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Rapid and Efficient Production of Human Functional Mast Cells through a Three-Dimensional Culture of Adipose Tissue–Derived Stromal Vascular Cells

Jenny Paupert,*,† Eric Espinosa,†,‡ Nicolas Cenac,§ Virginie Robert,*,† Patrick Laharrague,*,† Solène M. Evrard,¶,‖ Louis Casteilla,*,† Anne Lorsignol,*,† and Béatrice Cousin*,†

Mast cells (MC) are innate immune cells involved in many physiological and pathological processes. However, studies of MC function and biology are hampered by the difficulties to obtain human primary MC. To solve this problem, we established a new method to produce easily and rapidly high numbers of MC for in vitro studies using human adipose tissue, which is an abundant and easy access tissue. Stromal vascular fraction of adipose tissue, obtained from human abdominal dermolipectomy, was cultured as spheroids in serum free medium supplemented in stem cell factor. Using this method, we generated, within 3 wk, a highly pure population of connective tissue–type MC expressing MC typical peptidases (tryptase, chymase, and carboxypeptidase-A3) with a yield increasing over time. Stem cell factor was required for this culture, but unlike MC derived from CD34$^+$ cells, this culture did not depend on IL-3 and -6. MC obtained with this method degranulated following FcεRI cross-linking or stimulation by C5a, compound 48/80, and substance P. Interestingly, activation by anti-IgE of both white adipose tissue–MC and MC obtained from peripheral blood–derived CD34$^+$ pluripotent progenitor cells induced the production of PGs as well as proinflammatory cytokines (TNF-α, Il-6, and GM-CSF). In conclusion, we developed a new time saving and reproducible method to produce highly pure and functional human MC in 3 wk from human adipose tissue. ***The Journal of Immunology*****, 2018, 201: 3815–3821.**

Mast cells (MC) are immune cells located in virtually all organs at strategic locations close to host–environment interfaces, such as skin, vascular and mucosal barriers, or nerve endings (1). MC are easily distinguishable from other cells by their high content of basophilic and electronic dense secretory granules (2) containing a huge array of molecules such as histamine, serotonin, cytokines, or serine proteases. Besides these preformed mediators, MC also release de novo synthetized cytokines, chemokines, and eicosanoids. Via these mediators, MC are involved in incalculable biological processes such as allergy, vascular permeability control, pain, or tumor progression (2–6).

However, studies of MC biology are limited because of lack of reproducible in vitro models, especially in humans. Indeed, MC cell lines such as HMC-1, LUVA or LAD-2 do not behave like in vivo MC (7–9). HMC-1 cells do not possess the IgE receptor (FCεRI) (9), and immortalized LUVA cells do not respond to stem cell factor (SCF), which is required for the normal development of MC in vitro (8). To date, most in vitro studies involving MC are based on CD34$^+$ cells isolated from the bone marrow, cord blood, or peripheral blood and differentiated into functional MC (10). However, this method is a long lasting process that usually requires ∼10 wk to obtain pure mature MC. Primary human MC can also be obtained via their direct isolation from tissues such as skin (11). However, this method is limited by the scarcity of mature MC and the difficulties in obtaining fresh human tissues. The development of in vitro models that produce easily and readily high numbers of mature MC is therefore required for a better understanding of MC functions in health and diseases.

White adipose tissue (WAT) is an easy access tissue which is present throughout the human body and represents around 20% of the body weight in healthy humans. We and others have previously shown that in mice and in humans, WAT contains progenitors and/or mature MC (12, 13). MC accumulation has been described in the WAT in obesity and food allergy, but their roles are still debated (13–16).

In this study, we present an efficient method to produce mature and functional human connective tissue–type MC (MC_{TC}) within

*STROMALab, Université de Toulouse, CNRS 11 Équipe de Recherche Labellisée 5311, Établissement Français du Sang, École Nationale Vétérinaire de Toulouse, INSERM U1031, Université Paul Sabatier, 31100 Toulouse, France; †Université Toulouse III–Université Paul Sabatier, F-31062 Toulouse; ‡INSERM, U1037, Centre de Recherche en Cancérologie de Toulouse, F-31037 Toulouse, France; §Institut de Recherche en Santé Digestive, Université de Toulouse, INSERM, École Nationale Vétérinaire de Toulouse, Université Paul Sabatier, 31062 Toulouse, France; ¶Département d'Histologie et d'Embryologie, École de Médecine de Rangueil, Université Toulouse III–Université Paul Sabatier, 31059 Toulouse Cedex 9, France; and ‖Département de Pathologie et Cytologie, Centre Hospitalier Universitaire Toulouse, l'Institut Universitaire du Cancer de Toulouse Oncopole, 31100 Toulouse Cedex 9, France

ORCIDs: 0000-0001-9550-9296 (J.P.); 0000-0002-3512-0921 (E.E.); 0000-0002-1552-7812 (N.C.); 0000-0001-9998-6890 (V.R.); 0000-0002-1194-3456 (P.L.); 0000-0001-9669-8316 (S.M.E.).

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Address correspondence and reprint requests to Dr. Béatrice Cousin, STROMALab, Université de Toulouse, CNRS 11 Équipe de Recherche Labellisée 5311, Établissement Français du Sang, École Nationale Vétérinaire de Toulouse, INSERM U1031, Université Paul Sabatier, 4 Bis Avenue Hubert Curien, 31100 Toulouse, France. E-mail address: beatrice.cousin@inserm.fr

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Abbreviations used in this article: hSCF, human SCF; LOQ, limit of quantification; MC, mast cell; MC_T, mucosal-type MC; MC_{TC}, connective tissue–type MC; SCF, stem cell factor; SP, substance P; SVF, stromal vascular fraction; WAT, white adipose tissue.

3 wk from the stromal vascular fraction (SVF) of WAT. This method produces a homogeneous MC population expressing typical MC peptidases (tryptase, chymase, and carboxypeptidase A3) with good yield and high purity. These WAT-MC are able to degranulate upon FcεRI aggregation or following stimulation with C5a, substance P (SP), or compound 48/80. WAT-MC exhibit the same phenotype than MC derived from peripheral blood $CD34^+$ cells, and both release, upon activation by anti-IgE, identical eicosanoids and proinflammatory cytokines (TNF-α, IL-6, GM-CSF).

Materials and Methods

Abs

Abs used are listed in Supplemental Table I.

Isolation of the SVF

Twenty-four patients were used in this study. After informed consent obtained from the patients, s.c. WAT was obtained from the Department of Plastic Surgery at Rangueil University Toulouse Hospital and processed rapidly after surgery. This study was approved by the Institutional Review Board, Rangueil University Toulouse Hospital, and was performed in accordance with the principles of the Declaration of Helsinki (1964) and in agreement with French bioethics laws of July 7th, 2011. SVF was obtained as previously described (17). Briefly, 10 to 50 g of WAT was digested with collagenase A (Serva Electrophoresis, Heidelberg, Germany) under agitation for 45 min at 37°C. The cell suspension was filtered through a 100-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ) and centrifuged 10 min at 600 × *g*. The pellet was incubated in hemolysis buffer (155 mM NH_4Cl and 20 mM Tris, pH 7.6) to eliminate RBCs. Cells of the SVF were then centrifuged 10 min at 600 × *g* and counted, and their viability was assessed by trypan blue exclusion.

Cell culture

SVF cells were plated in StemSpan SFEM II (STEMCELL Technologies, Vancouver, Canada) supplemented with penicillin 100 μg/ml, streptomycin 100 μg/ml, amphotericin 0.25 μg/ml (Life Technologies BRL, Grand Island, NY), 100 ng/ml human SCF (hSCF) (PeproTech, Rocky Hill, NJ), and 2% methylcellulose (Sigma-Aldrich, Saint-Louis, MO) (18), at 1.7 × 10^5 cells per well in ultra-low attachment 96-well plates (Corning Costar, Corning, NY). One week later, spheroids were discarded and nonaggregated cells were kept in culture. Half of the medium was changed once a week.

To test the combination of SCF, IL-3, and IL-6 on MC growth and purity, human SVF cells were cultured after isolation in medium containing 100 ng/ml hSCF and 30 ng/ml human IL-3 (PeproTech) for 1 wk and then in medium supplemented with 100 ng/ml hSCF and 100 ng/ml human IL-6 (PeproTech). To test the effect of IL-4 on FcεR1 cell surface expression, 100 ng/ml IL-4 (PeproTech) was added to the medium for 1 wk after 3 wk of culture.

MC derived from peripheral blood $CD34^+$ cells were obtained as previously described (19). Briefly, $CD34^+$ cells were isolated from buffy coats (Etablissement français du sang) and grown in StemSpan medium (STEMCELL Technologies) supplemented with recombinant human IL-6 (50 ng/ml; PeproTech), human IL-3 (10 ng/ml; PeproTech), and 3% supernatant of Chinese hamster ovary transfectants secreting murine SCF (a gift from Dr. P. Dubreuil, Marseille, France; 3% corresponds to ∼50 ng/ml SCF) for 1 wk. Cells were then grown in IMDM Glutamax I, sodium pyruvate, 2-ME, 0.5% BSA, Insulin-Transferrin-Selenium (all from Invitrogen), ciprofloxacin (10 μg/ml; Sigma-Aldrich), IL-6 (50 ng/ml), and 3% supernatant of Chinese hamster ovary transfectants secreting murine SCF.

Toluidine blue staining

A total of 5 × 10^4 cells were cytocentrifuged onto glass slides and air dried. Slides were incubated in sulfuric ether solution for 30 min, rinsed with tap water, and stained with toluidine blue (VWR, Allison Park, PA). Slides were then rinsed twice with propanol 60% for 15 s. After dehydration and air drying, slides were examined under a light microscope (DM3000; Leica).

Immunocytochemistry

A total of 5 × 10^4 cells were cytocentrifuged onto Superfrost microscope slides and air dried. Slides were fixed with formalin 10% (Sigma), washed with PBS, and then incubated with PBS containing normal horse serum 2%, Triton X-100 0.2% for 1 h at room temperature. Slides were incubated overnight in a humidified chamber at 4°C with mAb against tryptase or with biotinylated mAb against chymase and then washed with PBS–Triton X-100 0.2%. For chymase detection, cytospins were incubated with streptavidin–Alexa 488 conjugate (Invitrogen, CA) and counterstained with DAPI. For tryptase immunostaining, OptiView DAB IHC Detection Kit (Roche, Switzerland) was used and nuclei were stained with hematoxylin. Observations were performed under a light microscope (DM3000; Leica) or Nikon Eclipse TE2000-5 inverted fluorescent microscope.

Flow cytometry

Cultured cells were stained in PBS containing FcR-blocking reagent (Becton Dickinson). For intracellular detection of tryptase and chymase, cells were first permeabilized with the Cytofix/Cytoperm kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Staining was performed by direct immunofluorescence with conjugated mouse anti–human mAbs, as compared with their matched isotype control mAb. Cell viability was assessed by DAPI labeling. Cells were then washed in PBS and analyzed on a fluorescence-activated cell sorter LSR Fortessa (BD Biosciences). Data were acquired with FACSDiva software (BD Biosciences) and analyzed with Kaluza 1.5a software (Beckman Coulter, Roissy Charles de Gaulle, France).

Degranulation assay

MC degranulation was determined by measuring the release of β-hexosaminidase as previously described (20). MC were sensitized with human IgE for 16 h; the cells were then washed and distributed in 96-well flat-bottom plates at a density of 1 × 10^5 cells in 50 ml of Tyrode's buffer. The cells were adapted to 37°C for 20 min and then treated with prewarmed of the indicated stimuli: PMA plus ionomycin (respectively, 0.1 and 1 μg/ml) (Sigma-Aldrich), SP (50 μm), compound 48/80 (50 μg/ml) (Sigma), C5a (50 ng/ml) (R&D Systems, Minneapolis, MN), or anti-human IgE (Santa Cruz Biotechnology, Dallas, TX) diluted in Tyrode's buffer for 45 min at 37°C. The release of β-hexosaminidase in the supernatants was measured by means of enzymatic colorimetric analysis with p-nitrophenyl N-acetyl-b-D-glucosamine (Sigma). To measure lipid mediators and cytokines production, cells were stimulated overnight at 37°C in medium as indicated above and cell supernatants were kept for measurements.

Measurements of lipid mediators

Supernatant lipid extraction was performed by adding 5 μl of internal standard mixture (LxA_4-d5 and LTB_4-d4 at 400 ng ml^{-1} in MeOH) and 300 μl of cold methanol to 500 μl of cell supernatants. Samples were centrifuged at 3000 rpm for 15 min at 4°C. Supernatants were collected and submitted to solid-phase extraction using HRX-50 mg 96-well (Macherey Nagel, Hoerd, France) as previously described (21). Solvent was evaporated under nitrogen and samples were dissolved with MeOH.

Liquid chromatography/tandem mass spectrometry measurement was used to quantify arachidonic acid metabolites: $6kPGF_{1\alpha}$, TXB_2, PGE_2, PGD_2, 8-isoPGA_2, 11β-PGF2α, PGF2α, LxA_4, LxB_4, LTB_4, 5,6-DiHETE, 15-HETE, 12-HETE, 8-HETE, 5-HETE, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET, and 5-oxo-ETE (21). To simultaneously separate 19 lipids of interest and three deuterated internal standards, liquid chromatography/tandem mass spectrometry analysis was performed on ultra-high performance liquid chromatography system (Agilent LC1290 Infinity) coupled to Agilent 6460 Triple Quadrupole MS (Agilent Technologies) equipped with electrospray ionization operating in negative mode. Reverse-phase ultra-high performance liquid chromatography system was performed using ZorBAX SB-C18 column (Agilent Technologies) with a gradient elution. The mobile phases consisted of water, acetonitrile, and formic acid (75:25:0.1; v/v/v) (A) and acetonitrile, formic acid (100:0.1, v/v) (B). The linear gradient was as follows: 0% B at 0 min, 85% B at 8.5 min, 100% B at 9.5 min, 100% B at 10.5 min and 0% B at 12 min. The flow rate was 0.35 ml/min. The autosampler was set at 5°C and the injection volume was 5 μl. Data were acquired in multiple reaction monitoring mode with optimized conditions. Peak detection, integration, and quantitative analyses were done using MassHunter Quantitative analysis software (Agilent Technologies). For each standard, calibration curves were built using 10 solutions at concentrations ranging from 0.95 to 500 ng/ml. A linear regression with a weight factor of 1/X was applied for each compound. The limit of detection and the limit of quantification (LOQ) were determined for the 27 compounds using signal to noise ratio. The limit of detection corresponded to the lowest concentration leading to a signal to noise over three, and LOQ corresponded to the lowest concentration leading to a signal to noise over 10. No values under the LOQ were considered.

Importantly, blank samples were evaluated, and their injection showed no interference (no peak detected) during the analysis.

Cytokine quantification

Cytokines (IL-6, TNF-α, GM-CSF) were measured by a multiplex immunoassay (BioLegend, San Diego, CA) according to the manufacturer's instructions. Data were acquired on a BD FACSCanto, and analysis was performed using LEGENDplex software (BioLegend).

Chymase and tryptase activity measurements

To quantify intracellular tryptase and chymase amounts, 1×10^6 MC were disrupted with 100 μl 1% Triton X-100 in PBS, and the cell lysates were immediately processed. Enzymatically active tryptase was measured spectrophotometrically by adding 50 μl of 0.5 mmol/l Tosyl-Gly-Pro-Lys-p-nitroanilide (T6140; Sigma-Aldrich) 100 mmol/l Tris-HCl (pH 8), and 100 mmol/l NaCI to 50 μl of cell lysate. Enzymatically active chymase was quantified by adding 50 μl of 1 mmol/l Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (S7388; Sigma-Aldrich), 300 mmol/l Tris (pH 8), and 1.5 mol/l NaCl to 50 μl of cell lysate. Changes in absorbances at 405 nm were immediately registered for 5 min at 30 s intervals at room temperature.

Statistics

All results are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 5 software, and Student *t* test was applied.

Results

Production of mature MC from human WAT in 3 wk

Culturing SVF of human s.c. WAT in ultra-low adherent plates and in serum-free medium supplemented in SCF led to the formation of spheroids between 5 and 7 d after seeding (Fig. 1A). As soon as spheroids were formed, dispersed cells surrounding the spheroids were observed (Fig. 1A). These cells formed a homogenous population after 3 wk in culture. Analyses by flow cytometry showed that this homogenous cell population expressed the MC cell surface markers CD45, CD117, and FcεR1 (Fig. 1B, 1C). In addition, the presence of metachromatic cytoplasmic granules and round unilobular nucleus identified by toluidine blue staining on cytocentrifuged cells confirmed the MC nature of the cultured cell type (Fig. 1D). MC purity reached 91.8 ± 2.1% after 3 wk in culture and remained as high for the following 3 wk (Fig. 1E).

Altogether, these results show that three-dimensional culture of human SVF cells led to the production of highly pure MC in only 3 wk without any prior purification.

Expansion of WAT-MC

With this three-dimensional culture, $5.3 \pm 3.2 \times 10^4$ MC/g of WAT were obtained after 3 wk, and this yield doubled every week, reaching $4.8 \pm 2.7 \times 10^5$ MC/g of WAT after 6 wk (Fig. 2A). WAT-MC stopped expanding shortly after 6 wk and could be maintained in culture for 2 mo. Interestingly, the yield increased as long as the spheroids remained compact. Indeed, if spheroids were seeded in fresh medium after 1 wk, WAT-MC were observed de novo around spheroids. These MC are called G2 for second generation (Fig. 2B). As shown in Fig. 2C, G2 WAT-MC underwent a similar expansion compared toMC produced initially (G1 WAT-MC). We were able to generate up to four successive generations of MC, leading to a total production of 10^6 MC/g of WAT after 6 wk.

To test whether cytokines classically added to expand $CD34^+$ cell–derived MC were able to increase WAT-MC yield, cultures were performed with or without SCF (100 ng/ml), IL-6 (100 ng/ml), and/or IL-3 (30 ng/ml) for 5 wk. As expected, in the absence of SCF, no MC were obtained in vitro (data not shown), demonstrating that this cytokine is required for MC development, as previously described (22). The effects of IL-3 and IL-6 on WAT-MC yield were tested by using the protocol classically described for $CD34^+$ cell–derived MC (10). SVF of human s.c. WAT was seeded in medium with SCF and IL-3 for the first week and

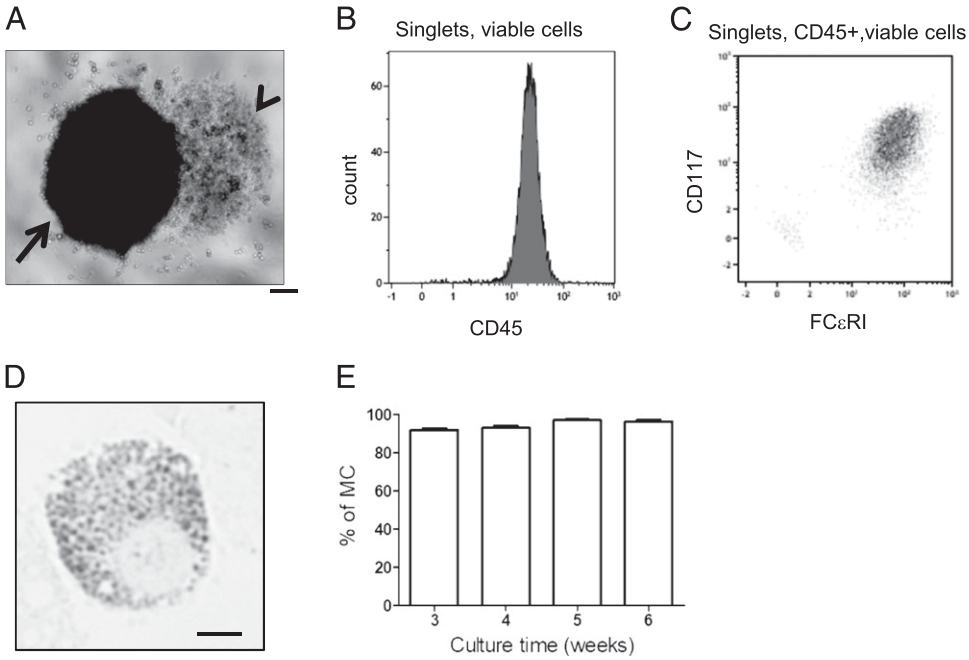

FIGURE 1. Generation of MC from SVF of human adipose tissue. (**A**) Seeding of the SVF cells from human WAT into a low adherent plates leads, after 1 wk, to the formation of a spheroid (arrow) surrounded by dispersed cells (arrow head). Scale bar, 200 μm ($n = 24$ donors). (**B**) Representative histogram of flow cytometry analysis showing cell surface expression of CD45 in cells surrounding the spheroid, gated on singlet living cells ($n = 24$ donors). (**C**) Representative dot plot of flow cytometry analysis showing cell surface expression of FcεRI and CD117 in cells surrounding the spheroid, gated on singlet $CD45^+$ living cells ($n = 24$ donors). (**D**) Toluidine blue staining of dispersed cells surrounding spheroids. Scale bar, 5 μm ($n = 4$ donors). (**E**) Percentage of MC ($CD45^+$, $CD117^+$, $FcεRI^+$ cells) among dispersed cells surrounding spheroids, quantified by flow cytometry each week from week 3 until week 6. Results are expressed as mean ± SEM and compared by using unpaired *t* test ($n = 3$ donors).

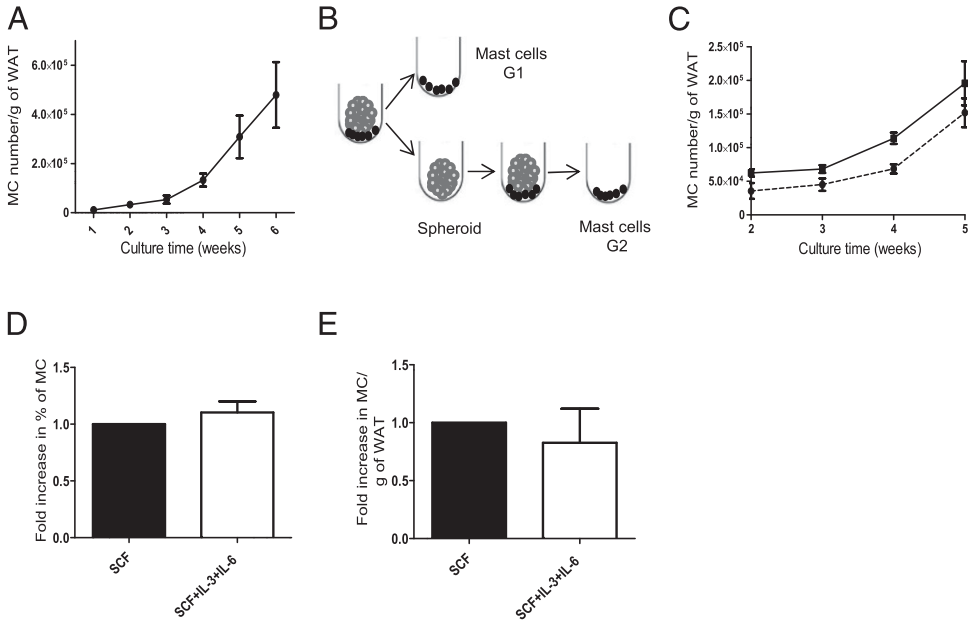

FIGURE 2. Expansion of WAT-MC. (**A**) Total number of MC ($CD45^+$, $CD117^+$, $FC\varepsilon RI^+$ cells) evaluated once a week, from week 1 to week 6, and expressed as MC number/g of WAT (10 g of WAT, $n = 4$ donors) (**B**) Spheroids continuously produced MC. Seeding of SVF into low adherent plates lead to the formation of a spheroid surrounded by MC. This first generation of MC is named G1. If spheroids were seeded in new fresh medium after washing them to discarded nonadherent cells, WAT-MC were observed de novo around the spheroids. These MC are called G2 for second generation ($n = 20$ donors). (**C**) Total number of WAT-MC G1 (black lines) and WAT-MC G2 (dashed lines) ($CD45^+$, $CD117^+$, $FC\varepsilon RI^+$ cells) from the same donors was evaluated once a week, from week 2 to week 5, and expressed as MC number/g of WAT ($n = 2$ donors). (**D** and **E**) SVF cells from the same donor were seeded in medium supplemented with SCF alone (SCF, black histogram) or with SCF + IL-3 for the first week and SCF + IL-6 for the following weeks (SCF+IL-3+IL-6, white histogram) ($n = 3$ donors). Purity of MC population (D) was evaluated after 3 wk in culture and expressed as percentage of MC ($CD45^+$, $CD117^+$, $FC\varepsilon RI^+$ cells) in cultured cells ($n = 3$ donors). Total number of MC per gram of WAT (E) was evaluated at 5 wk and expressed as percentage of values obtained with SCF alone ($n = 3$ donors). Results are expressed as mean ± SEM and compared by using unpaired t test ($n = 3$ donors).

then with SCF and IL-6 for the following weeks. Addition of IL-3 and IL-6 in the medium modified neither MC purity after 3 wk (Fig. 2D) nor WAT-MC yield evaluated after 5 wk (Fig. 2E).

These results demonstrate that SCF alone is required and sufficient for WAT-MC production in culture. In contrast, this cell culture system does not require IL-3 or IL-6, suggesting that WAT-MC produced in vitro differ from MC obtained from bone marrow $CD34^+$ purified cells.

WAT-MC are mature MC_{TC}

MC can be divided into two distinct populations based on tryptase and chymase expression. MC express either tryptase alone (mucosal-type MC [MC_T]) or tryptase and chymase MC_{TC} (23). As demonstrated by flow cytometry and immunofluorescence experiments, WAT-MC expressed typical MC_{TC} peptidases (i.e., tryptase, chymase, and carboxypeptidase A3) (Fig. 3A–C), and the mean fluorescence intensities relative to tryptase, chymase, and carboxypeptidase A3 were similar between WAT-MC and $CD34^+$ cell–derived MC (Fig. 3A). Moreover, tryptase and chymase activity were similar between both MC types (Fig. 3D, 3E). WAT-MC also expressed MrgX2, a G protein coupled receptor specific for MC_{TC} and that recognizes several basic secretagogues (Fig. 3F) (24). In addition, treatment of WAT-MC with IL-4 (100 ng/ml) for 5 d led to an increase in cell surface expression of FcεRI (Fig. 3G), demonstrating that these cells behave as mature MC and not as progenitors (25, 26).

Taken together, these results indicate that WAT-MC produced in vitro are mature MC_{TC}.

Mediators released from WAT-MC

WAT-MC functionality was characterized by the standard degranulation assay using Ag (DNP-HSA) after sensitization with IgE or SP, compound 48/80, or other polycationic molecules. WAT-MC, previously sensitized with IgE, showed a dose-dependent degranulation when stimulated with increasing concentrations of anti-IgE as detected by β-hexosaminidase release (Fig. 4A). MC_{TC}, in contrast to MC_T, can also be activated by basic compounds such as C3a, C5a, compound 48/80, or SP (25). Consistent with our previous results regarding the phenotype of cultured cells, stimulation of WAT-MC by C5a (50 ng/ml), SP (50 μm), or compound 48/80 (50 μg/ml) induced their degranulation (Fig. 4B).

Upon activation, MC release preformed and newly synthesized mediators. We thus quantified eicosanoids produced and released by WAT-MC after sensitization with IgE and stimulation with anti-IgE. Stimulated WAT-MC released PGs (PGE_2, PGD_2, 8-isoPGA$_2$, 15d-PGJ$_2$) (Fig. 4C). In contrast, stimulation of WAT-MC has no effect on HETE concentrations (data not shown) or other arachidonic metabolites, including LTB4 (Fig. 4C). Interestingly, the same pattern of arachidonic acid metabolites released after IgE sensitization and stimulation with anti-IgE was obtained with $CD34^+$ cell–derived MC (Fig. 4D). Moreover, as shown in Fig. 4E and 4F, WAT-MC as well as $CD34^+$ cell–derived MC produced TNF-α, IL-6, and GM-CSF following PMA/ionomycin activation or FcεRI aggregation.

Altogether, these results demonstrate that three-dimensional culture of SVF cells leads to the production of mature and functional human MC_{TC} within 3 wk.

Discussion

In this study, we report a novel time-saving and reproducible method to produce highly pure human MC in 3 wk from WAT. WAT-MC produced with this method are mature and functional MC_{TC}. WAT-MC behave like MC_{TC} obtained from peripheral blood–derived $CD34^+$ pluripotent progenitor cells, which is, to

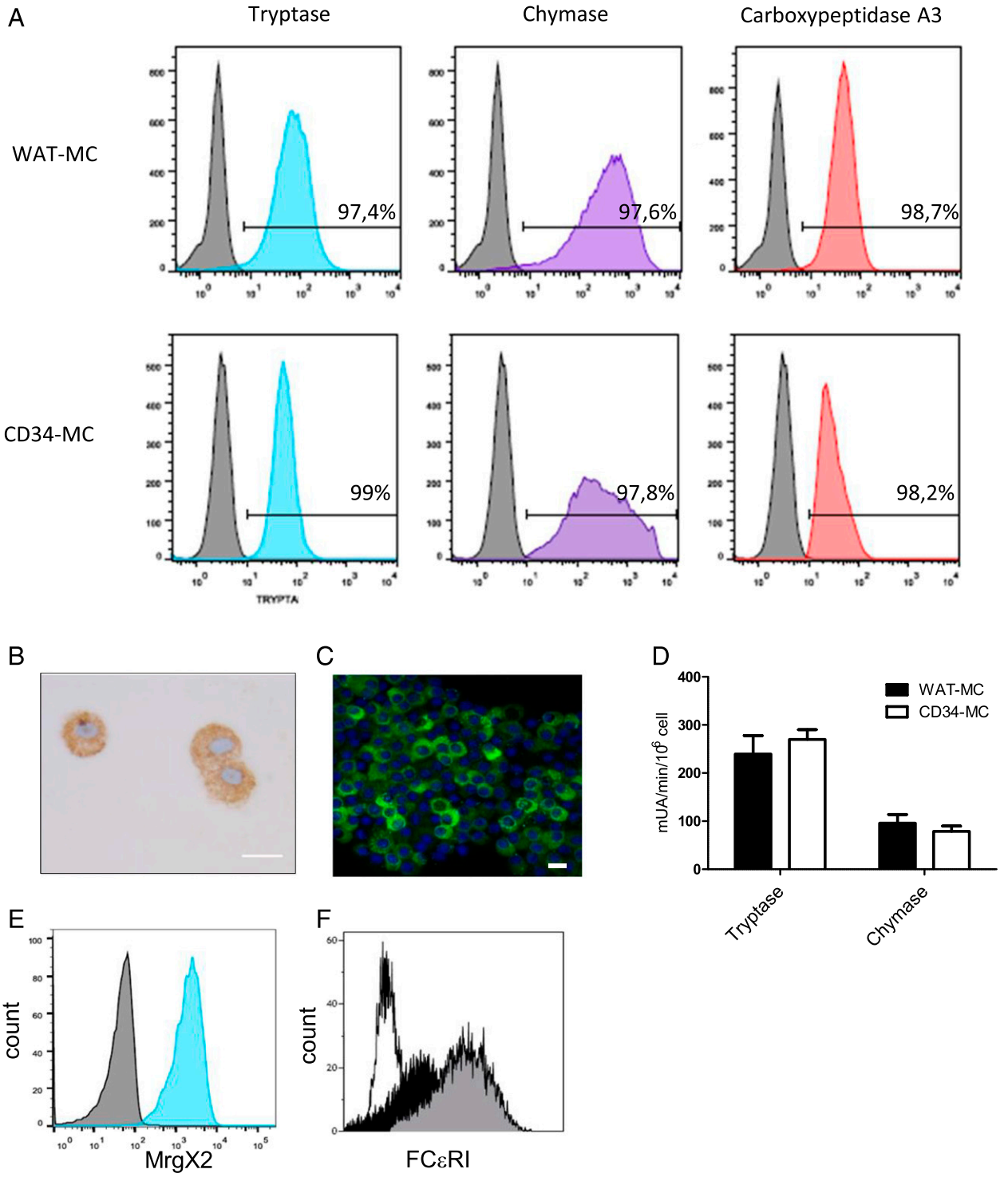

FIGURE 3. WAT-MC are mature MC_{TC}. (**A**) Representative histogram of flow cytometry analysis showing the expression of tryptase (blue), chymase (purple), and carboxypeptidase (red) by WAT-MC (upper panels) and MC from peripheral blood–derived $CD34^+$ pluripotent progenitor cells (lower panels), gated on singlet living cells. Gray histograms correspond to the isotype matched control (n = 1 or 3 donors). (**B**) Immunocytochemistry of WAT-MC using an Ab directed against tryptase. Nuclei are stained with hematoxylin. Scale bar, 20 μm (n = 2 donors) (**C**) Immunocytochemistry of WAT-MC using an Ab directed against chymase. Nuclei are counterstained with DAPI. Scale bar, 20 μm (n = 2 donors). (**D**) Tryptase activity measurement of WAT-MC (black plots) or peripheral blood–derived $CD34^+$ pluripotent progenitor cells (white plots). Results are expressed as mean ± SEM (n = 4 donors for WAT-MC and n = 5 donors for $CD34^+$ derived MC, each performed in duplicate). Chymase activity measurement of WAT-MC (black plots) or peripheral blood–derived $CD34^+$ pluripotent progenitor cells (white plots). Results are expressed as mean ± SEM (n = 4 donors for WAT-MC and n = 5 donors for $CD34^+$ derived MC, each performed in duplicate). (**E**) Representative histogram of flow cytometry showing cell surface expression of MrgX2 by WAT-MC, gated on singlet living cells. Blue and gray histograms correspond to the MrgX2 staining and its isotype-matched control, respectively (n = 2 donors). (**F**) Representative histogram of cell surface expression of FcεRI on WAT-MC after 1 wk in culture with or without IL-4 (100 ng/ml) (gray and black histograms, respectively), gated on singlet living cells. White histogram corresponds to the isotype-matched control (n = 3 donors, each performed once).

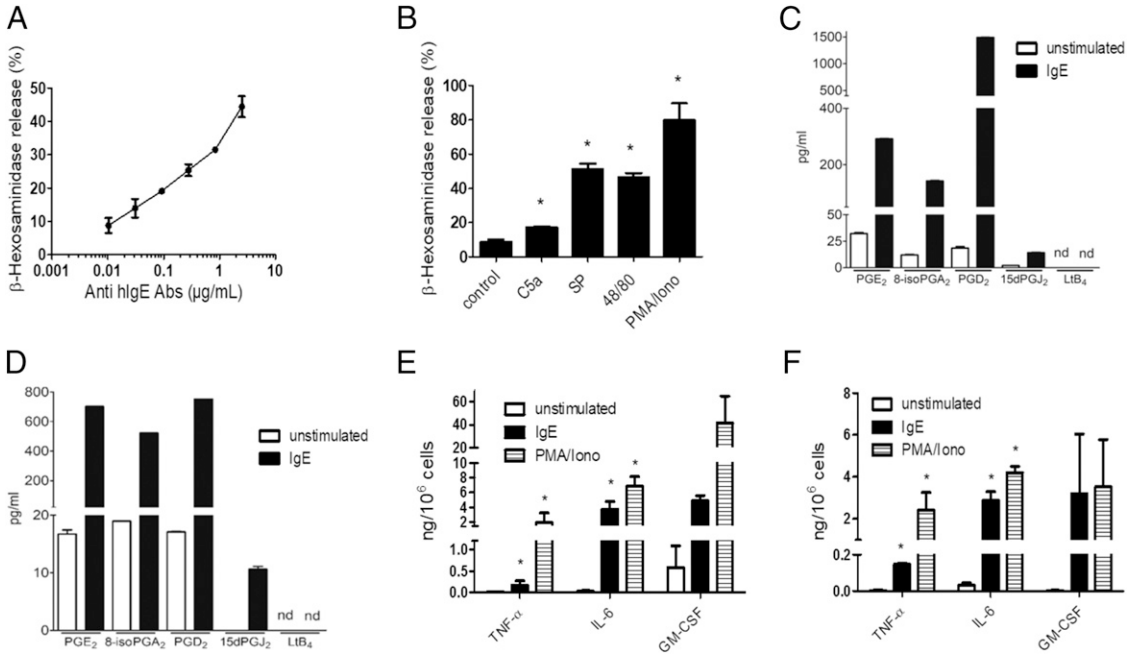

FIGURE 4. WAT-MC are functional. (**A**) Degranulation of WAT-MC assessed by measuring the release of β-hexosaminidase after sensitization with IgE and stimulation with increasing concentrations of anti-IgE Abs. Results are expressed as mean ± SEM (n = 3 donors, each performed in triplicate). (**B**) Degranulation of WAT-MC assessed by measuring the release of β-hexosaminidase after stimulation with C5a (50 ng/ml), SP (50 μm), compound 48/80 (50 μg/ml), and PMA plus ionomycin (respectively 0.1 and 1 μg/ml). Control cells correspond to unstimulated cells, and PMA/ionomycin was used as positive control. Results are expressed as mean ± SEM and compared by using unpaired t test (n = 3 donors, each performed in triplicate). *$p < 0.05$ compared with unstimulated cells. (**C** and **D**) Results of arachidonic acid metabolites production by WAT-MC (C) or CD34$^+$ derived MC (D) assessed by measuring their release after sensitization with IgE and stimulation with IgE (10 μg/ml, black plot) or not (white plot) (n = 1 donor, each performed in duplicate). (**E** and **F**) Results of TNF-α, IL-6, and GM-CSF production by WAT-MC (E) or CD34$^+$ derived MC (F) assessed by measuring their release after sensitization with IgE and stimulation with IgE (10 μg/ml, black plot) or stimulation with PMA plus ionomycin (respectively, 0.1 and 1 μg/ml). White plots represent production of unstimulated cells (n = 2 to 4 donors, each performed in triplicate; n = 2 for GM-CSF production). Results are expressed as mean ± SEM. *$p < 0.05$ between cells stimulated with anti-IgE or PMA plus ionomycin compared with unstimulated cells. nd, not detectable.

date, the gold standard strategy to generate fully mature and functional primary MC lines. Indeed, WAT-MC and CD34$^+$ derived MC exhibit similar phenotypes and release similar cytokines and lipid mediators following FcεRI aggregation. The amount of lipid mediators and cytokines produced by both MC types were in the same range, although they cannot be compared because they were not obtained from the same donor.

The method described here present two major advantages as compared with methods using CD34$^+$ progenitors. First, it is a time saving method. Indeed, we are able to generate an almost pure population of mature functional MC_{TC} in only 3 wk, whereas in most of studies, MC are generated after 9–14 wk in culture (10). In some protocols however, the culture is shorter, but in these cases MC purity is not high or MC obtained are a mix population between MC_T and MC_{TC}. For example, a protocol has been developed to generate MC in 3 wk using CD34$^+$ cells from discarded autologous stem cell concentrates, but their purity greatly varied among donors (from 55 to 90%), and only 4–20% of MC contained both tryptase and chymase (26). The shortest protocol with same range of MC_{TC} purity requires 9 wk of culture of CD34-positive cells from buffy coat (27). Second, we are not limited by the amount of starting materials because adipose tissue is an abundant and easily accessible tissue.

Our protocol, however, does not improve MC yield compared with protocols generating MC from CD34$^+$ cells. A total of 10^6 to 2×10^7 mature MC can be generated from 50 to 100 ml of blood or buffy coat in 9 to 14 wk (27–29). With our protocol, $5 \pm 3 \times 10^4$ MC/g of WAT were obtained after 3 wk, reaching $5 \pm 3 \times 10^5$ MC/g of WAT after 6 wk. This yield can be increased by at least 2-fold if spheroids are maintained in culture.

In our model, we observe that spheroids are able to continuously produce MC for four generations, suggesting that WAT-MC seem to derive from MC progenitors present in the spheroids. This observation is supported by the fact that we previously demonstrated that, in mice, MC in adipose tissue come from resident MC progenitors (12). Moreover, we have quantified the proliferation of mature MC in the spheroids by using BrdU incorporation assay, and our results show that ~10% of the mature MC population proliferate (data not shown). The lack of robust markers of MC progenitors allowing their identification in vivo did not allow us to conclude about the origin of WAT-MC.

Our culture conditions are based on what is currently used to obtain MC from CD34$^+$ cells. First, serum-free culture conditions have been used to prevent batch to batch variability in serum composition and thus increase reproducibility. Indeed, variations in proliferation and maturation of MC have been reported depending on the presence of serum in culture media. Serum almost suppresses MC development of CD34$^+$ cord blood cells and inhibits proliferation of skin derived MC when added at the beginning of the culture (30, 31). In contrast, it increases MC maturation when added at later stages (31). Second, we used IL-3 and IL-6, both known to increase the yield of MC derived from CD34$^+$ cells mainly by acting on early stage of differentiation (4, 10, 32, 33). The fact that IL-3 and IL-6 were not required for WAT-MC production suggests that MC progenitors present in the WAT are less immature than bone marrow CD34$^+$ cells. Moreover, this hypothesis is consistent with the faster production rate of WAT-MC compared with MC derived from CD34$^+$ cells.

In conclusion, we have developed a simple, robust, and time saving protocol to obtain mature and functional human MC_{TC} which behave

like MC derived from peripheral blood CD34$^+$ cells for all tested parameters. This short term culture protocol will be useful for the evaluation of MC biology in human as well as in mice and is a suitable tool to screen drug effect on human mature MC. In addition, because of the generalized localization and the easy access of WAT, this newly developed MC culture provides the possibility to study individual MC populations from patients in physiological or pathological conditions such as mastocytosis.

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Disclosures

The authors have no financial conflicts of interest.

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