Human macrophage lectin specific for galactose/N-acetylgalactosamine is a marker for cells at an intermediate stage in their differentiation from monocytes into macrophages

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Abstract

We studied the expression of a human macrophage lectin specific for galactose/N-acetylgalactosamine (hMGL) during macrophage differentiation. The expression of hMGL during the in vitro differentiation induced by human serum was examined by immunostaining and Western blotting with a specific mAb, MLD-1, as well as with RT-PCR analysis. hMGL was detected on cells at an intermediate stage of differentiation. These cells were round, slightly larger in size (12.7 ± 0.2 μm) than monocytes (9.8 ± 0.1 μm) and expressed the macrophage marker CD14, but lacked the dendritic cell marker CD1a. The highest levels of expression occurred after 2–4 days of culture. At this time point, MLD-1 prominently stained 20–40% of the cells. Monocytes cultured for 16 h or fully differentiated monocyte-derived macrophages were negative or weak for hMGL expression. Similar transient expression was also observed during granulocyte macrophage colony stimulating factor- or macrophage colony stimulating factor-dependent macrophage differentiation. The lectin was characterized as a functional endocytic receptor for glycosylated macromolecules, since the uptake of carbohydrate polymers was partially inhibited by the addition of MLD-1. The distribution of hMGL+ cells in normal human skin was found by immunostaining to be mainly in the upper dermis distant from vascular structures. More than 90% of the hMGL+ cells were double stained with anti-CD68 mAb and constituted ~20% of the CD68+ cells. We suggest that the dermal hMGL+ cells are a subset of differentiated cells derived from monocytes and that hMGL is a unique marker for cells at an intermediate stage of macrophage differentiation.

Introduction

Macrophages and related cells play important roles in innate immunity by capturing, clearing and transporting foreign and altered host components. Subsets of macrophages, as well as dendritic cells (DC) derived from peripheral blood monocytes, have accessory functions in that they present antigen to T cells and can thus directly regulate acquired immunity. To efficiently perform these functions, monocytes and macrophages acquire phagocytic and accessory functions during their differentiation, a process that is accompanied by a number of phenotypic alterations.

Monocyte differentiation is not a unidirectional event, but can follow at least two alternative pathways. Monocytes can differentiate either into immature DC under the influence of cytokines such as granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 (1,2) or into mature macrophages. In addition, the mature macrophages may be of at least two distinct types: M-macrophages that resemble peritoneal macrophages and are obtained by culturing monocytes with macrophage colony stimulating factor (M-CSF), and GM-macrophages that resemble alveolar macrophages and are
derived by culturing monocytes with GM-CSF in the absence of IL-4 (3). These three monocyte-derived populations are probably interchangeable with one another, most likely at intermediate differentiation stages. For example, M-macrophages and immature DC can be interchanged in vitro depending on which cytokines are added to the culture medium (4,5). It is likely that the commitment to differentiate into either macrophages or DC occurs only after passing through a particular intermediate stage. Previous studies have shown that macrophages at intermediate stages of differentiation are phenotypically and functionally distinct from monocytes and mature macrophages (5–9). However, characterization of such immature macrophages has been hampered by the absence of a suitable marker that facilitates their identification.

Various lectins are known to be expressed on macrophages (10–13) and we have particularly focused on the macrophage galactose/N-acetylgalactosamine (Gal/GalNAc)-specific C-type lectin (MGL). Both the human MGL (hMGL) and murine MGL are type II transmembrane glycoproteins with a single extracellular C-type carbohydrate recognition domain (14–16). In our work to characterize the carbohydrate specificity of MGL, we found that it recognizes carbohydrate structures possessing terminal Gal/GalNAc residues, particularly clusters of truncated O-linked carbohydrate chains such as those occurring on the Tn antigen. This binding capacity was confirmed by using the recombinant forms of both the hMGL and murine MGL (14,17,18).

MGL may participate in a number of cellular functions. In vitro studies have suggested that macrophages expressing MGL might recognize and capture malignant cells through a MGL–carbohydrate interaction (19,20). Macrophages expressing MGL, and an adoptively transferred T cell line that expresses MGL, are found to accumulate specifically in the tumor sites of mice with experimental lung metastases of tumors expressing MGL, and an adoptively transferred T cell line MGL±carbohydrate interaction (19,20). Macrophages with restricted expression of MGL may function in a similar manner. In vitromediated migration is the first example of myelogenous cell trafficking that is regulated not by the selectin system but by a type II C-type lectin (28,29). MGL might also be involved in cellular recognition during antigen processing and presentation to T cells. An example of a C-type lectin with this property is DC-SIGN, which is preferentially expressed on DC. DC-SIGN has been reported to bind to ICAM-3 on naive T cells, thus facilitating the formation of DC–T cell clusters (30). MGL might function in a similar manner.

To elucidate the biological role of MGL+ cells in the immune system and to understand the significance of carbohydrate recognition by MGL, it is essential to be able to identify and characterize MGL-expressing cell populations. Based on our previous observations, we hypothesize that MGL expression is restricted to a subset of monocyte/macrophage cells that are able to take up antigens efficiently and to migrate. In the present study, we have characterized a human macrophage subpopulation that expresses hMGL during monocyte–macrophage differentiation in vitro. We found that hMGL is expressed only transiently at an intermediate stage in the differentiation process. Immunostaining of human skin indicated that hMGL+ cells are present in the upper dermis, especially in sites distant from vascular structures.

**Methods**

**Antibodies**

Antisera against recombinant hMGL (rhMGL) were obtained as described in our previous article (14). The rhMGL protein composed of the putative extracellular region was synthesized in *Escherichia coli* strain BL21 (DE3) that had been transformed with the constructed plasmid pET-8c-rhMGL. Soluble rhMGL was purified by affinity chromatography on a column of galactose–Sepharose 4B as described (16). rhMGL was mixed with Freund’s complete adjuvant (Difco, Detroit, MI) and injected i.c. into female Japanese white rabbits. After three injections given at 2-week intervals, antiserum against rhMGL were obtained.

Hybridomas producing anti-hMGL mAb were established and details will be published elsewhere (Sano et al., in preparation). In brief, mice were injected i.p. with purified rhMGL (100 μg) mixed with complete Freund’s adjuvant. After repeated immunization, splenocytes were fused with myeloma line SP2/0. mAb that blocks hMGL–carbohydrate interaction (MLD-1) was established, obtained as ascites, purified with Protein G–Sepharose chromatography, and used in the present work. Labeling of mAb MLD-1 with digoxigenin was carried out with a digoxigenin protein labeling kit according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany). An alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody F(ab')2 fragment (Roche Diagnostics) was used to recognize the primary antibody. A mAb specific for the sulfo-Lea carbohydrate epitope (91.9H) was used as a negative control (31).

Mouse mAb against CD14 (RPA-M1, IgG2b), HLA-DR (LN-3, IgG2b) and CD11c (B-Ly6, IgG1) were obtained from Zymed (South San Francisco, CA), against CD68 (KP-1, IgG1) from Dako (Kyoto, Japan), and against CD1c (M241, IgG1) from Ancell (Bayport, MN). For flow cytometry, FITC-labeled anti-CD1a (B-BS, IgG1; Biosource International, Camerillo, CA), anti-CD14 (TU1, IgG2a; Dako, Kobe, Japan), anti-CD16 (DJ130c, IgG1; Dako), anti-CD33 (D3HL60-251, IgG1; Immunotech, Marseille, France), anti-CD80 (MAB104, IgG1; Immunotech), anti-CD83 (HB15a, IgG2b; Immunotech), anti-HLA-DR (Tu36, IgG2b; Becton Dickinson, Mountain View, CA) and unlabeled CD11b (B-1, IgG1; Nichirei, Tokyo, Japan)
and CD86 (BU63, IgG1, Ancell) were used. Unlabeled or biotinylated mouse IgG1 (Zymed), and mixture of fluorescein-labeled mouse IgG1 and IgG2a (Sigma) were used for negative controls.

Separation of peripheral blood monocytes
Human blood from healthy donors was kindly donated by the Tokyo Red Cross (Tokyo, Japan). Peripheral blood mononuclear cells were obtained after sedimentation with 3% dextran 200 (extrasyntetase) followed by discontinuous density gradient centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). After centrifugation at 250 g for 30 min, cells accumulating at the interface were collected, washed extensively with PBS to remove platelets and suspended in RPMI 1640 medium (Gibco, Gaithersburg, MD) containing 0.5% FCS (Biowhittaker, Walkersville, MD) and 0.5% pooled human serum. The cell suspension was added to either 24-well flat-bottom plates (MS-80240; Sumitomo Bakelite, Osaka, Japan) or Lab-Tek chamber slides (153437; Nalge Nunc, Naperville, IL) and cultured at 37°C for 1–2 h to allow the cells to adhere. Non-adherent cells were discarded by gentle washing, and the remaining cells were cultured in RPMI 1640 medium containing 5% FCS and 5% pooled human serum, or the same medium containing 7.5% FCS and 500 U/ml GM-CSF (Kirin Brewery, Tokyo, Japan) or 100 ng/ml of M-CSF (Morinaga Milk Industry, Tokyo, Japan) for up to 10 days (32).

Immunostaining of cultured monocytes
Following incubation for varying intervals, macrophages cultured in Lab-Tek chamber slides were washed twice with Dulbecco’s modified PBS (DPBS; PBS containing 0.91 mM CaCl₂ and 0.49 mM MgCl₂) and fixed with cold ethanol for 30 s. Following incubation for varying intervals, macrophages were treated with primary antibody for 40 min at room temperature, then with biotinylated goat anti-mouse Ig (Nichirei) for 30 min and finally with AP-conjugated streptavidin (Zymed) for 30 min. The staining was visualized using HistoMark Red (Kirkegaard & Perry, Gaithersburg, MD). The samples were mounted in Aqua Poly/Mount (Polyscience, Warrington, PA) and observed with microscopes (Olympus BX60, Tokyo, Japan/Nikon DIAPHOT, Tokyo, Japan).

RT-PCR
Total RNAs (4 μg) were prepared from monocytes at different time points in culture using Ultraspec RNA (Biotecx, Houston, TX). The RNA was reverse transcribed in 40 μl of 50 mM Tris-HCl (pH 8.3), 3 mM MgCl₂, 75 mM KCl, 1 mM dithiothreitol, 0.5 mM of each dNTP, 50 μg/ml oligo(dT)12–18 and 10 U/ml of Moloney murine leukemia virus reverse transcriptase for 1 h at 37°C. cDNA was boiled for 5 min and quenched on ice before amplification by PCR. The conditions for PCR were as follows: in a 20 μl reaction there was 0.5 μM of each primer, 250 μM each of dGTP, dATP, dCTP and dTTP (Amersham Pharmacia Biotech), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl₂, and 0.2 μl of AmpliTaq polymerase (Applied Biosystems, Tokyo, Japan). The primers were used as follows: hMGL: sense 5'-TCAAGACGAGGCAAGCGAGCAG-3', antisense 5'-CATGGACTGTGGT-3'; Reaction mixtures were incubated in a Perkin-Elmer DNA thermal cycler (hMGL: 40 cycles, denaturation at 94°C for 40 s, annealing at 63°C for 60 s, extension at 72°C for 90 s; GAPDH: 20 cycles, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s).

Detection of hMGL in macrophage extracts
Adherent day 4 macrophages were washed with PBS and detached from the plate with 10 mM EDTA/PBS treatment. The detached cells were collected and extracted for 60 min on ice with 1 ml of lysis buffer (DPBS containing 1% Triton X-100, 0.02% NaN₃, 0.1 mM aprotinin, 1 mM pepstatin A, 1 mM leupeptin and 1 mM PMSF; all purchased from Sigma)10⁶ cells. After centrifugation (15,000 r.p.m., 20 min), the supernatant containing the cell lysate was collected. Protein concentrations in the lysates were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Lysate proteins (300 μg/60 μl) were subjected to immunoprecipitation with the MLD-1 mAb or affinity isolation with GalNAc-conjugated agarose (30 μl suspension; Sigma) to precipitate the carbohydrate-binding molecules. MLD-1 was conjugated with Protein G-Sepharose (670 μl ascites/270 μl resin) prior to immunoprecipitation. Proteins bound to the resins were mixed with 60 μl of SDS–PAGE sample buffer and boiled for 5 min. Proteins extracted from the precipitate were subjected to SDS–PAGE (10% gel) under reducing conditions and transferred to a PVDF (Millipore, Bedford, MA) using the MilliBlot-SE system (Millipore). The membrane was treated for 60 min at room temperature with 10 mM sodium phosphate and 0.15 M NaCl (pH 7.2; PBS) that contained 3% BSA to block non-specific antibody binding. The membrane was subsequently incubated for 90 min at room temperature with rabbit serum against rhMGL (1/1000 dilution in PBS containing 0.2% Tween 20), followed by incubation for 90 min at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H + L) (Zymed) diluted at 1/1000 in PBS/0.2% Tween 20. The binding of the mAb was visualized using ECL Western blotting detection reagent and hyperfilm ECL (Amersham, Arlington Heights, IL). Reference standards included phosphorylase b, BSA, aldolase and carbonic anhydrase from Daiichi Pure Chemicals (Tokyo, Japan).

Flow cytometry
Macrophages cultured in 24-well microplates were harvested with rubber policemen, and suspended in PBS containing 3% BSA and 5% mouse or goat serum for blocking. The viability of the macrophages thus recovered was 98–9.0% (n = 4). The cells were then stained with a number of antibodies recognizing macrophage/DC markers for 60 min on ice. The cells were washed 3 times and then treated with goat anti-mouse IgG (G + A + M) if necessary. The cells were also stained with biotinylated MLD-1 to identify possible non-specific antibody binding. The cells were first stained with 10 μg/ml of the biotinylated antibody and then with phycoerythrin-labeled streptavidin diluted 300-fold (PharMingen, San Diego, CA). The staining was determined with an Epics Elite flow cytometer (Coulter, Miami, FL). Calibration of the machine was confirmed using Flow-Check fluorophores (Coulter). Macrophages (8000 cells) were ana-
Uptake of carbohydrate polymers by macrophages

Day 3 macrophages were harvested with rubber policemen and suspended in RPMI 1640 with 10% FCS (1 × 10^6 cells/100 μl). In some experiments, the cells were preincubated with mAb MLD-1 (5 μg/100 μl) for 30 min at this time. Subsequently, they were mixed with polyacrylamide polymers conjugated with FITC and monosaccharides (α-GalNAc and β-GlcNAc polymers, 1 μg/100 μl; Seikagaku, Tokyo, Japan). Incubations were performed at 37°C for 2 h. The cells were washed with DPBS containing PI (1 μg/ml; Sigma), and then with DPBS twice and fixed with 4% paraformaldehyde (Nakalai Tesque, Kyoto, Japan) for 10 min at 4°C. Uptake of the carbohydrate polymers was determined with an Epics Elite flow cytometer. The fixed macrophages (10,000 cells) were analyzed after gating on forward/side scatter and PI− cells. Similar experiments were performed with day 3 macrophages grown in Lab-Tek Chamber slides. Adherent cells were incubated with α-GalNAc polymers at the same concentration (2.5 μg/250 μl), incubated for 2 h, washed 3 times, fixed with 4% paraformaldehyde for 10 min and examined under a confocal microscope (MRC1024; BioRad, Hemel Hempstead, UK).

Tissue specimens

Fresh surgical biopsies of healthy skin were obtained from female breast cancer patients aged 35–72 who underwent surgery at the Juntendo Hospital, Tokyo, Japan. The samples were obtained after informed consent. The biopsies were snap-frozen in liquid nitrogen and kept frozen until use.

Detection of hMGL+ cells in human skin by immunostaining

Immunohistochemical detection of hMGL+ cells in human skin was performed using the mAb MLD-1 and HRP- or AP-conjugated goat anti-mouse IgG (H + L) (Zymed). The skin sections were first cut into 10 × 5 × 5 mm pieces and embedded in OCT compound (Miles, Naperville, IL). Frozen sections (10 μm) were fixed at 4°C with methanol for 30 s or with 2% paraformaldehyde for 10 min, depending on the
speci®city of the mAb used. The sections were then washed in DPBS, incubated for 60 min at room temperature with blocking solution (DPBS containing 3% BSA and 4% normal goat serum) and then treated for 16 h at 4°C with primary antibody at a concentration of 5±20 µg/ml depending on the antibody. After washing 3 times, the sections were treated for 60 min at room temperature with 5 µg/ml of AP- or HRP-conjugated goat anti-mouse IgG (H + L). After washing 3 times, mAb binding was visualized with HistoMark Red or with a combination of Fast Blue BB (Wako, Osaka, Japan) and naphthol (Dojin, Kumamoto, Japan) in the case of AP staining, or with diaminobenzidine tetrahydrochloride (Dojin) in the case of HRP staining. Cell nuclei were counterstained with Mayer’s hematoxylin. To quantify cell density in the skin, the stained cells in the upper dermis (~0.5 mm below the surface of the skin) were counted. The density was calculated as the number of the stained cells/mm² in the upper dermis.

Results

Transient hMGL expression during the differentiation of monocytes into macrophages in vitro

Human monocytes are known to differentiate into monocyte-derived macrophages (MDM) when cultured in media containing human serum. This is a well-established in vitro method used to obtain mature human macrophages. We examined the expression of hMGL on these macrophages at various stages of differentiation by immunostaining with an anti-hMGL mAb, MLD-1.

After incubation for 16 h (day 1), <1% of the cells in the monocyte fraction were hMGL+, but after 4 days the macrophages became strongly reactive with mAb MLD-1. The day 4 macrophages were round and signi®cantly larger in size (diameter: 12.7 ± 0.2 µm, n = 45) than monocytes cultured for 16 h (diameter: 9.8 ± 0.1 µm, n = 45) and were strongly stained with mAb MLD-1. Between 7 and 9 days later, the morphology of the monocytes had changed. They appeared to have become terminally differentiated macrophages (diameter: 25±50 µm) and did not appear to react with mAb MLD-1 (Fig. 1A and B). We also examined a similar transient expression of MGL in macrophages cultured in the presence of M-CSF or GM-CSF. The day 4 macrophages were strongly stained with mAb MLD-1, while monocytes cultured for 16 h were scarcely stained and mature macrophages were only weakly stained (Fig. 1C–F). To determine the time course of hMGL expression, we quanti®ed the proportion of strongly stained cells, designated MGL+ cells, relative to all nucleated cells at each incubation time. The period where the proportion of hMGL+ cells was maximal varied slightly from donor to donor, but the observed peak of expression was always between days 2 and 4 of culture. This is a period that corresponds to an intermediate stage in the differentiation of monocytes into macrophages (Fig. 1G). The transient expression of hMGL was further con®rmed in monocytes from three independent donors by RT-PCR analysis (Fig. 2A and B).
The presence of hMGL protein at the intermediate stage was further confirmed by Western blotting analysis of lysates derived from day 4 macrophages. Polyclonal anti-hMGL antiserum detected a 37-kDa component on the blot (Fig. 3).

Phenotypic analysis of hMGL + cells prepared in vitro

We further characterized day 4 macrophages by flow cytometry, focusing on the presence of other types of cells related to macrophages, such as monocyte-derived DC. The day 4 macrophages were mostly homogeneous with respect to hMGL expression. They were CD14+CD1a−, indicating that this preparation did not contain monocyte-derived DC. Furthermore, this population also expressed the mature macrophage marker CD16, but lacked expression of CD80 and CD83. These staining profiles further support the categorization of the cells in this population as macrophages (Fig. 4).

Uptake of carbohydrate polymers by macrophages via hMGL

One of the potential biological roles of endogenous lectins is to incorporate foreign substances into cells. Day 3 macrophages expressing a high level of hMGL were tested for their endocytic function by flow cytometry. A significant shift of the peak due to uptake of FITC-labeled α-GalNAc polymers was demonstrated at 37°C. The shift of the peak was partially inhibited by addition of mAb MLD-1, an anti-hMGL mAb that blocks the hMGL–carbohydrate interaction (Fig. 5A). Uptake of β-GlcNAc polymers, on the other hand, was only slightly inhibited by the same antibody (Fig. 5B). Increase in the fluorescence intensity was shown by confocal fluorescence microscopy to be caused by the uptake of α-GalNAc polymers (Fig. 5C–F). These results strongly suggest that hMGL is involved in α-GalNAc-dependent endocytosis of glycosylated macromolecules in immature macrophages (Fig. 5).

Detection of hMGL+ cells in skin

To explore the distribution of these immature macrophages in situ, we carried out immunostaining with the mAb MLD-1, anti-CD14 and anti-CD68 in frozen sections of normal skin derived from breast cancer patients. It should be noted that skin contains many tissue macrophages of diverse localization, such as perivascular macrophages and interstitial macrophages distributed in upper dermis (33). The hMGL+ cells were found to distribute predominantly in the upper dermis. The density of hMGL+ cells in the skin was 29 ± 5/mm² (n = 6), almost as high as the density of CD14+ cells (32 ± 5/mm²) and ~20% of the density of CD68+ cells (144 ± 12/mm²). The dermal hMGL+ cells were mainly distributed in sites distant from the perivascular regions (Fig. 6A). The distribution was different from that of CD68+ cells appearing in whole dermis (Fig. 6C).

Phenotypic characterization of hMGL+ cells in skin

The dermis is known to contain a variety of cells, including macrophages, dermal DC, lymphocytes, mast cells and other immune cells. To further phenotypically characterize the hMGL+ cells in the skin, we double stained the skin sections with digoxigenin-labeled mAb MLD-1 together with anti-CD68 or anti-CD1c which recognizes dermal DC. More than 90% (329 out of 355) of the hMGL+ cells were also stained with anti-CD68 mAb (Fig. 6E), strongly suggesting that they were derived from monocytes. Staining with a dermal DC marker, CD1c, indicated a partial (~50%, 210 out of 411) coincidence with hMGL expression (Fig. 6F). As there were also cells positive for CD1c that lacked hMGL expression (Fig. 6F), it is clear that hMGL+ cells and CD1c+ dermal DC do not overlap completely.

Discussion

Various C-type lectins are known to be specific markers for macrophages (10–13), DC (30,34–37) and NK cells (38).
These molecules are not only unique markers but are also potentially involved in regulating cellular recognition and trafficking. We have found that the C-type lectin MGL may be a specific marker for a unique subpopulation of cells of the monocyte–macrophage lineage, as MGL expression did not coincide with that of other typical macrophage markers in our previous study in mice (24). In the present study, we examined the expression of hMGL during the differentiation of monocytes into macrophages and demonstrated that hMGL is a marker for cells at an intermediate stage of macrophage differentiation. While the differentiation of monocytes into macrophages is a well-known phenomenon, the intermediate stages through which the cells pass have not been well defined. The hMGL+ cells appear to be at one of these intermediate stages. When human monocytes differentiated after 2–4 days of culture with human serum, they became hMGL+, after which hMGL expression declined. The hMGL+ cells were significantly larger than the adherent monocytes but much smaller than MDM (Fig. 1), indicating that they are at an intermediate stage of differentiation. Our results show that hMGL expression is only transient. Previous work has also suggested that monocytes pass through functionally and phenotypically distinct stages during their differentiation into macrophages. It is known, for example, that monocyte accessory functions become briefly up-regulated in the first 0.5–1.5 days of their differentiation into macrophages (6). Other markers such as CD36 may also be expressed at an intermediate stage, as elevation of CD36 expression parallels foam cell formation (7). Furthermore, in swine, macrophages at an intermediate stage are reported to be susceptible to virus infection (8,9). Human MGL may be used as a marker for such immature macrophages with unique functional capacities.

It would be interesting if MGL were expressed during the macrophage activation step, because expression of the macrophage mannose receptor, an endogenous lectin required for receptor-mediated endocytosis, is known to be selectively enhanced during the alternative macrophage activation induced by IL-4 (39,40). However, hMGL expression apparently depends on differentiation but not on activation, because the expression of hMGL was induced in the presence of M-CSF and GM-CSF (Fig. 1), cytokines that, along
with human serum, are known to induce macrophage differentiation rather than activation. Furthermore, IFN-γ and lipopolysaccharide, known as reagents for classical macrophage activation, did not influence the expression of hMGL (data not shown), in contrast to the reduced expression of the macrophage mannose receptor in response to these classical macrophage activators (39,41).

It will be important to know the molecular function of hMGL expressed on the surface of immature macrophages. It has been shown that murine MGL is mainly distributed in intracellular membranous structures (24). Our flow-cytometric analysis indicated that permeabilization of day 3 macrophages enhanced the staining intensity of MLD-1 (data not shown), as also shown by another group (42). The evidence thus suggests that hMGL is distributed both on the cell surface and in an intracellular pool, and that the molecule is recycled between the plasma membrane and intracellular compartments as an antigen capture molecule. We showed that uptake of a GalNAC-modified carbohydrate polymer, a mimic for glycosylated macromolecules, was partially inhibited by the addition of MLD-1, a mAb that blocks the interaction of hMGL and carbohydrates (Fig. 5). hMGL may therefore be a unique endocytic receptor for macromolecules to be processed for antigen presentation. Polymers with attached GalNAC residues are considered to be mucin mimetics. Mucins, and particularly MUC1 in its poorly glycosylated forms, are known to be efficient immunogens. The population of antigen-presenting cells responsible for induction of such immunity has not been properly identified, as digestion of mucin molecules is impaired in human DC (43), but we are currently investigating whether day 3 macrophages present antigen and elicit mucin-specific immunity. In the present study, the inhibition of glycan binding by MLD-1 was not complete. This might be because day 4 macrophages incorporate the carbohydrate polymer via both receptor-mediated endocytosis and pinocytosis. The confocal microscopic images in Fig. 5 also demonstrate that the addition of MLD-1 significantly interfered with carbohydrate polymer incorporation; therefore, we know that the hMGL-mediated endocytosis pathway does contribute to the incorporation.

Palucka et al. reported that day 5 macrophages are pluriplotent, as they can still differentiate into DC if they are cultured with GM-CSF and IL-4 (5). In vivo studies have shown that such macrophages (or macrophage-like cells) can also differentiate into DC after capturing antigens (44). As hMGL expression peaks at days 2–4 of culture, the hMGL+ cells may be at an uncommitted stage of differentiation. Our data from double immunohistochemical staining of skin sections are consistent with the notion that hMGL+ cells are or can differentiate into dermal DC. hMGL+ cells in the skin were monocyte-derived cells, as >90% of them also expressed CD68, but half of the hMGL+ cells also expressed the dermal DC marker CD1c (45,46). Thus, it is likely that the skin contains at least two types of hMGL+ cells, MGL+CD68+CD1c− immature macrophages and MGL+CD68−CD1c+ immature dermal DC (although we do not exclude the possibility that the hMGL is also expressed on other cell types). It is known that dermal DC resemble CD68+ monocyte-derived DC in their phenotype (2,45), which suggests that they are derived from monocytes or monocyte–macrophage lineage cells. As immature macrophages and dermal macrophages appear to be relatively plastic (5,44), we speculate that hMGL+ cells in the skin form a pluripotent reservoir that can supply both macrophages and DC for an immune response. Recently, we and others confirmed that MGL is expressed on human monocyte-derived DC and bone marrow-derived immature DC; but not on mature DC in human and mice (42, 47, 48). The plasticity of hMGL+ cells during their differentiation into macrophages and DC, and the expression pattern of hMGL during monocyte differentiation into DC, needs further study.

In conclusion, hMGL was found to be a unique marker for macrophages at an intermediate stage of differentiation from monocytes into macrophages. In human skin, cells expressing hMGL were found in the upper dermis, especially in sites distant from vascular structures. They are likely to be a subset of differentiated cells derived from monocytes.

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Abbreviations

AP  alkaline phosphatase
DC  dendritic cell
DPBS  Dulbecco’s PBS
Gal  galactose
GalNAC N-acetylgalactosamine
GlcNAC N-acetylglucosamine
GM-CSF granulocyte macrophage colony stimulating factor
h  human
HRP  horseradish peroxidase
M-CSF  macrophage colony stimulating factor
MDM  monocyte-derived macrophages
MGL  galactose/N-acetylgalactosamine-specific macrophage C-type lectin
PI  propidium iodide
r  recombinant

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