cell differentiation models to gain insights in the ontogeny of monocyte/macrophage in human. Using these two independent experimental approaches, we demonstrate that both hESC- and fetal liver-derived monocytes/macrophages arise through a similar developmental process and are specialized for specific homeostatic functions.

Monocytes/macrophages have been previously derived from hESCs by two approaches. One used hESC differentiation into hematopoietic cells on OP9 cells and led to the identification of a GM progenitor that differentiated into monocytes/macrophages. The second one used EB formation followed by monocyte/macrophage differentiation in the presence of M-CSF and IL-3. Here, we set up a stepwise culture method with defined combinations of cytokines, which allowed to generate functional mature monocytes and macrophages. Of interest, we identified two sequential waves of embryonic monopoiesis temporally linked with the emergence of primitive and definitive erythroid colonies suggesting that this experimental protocol reproduces the main stages of embryonic hematopoiesis. Clonogenic assays and cytologic examination confirmed the emergence of monocytes/macrophages from hESC through steps that included the sequential formation of mixed progenitors, monocyte precursors, mature monocytes and macrophages as described for bone marrow monopoiesis. To monitor this differentiation in details, we combined CD14 and CD16 markers, which discriminate the 2 major types of monocytes, CD14⁺CD16⁻ and CD14⁺CD16⁺ monocytes, in adult peripheral blood.
These cells have been shown to exhibit distinct phenotypes and functions although the CD14⁺CD16⁺ cell population seems quite heterogeneous. In addition, CD16 expression appears at the surface of CD14⁺CD16⁻ adult peripheral blood monocytes when induced to differentiate into macrophages upon M-CSF exposure (personal results). In hESC and fetal liver-derived cultures, these two markers identified 2 populations either CD14⁺CD16⁻ or CD14⁺CD16⁺. The first population clearly consisted of precursors capable to proliferate and to give rise to the second population in the presence of M-CSF, GM-CSF, IL-3 or a cocktail of cytokines. The CD14⁺CD16⁺ cells formed a homogenous population of monocytes and macrophages that did not proliferate. Interestingly, whatever the cytokine used and the time in culture, a CD14⁺CD16⁻ cell population was not observed. In contrast, in adult blood, the CD14⁺CD16⁻ monocytes represent the major blood population while the CD14⁺CD16⁺ subset accounts for only 10% of all adult monocytes. The difference in phenotype between embryonic/fetal and adult blood monocytes could reflect developmental changes in the monocyte/macrophage lineage. In order to answer this question, we compared the differentiation pattern of CD34⁺ cells from human fetal liver and adult blood into the monocyte/macrophage lineage using a similar cocktail of hematopoietic cytokines. This approach demonstrated that monocyte/macrophage differentiation from hESC and fetal liver was extremely close and occurred from a CD14⁺CD16⁻ progenitor in contrast to adult cultures where macrophage differentiation occurred through a CD14⁺CD16⁻ differentiation step. In addition, we confirmed results obtained on mouse embryonic monopoiesis that demonstrated the redundancy of M-CSF, GM-CSF and IL-3. In mice, a bone marrow progenitor with a M-CSFR⁺ CXCR3⁺ Flt3⁺ phenotype, termed MDP, give rise to several monocyte/macrophage subsets and dendritic cells. The hESC-derived CD14⁺CD16⁻
cells also expressed the CD115 (M-CSFR), FLT3 and CXCR3 gave rise to both dendritic cells and macrophages, and thus could be the equivalent of the MDP murine progenitor. This assumption will require further experiments performed at the unicellular level to be confirmed.

Our findings underscore the presence of a developmental regulation in human monocyte/macrophage differentiation. Mouse embryonic macrophages are specialized in phagocytosis, tissue remodeling and angiogenesis. Here, we show that the CD14+CD16+ embryonic and fetal monocytes/macrophages demonstrated a CD62L−, CCR2−, CCR5+, CX3CR1+ phenotype that overlapped with that of adult non-classical monocytes. Additionally, they exhibited a positive ratio between anti-inflammatory and pro-inflammatory cytokines suggesting M2 polarization and responded poorly to interferon γ and LPS. Our finding that embryonic/fetal macrophages secrete higher amount of TGF-β1 suggest a possible implication of additional paracrine/autocrine mechanisms that control the polarization of immune cells contributing to the maternal-fetal tolerance during embryonic development.

M2 markers identified in embryonic and fetal monocytes/macrophages include IGF1, LYVE1, MRC1, STAB1 and MMP12, which are expressed on their murine counterparts and on monocytes expressing the angiopoietin receptor Tie2 (TEMs) and human tumor-associated macrophages (TAMs). These latter cells promote tumor development by providing growth factors and remodeling factors that facilitate invasion and metastasis. The cytokines secreted at the highest level by the embryonic subset were either those regulating the degradation of extracellular matrix, like MMPs, Serpins and TIMPs or pleiotropic cytokines, like IGFs, NRG1,TGF-b known to act at the different stages of morphogenesis in mouse embryo. The impressive production of a wide range of
metalloproteases suggests that embryonic and fetal macrophages may have specific trophic functions to regulate tissue architecture and cell migration\textsuperscript{40-41}; human ES and fetal liver-derived macrophages particularly expressed a high level of MMP12, a differential marker between embryonic TEM and other embryonic macrophages\textsuperscript{17}. In addition hESC-derived monocytes/macrophages synthesized a large pattern of factors involved in angiogenesis that could even be up regulated in a hypoxic embryonic environment. One of the characteristics of adult TEMs is to promote angiogenesis in xenograft transplant\textsuperscript{19}. In a similar assay, we could not demonstrate a significant pro-angiogenic activity using our fetal liver and hES derived “TEMs”. This negative result may have three explanations: - the assay used, as in our hand a profuse angiogenesis was observed in the control group, - the exit of embryonic macrophages from the tumor or - their apoptosis after cytokine starvation. Nevertheless embryonic cells promoted the enlargement of the tumor vessels lumen, which might be related to MMP secretion\textsuperscript{38-39}. Similarly to adult macrophages, embryonic monocytes exhibited phagocytosis functions required for development\textsuperscript{42}.

It remains to determine whether monocyte/macrophage subsets and evolution with time reflect the plasticity of a unique cell population or the presence of distinct precursors. According to the first hypothesis, plasticity could be generated by epigenetic controls restricting monocyte/macrophage functions to tissue remodeling in the embryo and the fetus, as recapitulated by TAM, and widening monocyte/macrophage functions to inflammation in adults. According to the second hypothesis, distinct precursors would arise sequentially during development with a first wave being more specialized in M2 functions. Thus monocyte/macrophage differentiation from hESC might be a powerful tool to precisely understand the developmental mechanisms of the monocyte/macrophage
heterogeneity. It might thus also offer a valuable experimental model for *in vitro* studies of mechanisms sustaining tumor development.
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Author’s contribution statement:

OK: designed and performed experiments, analyzed data and wrote the paper

A.D. S.: performed culture experiments

S.H.: performed culture experiments

B.G., P.O.: performed xenograft model experiments

T.R.: performed qPCR experiments

M.R.: technical support

J.E.B.: performed phagocytosis experiments

A.L.D. provided fetus samples

B.L.: performed protein analysis experiments

S.B.: performed electron microscopy cell analysis

E.S.: discussed results

W.V.: supervised the research and wrote the paper

F. N.: concepted and designed experiments, supervised the research and wrote the paper

No conflict of interest in relation to the submission has to be declared.
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Figure Legends

Figure 1: Differentiation of human embryonic stem cells (hESC) into monocytes/macrophages. A) Description of the 3 step protocol designed to induce hESC differentiation into monocyte/macrophages. Step 1: Embryoid bodies (EB) formation with mesoderm specification in the presence of BMP4 and VEGFA. Step 2: hematopoiesis induction in the presence of SCF, TPO, FLT3-L and IL-3. Step 3: culture of CD14+ cells sorted between day 14 and day 21 in the presence of M-CSF, GM-CSF, FLT3-L and IL-3. B) hESC and derived cells were characterized by cell surface marker analysis using flow cytometry at indicated times of the culture. C) Methylcellulose-based colony-forming assay: (➡) typical primitive erythroid (Ery-P) and macrophage (Macro-P) colonies and definitive (BFU-E. CFU-G. CFU-GM. CFU-M. CFU-GEMM) colonies. Original magnification 20 X (Ery-P and Macro-P) and 10 X (definitive colonies). Cells were assessed for their ability to form erythroid and myeloid colonies in a standard methylcellulose assay at indicated times of the culture; C middle panel: percentages of indicated colonies; C right panel: absolute numbers of CFU-GM + CFU-M per 10^5 total EB cells. Mean +/- SD of 3 independent time course experiments with methylcellulose assays performed in triplicate and colonies enumerated at day 9-10. D) Proliferative potential of monocytic cells; the percent of CD14+ cells was measured at different time of the culture and related to the total number of cells, including adherent, floating and part from embryoid bodies cells. E) CD14+ cells from step 2 were sorted, cultured, harvested at different times and stained with May-Grunwald Giemsa. Typical morphological features of monoblasts (top panel), monocytes (middle panel) and macrophages (bottom panel). Pictures were taken with an X63 objective.
Figure 2. Differential expression of CD14 and CD16 allows the characterization of two subsets of hESC and fetal-derived CD14⁺ cells.

A) CD14 and CD16 expression on cells issued from day 21 hESC (top panel), day 10 fetal liver CD34⁺ cells (middle panel) and day 10 adult peripheral blood CD34⁺ cells (bottom panel). hESC were cultured as described in figure 1, fetal liver and adult CD34⁺ cells were culture in the presence of M-CSF(50 ng/ml), GM-CSF (20 ng/ml), IL-3 (100 U/ml) and FLT3-L (10 ng/ml). Blue: CD14^{high}CD16⁺ cells; purple: CD14^{low}CD16⁻ cells. Right panel: forward (FSC) and size (SSC) scatter properties of CD14⁺ cells (right panel).

B) Embryonic, fetal and adult CD14^{low}CD16⁻ cells were sorted and cultured in the presence of IL-3 or GM-CSF or M-CSF for 9 days before assessing cell surface expression of CD14 and CD16.

Figure 3: hESC and fetal liver-derived CD14⁺CD16⁺ cells demonstrate a non-classical monocyte phenotype.

Immunophenotype analysis of hESC (top left panel), CD34⁺ fetal liver (top right panel)-derived and CD34⁺ adult (bottom panel)-derived CD14⁺CD16⁺ cells. Percentages of cells expressing indicated markers within the CD14⁺ cells are shown. The majority of embryonic and fetal subsets expressed CCR5 and CX3CR1. When monocytic cells were generated from adult CD34⁺ cells, a marker of the inflammatory type, CCR2, was detected on a subset of cells. Gating was set according to the negative isotype controls. One of at least 3 representative experiments is shown. The corresponding cell frequencies as determined in three independent experiments are presented.
Figure 4. hESC and fetal liver-derived monocytic cells have a pattern of cytokine expression characteristic of an anti-inflammatory state

A) Gene expression profile of sorted CD14<sup>+</sup>CD16<sup>+</sup> from hESC (n=6) and fetal (n=4) CD34<sup>+</sup> cell culture. qPCR analysis shows the expression level of relevant genes of M1 and M2 polarization paradigm. Results were expressed as 1/ΔCT values (mean ± SEM) over endogenous controls 18S and Gapdh. B, C) Cytokine production was measured in supernatant from 3x10<sup>5</sup> hESC and fetal sorted CD14<sup>+</sup>CD16<sup>+</sup> cells, comparatively assessed with CD14<sup>+</sup>CD16<sup>+</sup> peripheral blood monocytes (n=4, for each population). Tested cells were either left untreated (basal) (B) or were stimulated with LPS and IFN-γ for 24 hours (C); resulted supernatants were subsequently analyzed using a Luminex assay. Results are expressed in pg/mL. Note that the response to LPS and IFN-γ in terms of inflammatory cytokine production is decreased from embryonic and fetal cells in comparison to adult. (D) Comparative analysis of TGF-β secretion from hESC, fetal-derived and adult peripheral blood CD14<sup>+</sup>CD16<sup>+</sup> cells. Note that in contrast with other cytokines, TGF-β was over secreted by embryonic and fetal cells in comparison to adult. Results are expressed in pg/mL. †indicates that the cytokine level was over the maximal range of the assay. Statistical differences between groups were calculated by pairwise t test. * p < 0.05 between the expression and production of some pro-inflammatory cytokines after stimulation with IFNγ and LPS by adult versus hESC and fetal-derived cells.
Figure 5. hESC and fetal liver-derived monocytic cells share a distinguishing signature of tissue remodeling activity

hESC and fetal-derived CD14^+CD16^+ cells were sorted and expression of genes typical of M2 polarization were analyzed in comparison with adult blood sorted CD14^+CD16^+ cells using a customized gene array (Applied Biosystems. Foster City. CA) (n=4, for each population).  A) Heat map representation of a common M2 gene signature shared by hESC and fetal -derived monocyte/macrophages.  B) The relative mRNA expression (delta CT) for receptor and chemokines tested is indicated. Expression of genes was normalized over endogenous controls 18S and Gapdh; higher the delta CT, lower the gene expression. C) The expression of metalloproteases in hESC and fetal cells is shown in terms of fold amplification versus expression in adult cell. Embryonic and fetal macrophages exhibited several fold up-regulation of a number of metalloproteases (MMP2, MMP7, MMP9, MMP12 and MMP14) *Increased expression of genes encoding metalloproteases and anti-inflammatory chemokines in hES-derived cells in comparison with adult cells by pairwise comparisons using t test of genes (p=0.01). A decreased expression of the Th1 chemokine CXCL9 was found in embryonic cells (p=0.05).

Figure 6. Functional activity of hESC and fetal liver-derived monocytic cells

A) Protein arrays analysis in culture supernatants of hESC, fetal-derived and peripheral blood CD14^+CD16^+ cells. Array signals from scanned X-ray film images were analyzed using digital image analysis software. Array images from 10 minutes exposure to X-ray film (left panel) and profiles created by quantifying the mean spot pixel densities (right panel) are shown. Top, right panel: note that the broadest pattern of expression associated
with various proteins acting on extracellular matrix and tissue remodeling was found in the CD14^{hi}CD16^{+} embryonic cells; bottom, right panel: the secretion of proteins involved in angiogenesis in the 3 populations is also shown. * Proteins significantly increased in hES-derived cells in comparison to fetal and adult cells using pairwise comparisons with t test (p<0.05). ** Proteins significantly increased in hES-derived cells in comparison to fetal and adult cells using pairwise comparisons with t test (p<0.05). B) Quantification of MMPs secretion by Luminex technology. Note the secretion of large amounts of MMP-12 in embryonic cells in comparison to fetal and adult cells (**; p=0.04). Results are expressed in pg/mL.

Figure 7. Angiogenic phenotype and function of hESC and fetal liver-derived CD14^{+}CD16^{+} monocytic cells.

A) Tie2 expression analysis. hESC and fetal-derived CD14^{+}CD16^{+} cells were co-stained with human Tie2 antibody and expression was studied by cytometric analysis; plots from one representative experience are shown; percent of Tie2 expressing cells are mean values of 3 independent experiences. Tie-2 was expressed by the majority of embryonic and virtually by all fetal monocytes/macrophages. B) Human glioma U87 cells were injected subcutaneously into nude mice alone (control) or with hESC and fetal CD14^{+}CD16^{+} cells (n=6. for each group). Developing CD34^{+} blood vessels were studied by immunochemistry at day 5-7. The vascular area was calculated by digital image analysis based on the quantification of mouse CD34-labeled vessels. Error bars indicate SD. Computer-assisted image analysis showed that the overall vascular area was not significantly greater in tumors co-injected with embryonic/fetal macrophages than in control tumors. C) Detection of human cells by flow cytometric analysis. After administration of human glioma U87 cells into nude mice alone (control) or with hESC-
derived and adult CD14^+CD16^+ cells (n=6, for each group). Tumors at day 7 were
dissociated into single cell suspensions and labeled with mouse CD31-PE and human
CD45-APC. One representative experiment is shown. Results are mean values of the
percent of human CD45^+ cells present in the tumors on days of analysis (n=6, for each
group). *p < 0.05: statistical difference between the residual human CD45^+ cells on days
of analysis after administration of adult and hESC-derived CD14^+CD16^+ cells. D) Morphology of vessel sections in tumors coinjected with adult (left panel) or hESC
CD14^+CD16^+ (middle and right) cells. Magnification 200 X. Note the larger vessel lumen
in case of embryonic cell co-administration (➡).
Klimchenko O et al, Figure 3

hESC-derived cells

Fetal liver CD34⁺-derived cells

Adult CD34⁺-derived cells
Klimchenko O et al. Figure 5

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MMP2*  MMP7*  MMP9*  MMP12*  MMP14*
Klimchenko O et al, Figure 6

A.

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B.

** Pixel density

Cytokine secretion

- hESC
- Fetal
- Adult

- VEGF
- MMP 9
- MMP 12
- MMP 7
Klimchenko O et al, Figure 7

A. hESC and Fetal scatter plots with CD14 and Tie 2 expression.

B. Flow cytometry graphs showing CD31 and CD45 expression for U87, U87 + adult CD14+C16+, and U87 + hESC derived CD14+C16+.

D. Microscopy images showing overall microvessel density for U87, U87 + hESC derived CD14+C16+, and U87 + fetal derived CD14+C16+.
Monocytic cells derived from human embryonic stem cells and fetal liver share common differentiation pathways and homeostatic functions