Human Macrophage Maturation and Heterogeneity: Analysis With a Newly Generated Set of Monoclonal Antibodies to Differentiation Antigens

By Reinhard Andreesen, Klaus J. Bross, Jürgen Osterholz, and Frank Emmrich

We have analyzed the expression of late differentiation antigens during terminal in vitro maturation of human macrophages (MØ) from blood monocytes (MO) in comparison to their distribution among mature MØ residing in various tissue sites. By immunizing mice with MØ derived from blood MO by culture on hydrophobic Teflon foils, monoclonal antibodies (mAbs) were developed (MAX.1, MAX.2, MAX.3, MAX.11) that reacted with lineage-restricted differentiation antigens. These antigens were expressed exclusively on MØ or were markedly increased after in vitro differentiation. The only overlap to another hemopoietic cell lineage was observed with MAX.3, which is shared by platelets and megakaryocytes. In the course of MØ maturation in vitro, the MAX.1 and MAX.3 antigens are detected within the cytoplasm two days before they appear on the cell surface. In contrast, the MAX.11 antigen is expressed simultaneously in the cytoplasm and at the cell surface, is found in varying degrees on a minor portion of blood MO and U937 cells, and is expressed rapidly at high density during early MØ differentiation in vitro. Among conventional mAbs that do not react with MO we found those against the transferrin (TF)-receptor, the BA-2, and the PCA1 antigen to label MØ. MØ matured in vivo and isolated from body fluids were positive with some but not all MAX mAbs. Distinctive patterns were observed with pulmonary MØ, exudate MØ from pleural and peritoneal effusions, synovial fluids, and early lactation milk. MØ from the alveolar space, for example, constantly expressed the MAX.2 antigen but not the MAX.3 antigen. Pleural effusion MØ, however, did not react with the MAX.1 mAb, but in most cases, it did react with the MAX.3 mAb. The detection of novel differentiation antigens, all expressed on monocyte-derived MØ but differently expressed on site-specific MØ in situ, underlines the remarkable heterogeneity among human MØ. The expression of these antigens is flexible because those MAX antigens that were not expressed in situ could be induced if cells from distinct tissue sites were cultured in vitro for several days. MAX mAbs may be of potential value to study both the sequential stages of maturation within the MØ lineage as well as differential developments induced by various culture conditions in parallel to environmental factors in vivo.

MATERIALS AND METHODS

Generation of MAX mAbs

Two-month-old BALB/c mice were injected five times intraperitoneally at weekly intervals with 5 × 10⁶ monocyte-derived MØ that were collected from hydrophobic Teflon bags after culture of blood-borne MO for at least ten days (for characterization of the cells see Table 1). Three and a half days after the last injection, immune spleen cells were fused with the HAT-sensitive line X63Ag8.653. Hybridoma supernatants were tested for binding to autologous B cells, T cells, MO, and monocyte-derived MØ by a cell-ELISA (enzyme-linked immunosorbent assay) and by the immunoperoxidase slide technique as described later. Finally, five hybridomas were selected because of their ability to discriminate between MO and MØ. They were subjected to recloning and further expansion. Cytofluorometric analysis (ORTHO 50-11) was used to confirm the reactivity pattern as evaluated by cell-ELISA and the immunoperoxidase method.

MAbs to Cell Surface Antigens Used in This Study

OKT3, OKT4, OKT8, OKT6, OKT9, OKM1, and OKIa1 were purchased from Ortho Pharmaceutical Corp, Raritan, NJ; antihuman...
monocyte from Bethesda Research Lab Inc, Gaithersburg, MD; Coulter Clone B1, BA-1, BA-2, My4, and PCA1 from Coulter Immunology, Hialeah, Fla; Leu-2, Leu-3a, Leu-3b, Leu-7, and antihuman transferrin receptor from Becton Dickinson, Erembodegem, Belgium; antihuman IgG1, IgG2, IgG3, and IgG4 from Bio-Yeda Ltd, Rehovot, Israel; Ki-1, Ki-M1, and Ki-M5 from Behringwerke, Marburg, FRG; and anti-C3b from Dakopatts, Copenhagen. MØ S39 was a generous gift from Dr R. Winchester, Orthopedic Institute, Hospital for Joint Diseases, New York, and anti-Tac from Dr T. Waldmann, NIH, Bethesda, Md. A polyclonal rabbit antitransferrin antibody was purchased from Behringwerke AG.

Cells

Blood monocytes. Buffy coat cells from normal blood donors or whole blood from patients with acute and chronic monocytic leukemias (classified by morphology, cytochemistry, and surface antigen analysis) were diluted 1:1 with phosphate-buffered saline (PBS) and centrifuged over Ficoll-Hypaque (F/H) to separate mononuclear cells (MNCs) from granulocytes and red cells. MO were separated from lymphocytes usually by adherence: 3 x 10^6 MNCs/mL RPMI 1640 (supplemented with l-glutamine, antibiotics, 5 x 10^{-5} mol/L 2-mercaptoethanol, and vitamins) plus 20% fetal calf serum (FCS) were incubated for 60 minutes in plastic Petri dishes. The nonadherent cells were removed by repeated washings with warm serum-free medium and the adherent cells (>90% MO as revealed by morphology and phenotype analysis) incubated overnight in RPMI 1640 (supplemented with MEM vitamins, penicillin 50 U/mL, streptomycin 50 μg/mL, 2-mercaptoethanol 5 x 10^{-5} mol/L, l-glutamine 2 mol/L) plus 10% human AB-group serum. MO could then be recovered from the dishes at 4°C by vigorous pipetting. In some experiments, the MNCs were suspended in 0.1% EDTA containing PBS and loaded onto an elutriator centrifuge (J2-21, standard chamber, Beckman, Stockholm) with a flow rate of 10 mL/min. Lymphocytes were removed at 10 mL/min and 12.4 mL/min with 200 mL and 100 mL of PBS, respectively. The MO-enriched cell fraction was then collected at a flow rate of 14.0 mL/min with 150 mL PBS and contained >70% MO as judged by phenotype analysis.

Alternatively, MNCs were layered on top of a continuous Percoll (Pharmacia Fine Chemicals, Freiburg, FRG) gradient with densities ranging from 1.06 g/mL to 1.08 g/mL and centrifuged for 20 minutes at 1,000 g. MO-enriched fractions of about 70% purity could be collected from a band just below the dead cells that did not enter the gradient.

Regardless of the isolation procedure used, MO were then placed into rectangular bags of hydrophobic Teflon foils (Biofolie 25, Heraeus, Hanau, FRG) at a concentration of 3 x 10^7 cells/mL RPMI 1640 plus 10% human AB serum. MO could then be harvested from the bags and taken for phenotype analysis.

granulocytes. After F/H centrifugation of diluted buffy coat cells, the pelleted granulocytes and red cells were resuspended in equal volumes of PBS and hydroxyethylstarch. After sedimentation for 30 minutes at 1 g the granulocyte-rich supernatant was centrifuged and the remaining erythrocytes lysed by five-minute exposure to 0.16 mol/L ammonium chloride and 0.17 mol/L trishydroxymethylaminomethan (Tris, Merck, Darmstadt, FRG) at 37°C.

Megakaryocytes. Megakaryocytes were identified by morphology in bone marrow MNC suspensions obtained from healthy volunteers by posterior iliac crest aspiration and subjected to F/H centrifugation.

Thrombocytes. Thrombocytes were collected from the plasma supernatants after F/H centrifugation and sedimented for 10 minutes at 800 g.

T and B cells. Cells were enriched by rosetting MNCs with AET-treated sheep erythrocytes and subsequent centrifugation over F/H. T cells were activated by incubation with concanavalin A (2 μg/mL) for 72 hours.

Pulmonary MØ. Macrophages were collected from the broncho-alveolar lavage fluids of healthy volunteers and patients undergoing diagnostic bronchoscopy after giving informed consent.
**Pleural and peritoneal MΟ.** Macrophages were isolated from effusion fluids secondary to heart failure, portal hypertension, or malignancies.

**Synovial MΟ.** Macrophages were collected from samples obtained by diagnostic puncture of knee joints.

**Milk MΟ.** Macrophages were obtained from breast milk samples taken at day 3 to day 9 postdelivery.

**Human tumor cell lines.** DHL-1 (diffuse histiocytic lymphoma, from Dr M. Scott, Stanford University, Calif), U937 (histiocytic lymphoma, from Dr K. Nielsen, Wallenberg Lab, Uppsala, Sweden), HL-60 (promyelocytic leukemia, from Dr R. Vogler, Emory University, Atlanta), LBL6078 and LBL3283 (B lymphoblastoid cells), Mel-1 and Mel-2 (melanoma xenografts in nude mice, from Dr H. Fiebig, Medizinische Klinik, Freiburg, FRG), and Colo38 (melanoma cells, from Dr G. Moroni, University of Napoli, Italy).

**Immunoperoxidase Slide Technique**

For surface marker analysis, the cells were washed three times with cold PBS and transferred onto glass slides coated with 0.1% alcian blue in hydroxyethylstarch. After attachment of the cells to the negatively charged glass surface, they were fixed at 4°C with 0.05% glutaraldehyde in 0.1 mol/L PBS containing 10% glucose. They were then washed with a glycine containing medium and preincubated with a gelatine containing medium to prevent immunoglobulin binding to the glass surface. The staining procedure included the following steps: (1) 15-minute incubation with mAbs diluted in gelatine containing medium; (2) five-minute incubation with mAbs of different Ig class that did not react with the cells to be tested; (3) another five-minute incubation with swine anti-rabbit Ig (Dakopatts) absorbed as described; (4) incubation with peroxidase-antiperoxidase complex from rabbits (Dakopatts) diluted 1:20; (5) incubation with diaminobenzidine-H2O2 for ten minutes, followed by postfixation with OsO4. The slides were covered with a solution of 80% serum, and 10% glycerine, which was also used for dilution of mAbs, rabbit antimouse Ig, swine antirabbit Ig, and the peroxidase-antiperoxidase complex. The staining procedure was performed as described but each incubation was extended to 15 minutes.

**RESULTS**

To develop mAbs directed against lineage-restricted human MΟ differentiation antigens, we used monocyte-derived MΟ cultured in hydrophobic Teflon foils. This method prevents the spreading of MΟ on the surface, and thus allows the recovery of cells without damage. Cells cultured in this way meet all the morphologic, cytochemical, antigenic, and functional criteria for MΟ ex vivo (see Table 1 for full characterization, and also refs 12, 20, 22). In this report we give detailed information on five mAbs of the MAX series that discriminate between MΟ and MΟ. The reactivity pattern of these antibodies is described in Table 2. Two mAbs (MAX.1 and MAX.2) were reactive with membrane structures that are selectively expressed on MΟ at differentiation stages beyond the blood monocyte level and not found on any other cell type studied. MAX.3 is a similar mAb, with the exception that it also reacted with platelets and megakaryocytes. MAX.11 is infrequently expressed on a minor subset of blood MΟ and U937 cells. MAX.1, MAX.2, and MAX.3 each precipitated membrane molecules of distinct molecular weight: MAX.1 precipitated a 64-kd protein, MAX.2 a 200-kd, and MAX.3 a 68-kd molecule (F. Emmrich and R. Andreesen, manuscript in preparation).

The expression of MAX. antigens in vitro was independent of the separation procedure used to isolate MΟ from other MNCs (adherence, elutriation, or density centrifugation) and was also independent of the culture substrate used (ie, grown as a monolayer on glass or as a suspension in Teflon reagent on the antigenic determinants was tested by adding mAbs to membrane supernatants were diluted 1:10. All cell lines were tested at least three times.

| Table 2. Cellular Distribution of Surface Antigens Reactive With MAX. Monoclonal Antibodies |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Monoclonal Antibody Designation | Cells | MAX.1 | MAX.2 | MAX.3 | MAX.11 |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Monocyte-derived MΟ | + | + | + | + | + |
| Adherent monocytes (day 1) | - | - | - | - | var |
| Granulocytes | - | - | - | - |
| Platelets/megakaryocytes | - | - | + | - |
| B cells | - | - | - | - |
| T cells | - | - | - | - |
| Activated T cells | - | - | - | - |
| Permanent cells lines | - | - | - | - |
| L-428 (Hodgkin cells) | - | - | - | - |
| U-937 (histiocytic lymphoma)* | - | - | - | + |
| DHL-1 (histiocytic lymphoma) | - | - | - | - |
| HL-60 (promyelocytic leukemia) | - | - | - | - |
| B lymphoblastoid cells† | - | - | - | - |
| Melanoma cells‡ | - | - | - | - |

Cell distribution was evaluated by immunoperoxidase staining; hydrosupernatants were diluted 1:10. All cell lines were tested at least three times: MΟ, MΟ (10- 15-day-old MΟ cultures), B cells (non-rosetting cell population containing 55% B1° cells, MΟ and less than 2% OKT3° T cells) and T cells (>90% purity) were obtained from ten donors, whereas activated T cells (about 40% la° blast cells), platelets, and granulocytes were from three donors. + +, more than 90% of cells reactive; +, +, more than 90% to 15% of cells reactive; +, more than 90% to 15% reactive; var, reactivity varied among experiments from negative to up to 20% positive cells; -, no reactivity.

*Varying portions of positive cells on repeated tests with the same line (15-80%).
†LBL 6078 and LBL 3283.
‡Xenotransplanted tumors Mel-1 and Mel-2 (tested either freshly prepared from tissue specimens or after three or four serial passages in vitro) and permanent cell line Colo 38.

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During MO maturation in vitro, the differentiation antigens defined by MAX mAbs were expressed consecutively as shown in Fig 1A through D. Whereas nearly all mature MO reacted with MAX.1 and MAX.3, only up to 70% of the cells were positive for the MAX.2 antigen. MAX.11 antigen appeared to be the first antigen to be fully expressed (after three days) and MAX.3 the latest antigen not being detectable in three-day-old MO cultures but was fully expressed by day 9.

MAX.1 and MAX.3 antigen appeared in the cytoplasm two to three days earlier than they were expressed on the cell surface (Fig 2), whereas cytoplasmic and surface labeling of MAX.11 antigen coincided (not shown). MAX.2 antigen seemed to be sensitive to the methanol treatment that was required for intracellular staining. This treatment completely abrogated the reaction of glutaraldehyde-fixed cells with this antibody. MAX.1 and MAX.3, but not MAX.2, reactivity was lost after fixation at room temperature.

In addition, a series of conventional mAbs that do not react with blood MO was tested routinely as control for binding to in vitro-matured MO (OKT3, OKT4, OKT6, OKT9 directed to the TF-receptor, Leu-2, Leu-3a, Leu-3b, Leu-7, B1, BA-1, BA-2, PCA1, anti-Tac, Ki-1). Interestingly, mAbs directed to the TF-receptor,23 and the BA-2 antigen24 labeled mature MO to varying degrees (Fig 3). Only two days later, during in vitro differentiation, TF molecules were detected on the cell membrane (Fig 3A). The PCA1 antigen25 also appeared to be expressed only on mature MO with 17% to 72% of cells reacting (not shown). We observed in some experiments a weak reactivity of MO with OKT4 and anti-Tac that, however, did not depend on the stage of maturation.

In order to investigate the expression after in vivo maturation of the differentiation antigens described, we isolated MO from various body cavities (Table 3).

A considerable heterogeneity was found concerning membrane antigen expression in MO from different sites and also between different individuals. Some generalizations seem to be possible: MAX.3 was absent from pulmonary and breast milk MO but present on most pleural and some peritoneal MO. MAX.1 could be detected only on minor portions of pulmonary and peritoneal MO but never on pleural or breast milk MO. However, if pulmonary and peritoneal MO with distinct surface phenotypes were cultured on hydrophobic Teflon foils, the cells readily expressed these MAX antigens that were missing in situ (Table 4).

Up to now no significant difference could be detected in the MAX antigen expression on pulmonary MO from smokers and nonsmokers, whereas with pleural and peritoneal...
HUMAN MACROPHAGE ANTIGENS

Fig 3. Expression of lineage-restricted differentiation antigens during macrophage maturation in vitro: the TF-receptor and surface TF (A) and the BA-2 antigen (B). For experimental details, see Fig 1.

MO, a great variability among patients in terms of MAX antigen expression was seen. This is possibly due to the different diseases that had led to pleural or peritoneal effusions, a problem that will be studied in more detail. No different diseases that had led to pleural or peritoneal antigen expression was seen. This is possibly due to the details. see Fig 1.

MAX.1, MAX.2, and MAX.3, but could be induced in culture to express MAX.2 (R. Andreesen and H. G. Leser, to be published).

Studies concerning in situ localization of MAX differentiation antigens using a sensitive four-layer immunoperoxidase technique are in progress.

DISCUSSION

We have analyzed the expression of antigens on human mononuclear phagocytes as they undergo terminal maturation. Differentiation from monocyte precursor cells as a more or less homogeneous population in the peripheral blood leads to heterogeneous mature cells residing in various anatomical sites of the organism. Despite the changes in morphology, biochemistry, and function that occur while MO transform into mature cells, surprisingly little is known about the loss or acquisition of surface antigens beyond the blood monocyte stage of differentiation. With the new mAbs, we have identified two molecules (MAX.1 and MAX.2 antigens) on mature MO which are absent from MO and any other cell type tested so far, and a third molecule (MAX.3 antigen), which is shared only by platelets and megakaryocytes. Other antigens have been reported that are shared by the megakaryocyte and MO lineages, such as the MPA antigen, which differs, however, from MAX.3 as it is present on freshly harvested blood MO. As has been described for immunoglobulins during B cell differentiation, these maturation-related molecules are first detected in the cell cytoplasm before they appear on the outer cell surface.

All three antigens seem to be unique and different from any other reported so far: the PAM1 antigen detected by Biondi et al.,28 like MAX.2, also binds to a 200-kd structure that, according to their studies, is not expressed on monocyte-derived MO and is restricted to pulmonary MO; the BMM1 mAb reported by the same authors to be specific for

Table 3. Heterogeneity of Human Macrophages as Defined by Surface Antigen Analysis

<table>
<thead>
<tr>
<th>Source of Macrophages</th>
<th>MAX.1</th>
<th>MAX.2</th>
<th>MAX.3</th>
<th>MAX.11</th>
<th>OKT9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal blood (n = 6)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>21 ± 17</td>
<td>None</td>
</tr>
<tr>
<td>Monocytic leukemia (n = 5)</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>57 ± 28</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Monocyte-derived (Teflon bag, n = 6)</td>
<td>&gt;90</td>
<td>61 ± 10</td>
<td>&gt;90</td>
<td>&gt;90</td>
<td></td>
</tr>
<tr>
<td>Pleural effusion (n = 8)</td>
<td>&lt;1</td>
<td>23 ± 21</td>
<td>51 ± 28</td>
<td>41 ± 27</td>
<td></td>
</tr>
<tr>
<td>Ascites fluid (n = 12)</td>
<td>(2–61)</td>
<td>(15–80)</td>
<td>(10–80)</td>
<td>(0–10)</td>
<td></td>
</tr>
<tr>
<td>Alveolar lavage fluid (n = 14)</td>
<td>17 ± 18</td>
<td>16 ± 18</td>
<td>60 ± 24</td>
<td>46 ± 28</td>
<td></td>
</tr>
<tr>
<td>Breast milk (n = 4)</td>
<td>(0–50)</td>
<td>(18–95)</td>
<td>(10–90)</td>
<td>(0–45)</td>
<td></td>
</tr>
<tr>
<td>Synovial fluid (n = 4)</td>
<td>7 ± 6</td>
<td>84 ± 8</td>
<td>&lt;1</td>
<td>62 ± 30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0–16)</td>
<td>(10–94)</td>
<td>(10–94)</td>
<td>&gt;90</td>
<td></td>
</tr>
</tbody>
</table>

Monoclonal antibody reactivity was evaluated by immunoperoxidase staining on alcin blue-coated slides; MAX. mAbs were diluted 1:10 (hybridoma supernatant). OKT9 1:100 (ascites fluid). Values are given as mean percentage of positive monocyte-macrophages identified by morphology and in comparison by binding of monococyte-specific mAbs. The range within individual values is given in parentheses. MO were assayed for reactivity with mAbs in freshly prepared mononuclear cell suspensions where they represented 12% to 34% of total cells. All leukemic MO populations tested were of more than 90% purity. Except for alveolar lavage fluids, which always contained more than 80% of MO, the MO content in other body fluids varied from 18% to 68%.
breast milk MΦ in fact reacted with minor subpopulation of a T cell line (HPB-ALL) and a minor portion of blood MΟ; the 27F9 antigen reported by Zwadowski et al.29 is present on melanoma cells, differs in the molecular weight, is expressed only up to 70% on monocyte-derived MΟ, and can be detected on Kupffer cells. MAX.11 reacts with a lineage-restricted molecule that, however, is found to variable degrees on a minor portion of blood MO and U937 cells but is rapidly expressed on more than 95% of MΟ at day 3 in Teflon cultures of MO. Somewhat similar seems to be the Mo3 antigen30 that is found, although at low density, on the majority of blood MO and shows increased expression after overnight incubation.30

Other groups have developed mAbs specific for the monocyte-macrophage lineage but that are not able to distinguish between MO and mature MΟ: MΟ P15 and MΟ S39 by Dimitriu-Bona et al.,17 Mo2 by Todd and Schlossmann,49 some of the FMC series by Hancock et al.,31 the PHM3 by Becker et al.,32 the 3C10 and 1D9 by Van Voorhis et al.,33 and the KiM1 by Radzun and Parwaresch.44

The MΟ antigens described here do not seem to be the result of culture artifacts as (1) they all could be detected, although in a distinctive pattern, on in vivo-matured MΟ and in vitro,21,35,38 (b) the bactericidal activity39 and hydrogen peroxide metabolism,40 (c) the accessory cell function4 and the capacity to kill tumor cells in vitro,21,35,38 (3) in vitro culture, it supports the hypothesis that local factors in the microenvironment control the distinct phenotypes observed. This seems to be more likely than the existence of MΟ subsets, which are committed to terminal differentiation into pulmonary, plural, or peritoneal exudate MΟ, respectively.

The detection of antigens unique for late in vitro differentiation stages of MΟ and at the same time differentially expressed on subtypes of mature MΟ of various anatomical sites might be of functional significance, as both maturation in vitro as well as generation of site-specific heterogeneity in vivo seems to be accompanied by quantitative changes in certain MΟ functions: (a) the capacity to kill tumor cells in vitro,21,35,38 (b) the bactericidal activity39 and hydrogen peroxide metabolism,40 (c) the accessory cell function41 and production of interleukin 1,12,43 and our own unpublished results.

MΟ differentiation in vitro is followed by an increase in ferritin synthesis,21 a change in the glycolipid pattern (M. Rössle and R. Andreesen, submitted for publication), and in cell adhesion behavior,44 the induction of cAMP-dependent protein kinase A45 and calmodulin-binding protein,46 and the expression of ecto-5'nucleotidase47 and creatine kinase.48 Thus, it has to be established whether the expression of the MAX. antigens is related to some of those features. This is subject to current investigations. Preliminary data seem to indicate that MAX. antigens are not involved in tumor cytotoxicity or in oxidative metabolism and Fc-receptor-mediated phagocytosis (to be published).

Besides lineage-restricted differentiation antigens, we found also nonlineage-restricted antigens that are newly expressed (TF-receptor, BA-2, TF-R, and PCA1). The TF-receptors may serve to deliver iron to intracellular stores. The detection of cell surface TF two days later than the expression of TF receptor in culture has to be explained. It may be due to incomplete expression of TF-receptors not yet capable of binding TF. Alternatively, membrane-bound TF by itself might be a MΟ product that is detected independent of the TF-receptor. Data from biosynthetic labeling experiments indicate that TF is synthesized by mature MΟ and probably processed to surface membrane sites distinct from the TF-receptor reactive with the OKT9 mAb (R. Andreesen and G.
Bauer, manuscript in preparation). Both the TF-receptor and surface TF could function to facilitate tumor cell–Mφ interaction, as has been shown for natural killer cell activity. Together with the MAX series, mAbs to the TF receptor, the BA-2, and the PCA-l antigen seem to be useful for a phenotypic followup of Mφ terminal maturation. The novel differentiation antigens described here may also serve to indicate distinct pathways of differentiation, although their functional significance has yet to be established.

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R Andreesen, KJ Bross, J Osterholz and F Emmrich

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