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Ly-6C is a monocyte/macrophage and endothelial cell differentiation antigen regulated by interferon-gamma*

Using a new Ly-6C-specific antibody (Monts-1) we show that this class of antigens are differentially expressed on monocytes/macrophages and endothelial cells. Recently elicited peritoneal exudate Mac-1$^+$ mononuclear cells, as well as Mac-1$^+$ mononuclear cells in the bone marrow and in the peripheral blood, express high levels of Ly-6C. Ly-6C$^+$ mononuclear Mac-1$^+$ cells are absent in normal uninflamed skin, but are present in high numbers in skin lesions 3 days after the s.c. injection of lipopolysaccharide, concanavalin A or complete Freund's adjuvant. In addition, large Ly-6C$^+$ mononuclear cells are predominant in chronic granulomas induced by complete Freund's adjuvant. Resident macrophages in a variety of tissues express low levels or in many cases do not express Ly-6C. Two out of three monocyte-like cell lines are Ly-6C$^+$, whereas macrophage-like cell lines are negative. Ly-6C$^+$ monocytes/macrophages lose the Ly-6C antigen within 24 h after *in vitro* culture. Ly-6C$^-$ cultured monocytes and Ly-6C$^-$ monocyte-like cell lines, but not fully differentiated macrophages and macrophage-like cell lines, can be induced to express the Ly-6C antigen by interferon-γ. A population of small vessel endothelial cells in diverse tissues also express high levels of Ly-6C. The present findings suggest that the Ly-6C antigen family, shown by others to be involved in T cell activation, may have more general importance in immune responses and cellular differentiation than previously appreciated.

1 Introduction

Monocytes (MO) are believed to accumulate in various tissues where they undergo tissue-specific differentiation, ultimately giving rise under normal circumstances to resident macrophages (MΦ). The resident MΦ is thought to be important in homeostasis and, through presentation of antigen, in immune reactions. Due to the location of these cells throughout the host, they are also likely to serve as a primary non-specific host effector cell against many infectious agents. Inflammation results in increased extravasation of peripheral blood MO, as well as their accumulation and activation in the inflamed tissues. The "activated" MO-derived MΦ exhibits enhanced anti-microbial, tumoricidal and secretory activities, that are most likely crucial for effective host responses to agents that are not eliminated by the resident MΦ (reviewed in [1]).

In the mouse, the MO, resident MΦ and "activated" MΦ phenotypes are normally characterized by morphological, enzymatic and/or functional criteria. Antibodies have been raised against these cells [2–7], but few of these antibodies are effective in specifically separating these phenotypes. Additional antigenic markers would greatly facilitate *in vivo* studies of the cellular events that occur during MO/MΦ differentiation and activation, events that are only partially understood.

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Abbreviations: APCy: Allophycocyanin **PEC:** Peritoneal exudate cells **PC:** Peritoneal cells **IF:** Immunofluorescence **LPS:** Lipopolysaccharide **Con A:** Concanavalin A **CFA:** Complete Freund's adjuvant **IFN:** Interferon **FITC:** Fluorescein isothiocyanate **FACS:** Fluorescence-activated cell sorter **MO:** Monocyte(s) **MΦ:** Macrophage(s) **PMN:** Polymorphonuclear (cells)

As one approach to define the antigenic changes in MO and MΦ induced by inflammation, we have generated monoclonal antibodies against complete Freund's adjuvant (CFA)-inflamed mouse lymphoid tissue and screened for inflammation-specific antibodies that recognize MO/MΦ subsets. Here we describe the production of one such antibody, Monts-1, which we show to recognize a Ly-6C antigen. Ly-6C antigens are encoded by a complex multigene family and have primarily been defined as novel T cell-activating determinants [8–14]. Using Monts-1, we establish that Ly-6C is an immune-regulated MO/MΦ and endothelial cell differentiation antigen.

2 Materials and methods

2.1 Animals

BALB/c, AKR, C57BL/6, C3H and DBA/2 mouse strains were obtained from the Institute for Medical Research at San Jose, CA, and from the Jackson Labs., Bar Harbor, ME. Wistar rats were obtained from Simonsen, Gilroy, CA.

2.2 Immunization and hybridoma production

Twenty mice received footpad injections of 0.1 ml of CFA (Sigma, St. Louis, MO) and 3 days later their brachial, axillary and popliteal lymph nodes were collected. The inflamed lymph nodes were minced and gently pressed through wire mesh. Lymph node stromal fragments were collected from the mesh, washed in HBSS (Applied Scientific, San Francisco, CA) and isolated by 1 × g sedimentation for 10 min. The stroma, which included endothelium, MO/MΦ and some lymphocytes, was mixed with precipitated potassium aluminum sulfate as adjuvant. The precipitate was injected i.p. into a 3-mth-old Wistar rat. The immunization procedure was repeated 3 times at 3- to 4-wk intervals. Four days prior to the fusion the rat was boosted i.p. with inflamed lymph node stroma in normal saline. The rat spleen cells were fused to hypoxanthine,aminopterin,thymidine-sensitive mouse Sp2/0 myeloma

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cells [American Type Culture Collection (ATCC), Rockville, MD] as previously described [15]. After 10 days the supernatants from the hybridoma cultures were screened for inflammation/MO/MΦ-specific antibodies. Screening was done by immunofluorescence microscopy on acetone-fixed frozen tissue sections of CFA-inflamed and uninflamed peripheral lymph nodes using fluorescein isothiocyanate (FITC)-conjugated goat anti-rat Ig (Sigma, St. Louis, MO) as second stage.

One inflammation-specific antibody-producing hybridoma was selected (Monts-1), subcloned twice by limiting dilution, expanded and adapted to HB101 serum-free medium (New England Nuclear, Boston, MA). Antibody was precipitated from these supernatants with 50% saturated $(NH_4)_2SO_4$ and extensively dialyzed against phosphate-buffered saline (PBS). The antibody was purified and conjugates were made as described [16]. The isotype of Monts-1 was determined by an Ouchterlony anti-rat Ig isotype kit (Sigma) and was shown to be of the IgG_{2a} isotype.

2.3 Antibodies and cell lines

Table 1 lists the antibodies used in this study. Purification and conjugation of antibodies to biotin, FITC or allophycocyanin (APCy) was as described [16]. The J774.1 [24], WEHI-265.1 [25], WEHI-274 [25], RAW264 [26], WEHI-3 [24], PU5-1.8 [24] and WEHI-78/24 monocytoid cell lines were obtained through ATCC or from R. Coffman, DNAX, Palo Alto, CA. The lines were grown in cRPMI (RPMI; Applied Scientific + 10% fetal bovine serum and antibiotics) at 37 °C under 6.5% CO_2 and characterized by the following criteria: phagocytosis of yeast or fluorescent beads, adherence to plastic, morphology in cytosmears and by Mac-1 [2] staining. The Mac-1 antigen is expressed at high levels on thioglycollate-elicited inflammatory MO/MΦ. Fully differentiated resident tissue MΦ and polymorphonuclear neutrophils (PMN) express lower levels of the Mac-1 antigen [2]. Cell lines that were nonadherent, slightly phagocytic, had few cytoplasmic vacuoles and were Mac-1$^+$ phenotypically were considered monocyte like: these were the WEHI-78124, WEHI-265, and WEHI-274 lines. The cell lines that were actively phagocytic, strongly adherent and Mac-1$^+$ were considered MΦ like: these were the J774.1 and RAW264 lines. The WEHI-3 and PU5 cell lines gave intermediate phenotypes. Our criteria of MO and MΦ phenotype gave results similar to the original descriptions of the cell lines.

2.4 Cell suspensions

Peritoneal cells (PC) were collected from unstimulated BALB/c mice and mice 2 and 4 days after the i.p. injection of 2 ml of Brewer's thioglycollate broth (Sigma). Bone marrow cells were collected from mouse femurs. Spleens and lymph nodes were also collected, minced and gently pressed through wire mesh, and leukocytes were obtained. In all cases the cell suspensions were washed in Hanks' balanced salt solution and resuspended at 2×10^7 cell/ml for immunofluorescence staining or at other cell concentrations for *in vitro* culturing (see below).

2.5 *In vitro* treatment of MO/MΦ and monocytoid cell lines with IFN-γ

Two-day thioglycollate-induced peritoneal exudate cells (PEC) and resident PC, and the monocytoid cell lines described above, were resuspended at 1×10^6 cells/ml in cRPMI and plated into the wells of 96-well plates in 0.1 ml/well. The PEC were allowed to adhere and the monolayers were washed to remove nonadherent cells. The cells were incubated with different doses of recombinant murine interferon-gamma (IFN-γ, Genentech, San Francisco, CA). The cells were cultured for 48 h and the expression of the Monts-1 antigen was determined by immunofluorescence. In some experiments, the expression of Ia antigens was also examined after the IFN-γ treatment.

2.6 Preparation of skin lesions

An s.c. injection of emulsified CFA, or 50–100 μl of 1 mg/ml concentrations of lipopolysaccharide (LPS; *E. coli* 111:B4, Sigma) or concanavalin A (Con A; Sigma) was made on the backs of mice. Three days later inflamed tissues were harvested, frozen and sectioned for immunofluorescence staining. For chronic granuloma production, the CFA tissues were harvested 3 wks after the injection.

2.7 Immunoperoxidase staining

Acetone-fixed 6–12-μm frozen sections were incubated with Monts-1 in PBS (50 μg/ml), washed in PBS and incubated with horseradish peroxidase-conjugated rabbit anti-rat IgG (Dako,

Table 1. List of antibodies used in this study including their sources and specificities

a) Natural killer cells.
b) A. M. Stall, S. Fiering and L. A. Herzenberg, unpublished.

Copenhagen, Denmark) used at 1:40 and containing 5% normal mouse serum to block reactivity with endogenous Ig. Sections were then washed in PBS treated with diaminobenzidine/H_2O_2 solution to detect bound second-stage Ig and then incubated in 0.5% copper sulfate in saline to enhance the reaction product. Sections were lightly counterstained with hematoxylin.

2.8 Immunofluorescence staining and fluorescence-activated cell sorter (FACS) analysis

Immunofluorescence staining of cells was carried out in microtiter plates as described [16]. Briefly, 500000 cells were incubated with FITC and biotin-conjugated antibodies for 20 min on ice for two-color staining. For three-color staining, a third APC-conjugated antibody was added. After washing, bound biotin antibodies were revealed by incubation with avidin-Texas red. Finally, cells were resuspended in medium containing propidium iodine (1 µg/ml) to stain dead cells. FACS analysis was performed on a FACS II (Becton Dickinson, Sunnyvale, CA) as described [16]. Data were collected from 20000–30000 viable cells, and are presented as 5% probability contour plots or histograms.

2.9 Western blot SDS-PAGE analysis

Lysates of bone marrow cell suspensions were prepared by incubating 2×10^7 isolated bone marrow cells from the femurs of BALB/c mice in 0.5 ml lysis buffer (2% Nonidet-P40, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 1 mM phenylmethylsulfonyl fluoride in 50 mM Tris-HCl, pH 8.0) for 30 min on ice. Lysates were clarified by centrifugation at $20000 \times g$ for 10 min, mixed with an equal volume of $2\times$ reducing or nonreducing solubilization buffer, run on a 17% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel according to the method of Laemmli [27], and transferred to nitrocellulose with a Bio-Rad (Richmond, CA) transblot apparatus according to the manufacturer's directions. Filters were incubated with 50% horse serum in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween-20) for 1 h. Using a 25-lane mini-blot apparatus (Immunonetics, Cambridge, MA), the filters were then incubated for 30 min with either Monts-1, 6C3, Al-21 or an isotype control antibody, Monts-4, which recognizes a determinant expressed on resident MΦ (Jutila, M. A. et al., in preparation), at 50 µg/ml concentrations. The strips were then washed in TBST, incubated with goat anti-rat Ig-alkaline phosphatase conjugate (Sigma, A-9654), diluted 1:200, and then washed again. The blots were developed by addition of substrate solution from Promega Biotech, (Madison, WT).

In pre-clearing experiments, Monts-1 and the control antibody RB6-8C5 were incubated at a concentration of 100 µg/ml at 4°C for 30 min with the bone marrow lysates described above. Afterwards the lysates were incubated in a similar manner with rabbit anti-rat Ig at a concentration of 100 µg/ml. Protein A coupled to Sepharose beads (Sigma) was used to precipitate out the antibody-antigen complexes, and the supernatant fluids were collected after centrifugation at $20000 \times g$ for 5 min. The procedure was repeated twice, and the lysates were run on a 17% gel and Western blots were done as described above.

3 Results

3.1 The Monts-1-defined antigen is Ly-6C

Western blot SDS-PAGE analysis using Monts-1 was performed on lysates of normal mouse bone marrow cells (shown below to express high levels of the Monts-1 antigen). Under nonreducing conditions, Monts-1 recognized a 14–17-kDa M_r molecule (Fig. 1, lane 3). Monts-1 recognized an identical band in lysates of monocytoid cell lines (data not shown). The antigenic determinant recognized by Monts-1 was lost under reducing conditions.

The molecular mass (M_r) of the Monts-1 antigen was similar to that described for molecules encoded by the Ly-6 loci [8–10, 20]. To explore this similarity, we employed two antibodies against Ly-6C, Al-21 (A. M. Stall, S. Fiering and L. A. Herzenberg, unpublished observations) and 6C3 [20]. Al-21 and 6C3 identified bands in Western blots identical to the determinant seen by Monts-1 (Fig. 1, lanes 1 and 2). Pre-clearing bone marrow cell lysates with Monts-1 removed the Al-21 and 6C3-defined antigens without influencing levels of the irrelevant 25–30-kDa antigen defined by RB6-8C5 (data not shown). Pre-clearing with RB6-8C5 did not alter the reactivity of Monts-1, Al-21 or 6C3. Furthermore, two-color immunofluorescence staining of inflamed lymph node tissue sections showed that all Monts-1$^+$ cells, including endothelial cells, were also Al-21$^+$ and 6C3$^+$.

Finally, Monts-1 stained a subpopulation (approximately 40–50%) of Ly-2$^+$ T cells in both the spleen and lymph nodes in BALB/c mice without reacting with L3T4$^+$ cells, but in C57BL/6 mice the antibody stained a subpopulation (approximately 40–50%) of both Ly-2$^+$ and L3T4$^+$ T cells. These strain differences in T subset reactivity are characteristic of Al-21

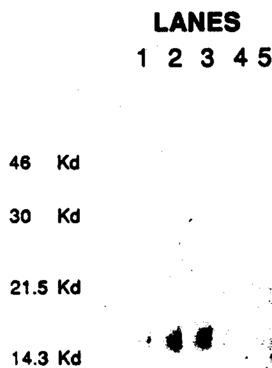


Figure 1. Monts-1 defines a 14000–17000-kDa M_r antigen identical to Ly-6C in Western analysis. A lysate of bone marrow cells was run on a 17% SDS gel and then transferred to nitrocellulose. The blot was probed with Monts-1, with the anti-Ly-6C antibodies 6C3 or Al-21, or with a class-matched negative control. Lanes 1, 2 and 3 were treated with Al-21, 6C3 and Monts-1, respectively. Lane 4 was treated with the isotype control antibody (Monts-4). Lane 5 was probed with second-stage antibody alone. Standards were ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa).

(Fiering, S. and Herzenberg, L. A., unpublished observations) and of Ly-6C [13]. The staining of bone marrow cells (see Fig. 5) is exactly as described for antibodies that recognize Ly-6C alloantigens [20]. Of interest was that Monts-1 did not stain B cells ($B220^+$, IgM^+) in normal lymph nodes, but did stain B cells in CFA-inflamed lymph nodes. The level of Monts-1 antigen on T and B cells ranged from one-tenth to one-hundredth the level on MO (described below).

The Western blot results, the preclearing experiments, the differential expression of the Monts-1 antigen on T cells in the BALB/c and C57BL/6 mice, and the two-color immunofluorescence analyses demonstrate that the Monts-1-defined molecule is Ly-6C.

3.2 The expression of Ly-6C on normal and inflamed tissues

Immunoperoxidase studies using Monts-1 on acetone-fixed tissue sections showed that Ly-6C was expressed at high levels in CFA-inflamed lymph nodes. The staining in inflamed tissues contrasted with lower and more variable staining in uninflamed lymph nodes (Figs. 2A and B). Monts-1 reactivity on lymph node sections was confined to cells within the T cell region and was only faintly seen in the MΦ-rich areas of the sinus and subcapsule. $Ly\text{-}6C^+$ cells were present in the red pulp of the spleen. Marginal zone MΦ and interdigitating cells of the T cell area in the splenic white pulp did not stain with Monts-1. Faint staining was observed in the medulla of the thymus. The liver was negative (data not shown). In addition to staining leukocytes in normal and inflamed lymph nodes, Monts-1 intensely stained a subpopulation of small vessel endothelial cells and this staining was not influenced by inflammation (Figs. 2A and B). Monts-1 stained cells in BALB/c, AKR, C3H, DBA and C57Bl/6 mouse strains, but not in human tissues.

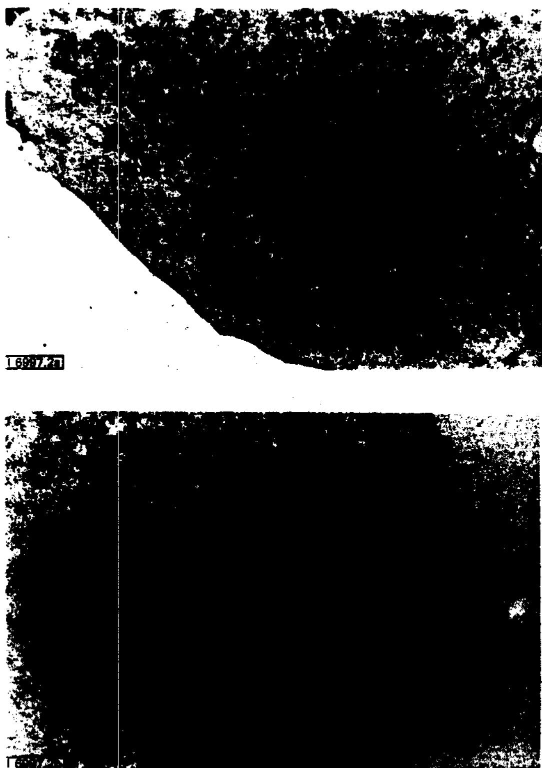


Figure 2. Immunoperoxidase staining using Monts-1 on acetone-fixed sections of normal and inflamed lymph node tissues. Monts-1 intensely stains many cells in the T cell region (paracortex, PC) of inflamed lymph nodes (A), but shows less intense reactivity on cells in uninflamed lymph nodes (B). Small vessel endothelial cells (arrow) express Ly-6C in both uninflamed and inflamed tissues. Magnification × 100.

Figure 3. Immunofluorescence staining using Monts-1 (A) and F4/80 (B) on acetone-fixed sections of 2-wk-old CFA-induced granuloma tissue. Monts-1 and F4/80 stain many large MO cells (arrow). Magnification × 200.

The influence of inflammation on the accumulation of $Ly\text{-}6C^+$ mononuclear cells was further studied in the skin. $Ly\text{-}6C^+$ cells were not present in normal uninflamed skin; however, 3 days after the s.c. injection of either LPS, Con A or CFA there was an influx of $Ly\text{-}6C^+$ mononuclear cells (data not shown). Monts-1 also stained many large mononuclear cells in 3-wk CFA-induced granuloma tissue (Fig. 3A). The expression of Ly-6C in the chronic granulomas was similar to the expression of the MO/MΦ-specific antigen recognized by the F4/80 antibody [3] (Fig. 3B). Many of the $Ly\text{-}6C^+$ cells in the granuloma were shown to be $Mac\text{-}1^+$, as well, in two-color immunofluorescence studies using biotin-Mac-1 and FITC-Monts-1 (data not shown). Quantitative FACS analysis using anti-Mac-1 and Monts-1 on inflammatory cell suspensions is presented below.

3.3 MO, monocytoid cell lines and a subset of MΦ express high levels of Ly-6C

The expression of both Ly-6C and the Mac-1 antigen by cells in granulomas, in combination with the apparent similarity of Monts-1 and F4/80 in these tissues, suggested that Monts-1 recognizes a Ly-6C antigen on some MΦ. Therefore, a number of MO/MΦ cell lines were screened for expression of Ly-6C. The cell lines, phenotypically characterized as MO like or MΦ like as described in Sect. 2.3, were studied both by immunostaining of acetone-fixed cytospins to assess cytoplasmic reactivity and by FACS to quantitate cell surface antigen. MO-like WEHI-265 was Ly-6C$^+$ after staining of cytospins and the cell line also expressed high levels of surface antigen. MO-like WEHI-78/24 expressed cytoplasmic antigen, but no cell surface antigen. MO-like WEHI-274, MΦ-like RAW264 and J774.1, and the cell lines with intermediate phenotypes, WEHI-3 and PU5, did not express significant levels of either cytoplasmic or cell surface antigen (Table 2).

The expression of Ly-6C on resident and inflammatory murine PC was also examined. Interestingly, Monts-1 intensely stained most 2-day thioglycollate-elicited PEC (a population of MO/MΦ and PMN), but reacted less with more differentiated resident MΦ present in the uninflamed peritoneal cavity (Table 2).

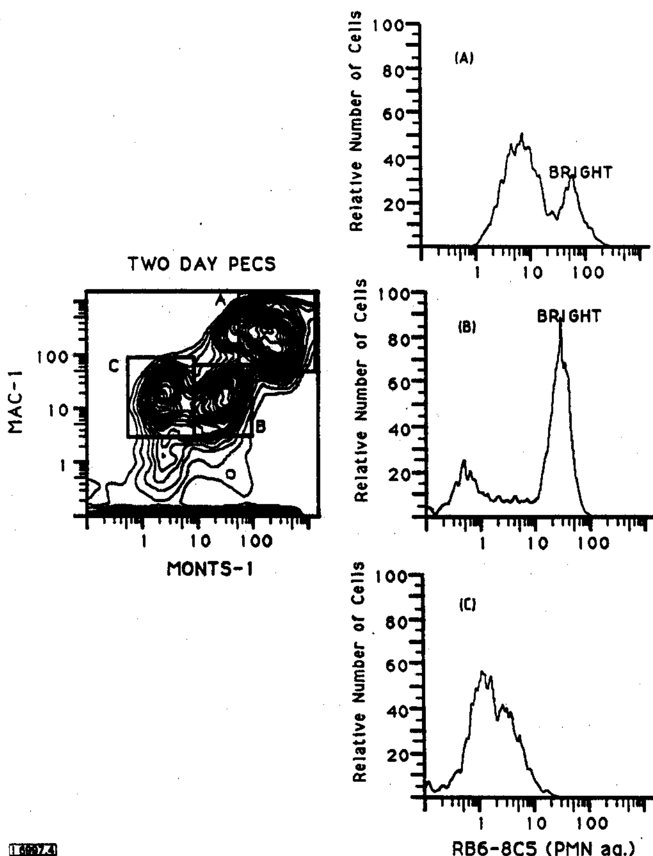


Figure 4. Three-color FACS analysis (FITC-RB6-8C5), APCy-Mac-1 and biotin-Monts-1/Texas red avidin) of 2-day thioglycollate-elicited PEC demonstrating Ly-6C on distinct Mac-1 and RB6-8C5-defined cell populations. The majority of the Monts-1^{hi},Mac-1^{hi} cells (A) are stained only weakly with FITC-RB6-8C5 (MO/MΦ), whereas the majority of the Monts-1^{med}, Mac-1^{med} cells (B) are RB6-8C5 bright (PMN). Monts-1^{dull}, Mac-1^{med} cells (C) were RB6-8C5$^-$ or very dull (presumably more differentiated MΦ).

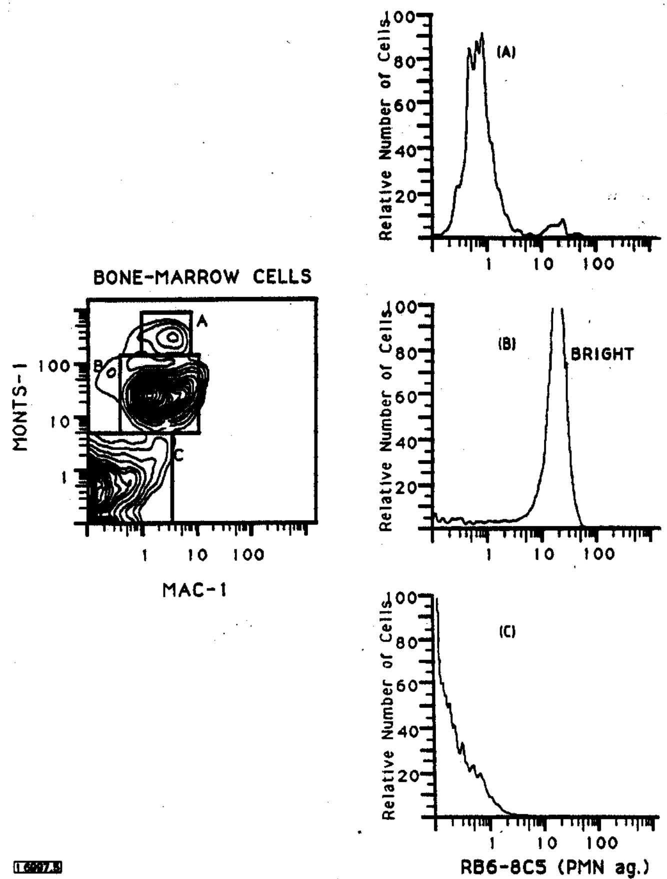
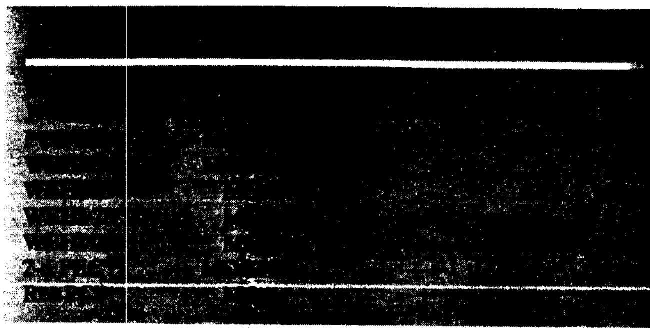


Figure 5. FACS analysis of bone marrow cells demonstrating high levels of Ly-6C on distinct Mac-1$^+$ cell populations. Immunofluorescence staining was as described in Sect. 2.8. As with the 2-d PEC, the Monts-1^{hi} Mac-1$^+$ cells (A) are RB6-8C5 dull or negative (early monocytes) and the Monts-1^{med}Mac-1$^+$ cells (B) are 8C5 bright (PMN or early myeloid cells). The Monts-1 dull cells (C) are Mac-1$^-$ and RB6-8C5$^-$.

FACS analysis was performed on the 2-day thioglycollate-elicited PEC, which included 30–50% MO/MΦ and 40–60% PMN based on Wright's stain morphology, to characterize the cell types that express Ly-6C, and to establish levels of expression. Anti-Mac-1 and RB6-8C5, an antibody that recognizes a determinant expressed at high levels on the PMN [17, 18], were used to assist in identifying MΦ and PMN. Three-color FACS analysis was carried out after staining with biotin-Monts-1 followed by avidin Texas red in combination with APCy-Mac-1 and FITC-RB6-8C5. Most PEC were Mac-1$^+$, and all of the Mac-1$^+$ cells expressed Ly-6C (Fig. 4, two-dimensional contour plot). Distinct populations were evident; one displayed high levels of Ly-6C and the Mac-1 antigen (Monts-1^{hi}, Mac-1^{hi}), another 5–10-fold lower levels of both antigens (Monts-1^{med}, Mac-1^{med}) and a third expressing 100-fold lower levels of Ly-6C and moderate Mac-1 antigen levels (Monts-1^{dull}, Mac-1^{med}). The distinct Mac-1$^+$ populations are consistent with thioglycollate-elicited inflammatory MO (Mac-1^{hi}), PMN (Mac-1^{med}) and differentiated resident MΦ (Mac-1^{med}) [2]. Most of the Monts-1^{med}, Mac-1^{med} cells had high levels of the RB6-8C5 antigen (Fig. 4B), and are, therefore, PMN. In contrast, most of the Monts-1^{hi}, Mac-1^{hi} population did not bear these high levels of the RB6-8C5 antigen (Fig. 4A), and thus are predominantly MO/MΦ. Monts-1^{dull}, Mac-1^{med} cells were negative or expressed very low levels of

Table 2. Reactivity of Monts-1 on MO/MΦ and monocytoid cell lines



a) MO and MΦ phenotype determined as described in Sect. 2.3.
b) Staining of acetone-fixed cytospins, which detects both cell surface and cytoplasmic antigen. A total of 200 cells were counted by immunofluorescence microscopy and the percentage of Monts-1^+ cells was determined and recorded as follows: – (negative), ± (< 10% positive), + (10–20% positive), ++ (20–50% positive) and +++ (> 50% positive).
c) Cell surface staining determined by FACS analysis. The modal fluorescence of cells stained with Monts-1 after subtraction of the fluorescence of cells stained with a class-matched control antibody (see Sect. 2.8).
d) PEC from mice 2 days after the injection of thioglycollate (2-d PEC).
e) Resident PC (Res PC).

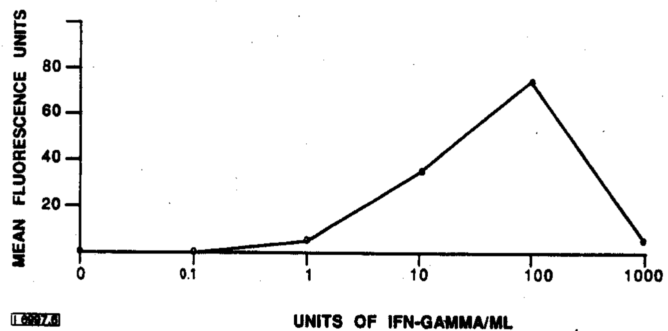


Figure 6. Induction of Ly-6C by IFN-γ. WEHI-274 cells were cultured for 48 h in the presence of the indicated concentrations of IFN-γ, and were then stained with FITC-Monts-1. Modal Monts-1 fluorescence above background is presented in arbitrary fluorescence units. Background, defined as the modal fluorescence of cells stained with FITC-53.2.1, a class-matched control antibody, was 2–3 units and was not influenced by IFN-γ.

the RB6-8C5 antigen (Fig. 4C); this population may include more differentiated MΦ, known to display lower levels of the Mac-1 antigen compared with newly arrived inflammatory MO/MΦ. Negative control staining of the Monts-1^{hi} cells with a control rat IgG_{2a} antibody (anti-Ly-1) and with avidin texas red alone was dull to negative. Thus the majority of Monts-1^{hi} 2-day thioglycollate-elicited PEC are MO/MΦ, and they express 10 times the level of Ly-6C present on PMN and also lymphocytes (data not shown).

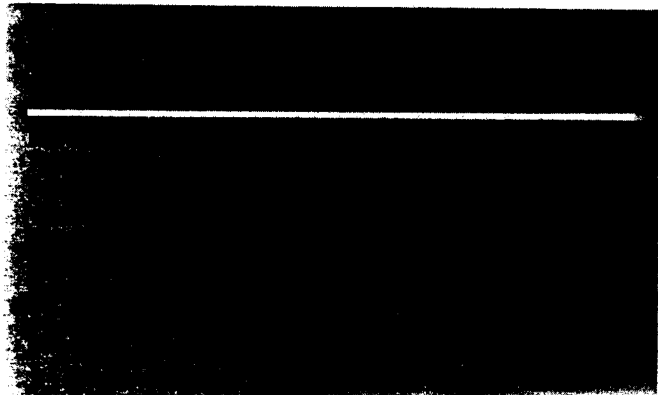
Similarly, Mac-1^+ cells in the bone marrow expressed high levels of Ly-6C (Fig. 5). Like the 2-day PEC, distinct Monts-1^+, Mac-1^+ populations were detected. The Monts-1^{med} population expressed high levels of the RB6-8C5 antigen (Fig. 5B), most likely representing PMN, whereas the Monts-1^{hi},Mac-1^+ cells were negative or expressed very low levels of the RB6-8C5 antigen (Fig. 5A). Mac-1^-, RB6-8C5$^-$ bone marrow cells were also Monts-1^-. The Monts-1^{hi} population represented about 5–10% of the total bone marrow cell suspension. Ly-$6C^+$ Mac-1^+ mononuclear cells comprised about 1% of the peripheral blood leukocyte cell population, consistent with the percentage of circulating MO in BALB/c PBL [17].

In summary, Ly-6C is expressed on Mac-1^+ mononuclear cells in the bone marrow, in peripheral blood, in skin inflammatory reactions and in the peritoneal cavity early during acute inflammation. In contrast, resident MΦ in a variety of tissues express much lower levels of Ly-6C. Along with the finding that only MO-like, but not MΦ-like cell lines express Ly-6C, the data suggest that Ly-6C is a marker of particular stages of MO/MΦ differentiation and/or activation.

3.4 Induction of Ly-6C by IFN-γ

Although 2-day thioglycollate-induced PEC are predominantly Monts-1^+, culture of PEC *in vitro* leads to loss of Ly-6C within 24 h (data not shown). This antigen loss *in vitro* contrasts with apparent continued high-level expression by MΦ in immune inflammatory sites *in vivo*, for example, within CFA-induced granulomas. These observations suggested that the expression of Ly-6C on MΦ might be regulated by inflammatory cytokines. Therefore, we investigated whether recombinant murine IFN-γ could induce Ly-6C *in vitro*. In initial experiments, the Monts-1^- MO-like cell line WEHI-274 was cultured with different doses of IFN-γ. The expression of Ly-6C after 48 h was then examined by FACS analysis. Fig. 6 presents the results of a representative experiment assessing

Table 3. Expression of the Monts-1 antigen on untrated and IFN-γ-treated MO/MΦ and monocytoid cell lines[a)]



a) The indicated cells were incubated at 37 °C for 48 h in the presence or absence of 100 units IFN-γ/ml. The results of cell surface staining assessed by immunofluorescence microscopy. A total of 200 cells were counted and the percent Ia and Monts-1^+ cells was determined and recorded as follows: – (negative), ± (< 10% positive), + (10–20% positive), ++ (20–40% positive) +++ (40–60% positive) and ++++ (> 60% positive). The values were obtained from two separate experiments.
b) MO and MΦ phenotype determined as described in Sect. 2.3.
c) Ia antigens detected by the M5/114.15.2 antibody, which recognizes an allotypic determinant encoded by the I-A and I-E subregions [19].
d) Clonal variant of the WEHI-265 cell line that expresses low levels of the Monts-1 antigen.
e, f) See Table 2.

the modal fluorescence of FITC-Monts-1-stained cells treated with various concentrations of IFN-γ. Peak induction of antigen was observed at 100 units IFN/ml. There was no significant change in background staining associated with IFN-γ treatment. Similar results were obtained in four independent experiments.

The optimal dose of IFN-γ (100 units IFN/ml) was then used to stimulate the other MO/MΦ cell lines, cultured 2-day thioglycollate-elicited PEC and resident PC. Expression of Ia antigen was also assessed. The results are shown in Table 3. IFN-γ induced or greatly increased the expression of Ly-6C on the Monts-1 dull or negative MO-like cell lines (WEHI-78/24, and a Monts-1 low variant of the WEHI-265 cell line) and on the cultured 2-day thioglycollate-elicited PEC. In contrast, stimulation with IFN-γ did not induce or significantly alter Ly-6C expression by the MΦ-like cell lines (RAW264 and J774.1), by WEHI-3 or by resident PC even though Ia was induced more effectively on these cells than on the MO-like cell lines.

4 Discussion

Using a Ly-6C-specific monoclonal antibody, Monts-1, we show that Ly-6C is a MO/MΦ differentiation antigen regulated by IFN-γ. Bone marrow, peripheral blood and early inflammatory Mac-1$^+$ MO and MO/MΦ within CFA-induced granulomas express the highest levels of Ly-6C recognized by Monts-1. Fully differentiated resident peritoneal MΦ and cultured MO-derived MΦ are Monts-1 dull to negative. Resident MΦ in the liver, spleen, thymus and uninflamed lymph node do not express detectable levels of Ly-6C. Two MO-like cell lines express Ly-6C, but the MΦ cell lines RAW264 and J774.1 are negative.

A role for immune cytokines in expression of Ly-6C was initially suggested by the high-level expression of the Ly-6C determinant on cells within granuloma tissue and the absence, or very low expression, of the antigen on resting MΦ in the peritoneum and other tissues. Consistent with this, recombinant murine IFN-γ-induced Ly-6C$^-$ MO and MO-like cell lines to express Ly-6C *in vitro*. IFN-γ is known to induce MO/MΦ activation resulting in a number of increased or altered functions [28–32]. Two aspects of the present findings are of particular interest in this regard. First, the ability of IFN-γ to induce Ly-6C is entirely dependent upon the differentiated state of the cell: IFN-γ had very little or no effect on the expression of Ly-6C on fully differentiated MΦ and MΦ-like cell lines, even though it induced the expression of Ia antigens on these cells. These observations require the existence and differential utilization during MO maturation of at least two pathways of activation by IFN-γ, one leading to induction of Ia and the other to expression or retention of Ly-6C. Second, the demonstration that IFN-γ induces and/or enhances the expression of Ly-6 antigens on MO/MΦ is reminiscent of the induction of this class of antigens on T cells triggered with IFN-γ [33, 34], and suggests that the regulation of these antigens on diverse cell types may involve similar pathways.

The Ly-6 loci encodes at least 5 distinct epitopes distinguished by their patterns of tissue distributions as (a) an antigen on activated T and B cells (Ly-6A.2 and Ly-6E.1, (b) a bone marrow-restricted determinant, presumably on granulocytes (Ly-6B.2), (c) a determinant shared by bone marrow and lymphocytes (Ly-6C.2), (d) a determinant found on most peripheral lymphocytes (Ly-6D.2) and (e) a fifth specificity expressed on bone marrow, thymus and B cells (ThB) [8, 9]. Antibodies to Ly-6 antigens on T cells, including Ly-6C, have been shown to effect cell activation. Both activation and inhibition of activation have occurred when T cells are incubated with different Ly-6-specific antibodies [10–14]. Thus, it has been proposed that Ly-6 antigens may be critical for normal T cell maturation and activation. In combination with the proposed involvement of these antigens in T cell activation, expression of Ly-6C on MO, as demonstrated here, suggests broader roles for Ly-6 in immune cell activation and differentiation. Interaction of Ly-6 antigens on the MO with their natural ligand(s) may contribute to MO "activation", influencing antigen-presenting, tumor cytolytic and anti-microbial capabilities. The role of Ly-6 antigens in MO activation and function is currently being studied.

An earlier study reported the possible expression of Ly-6 on MΦ in BCG reactions leading to the proposal that Ly-6 is induced on MΦ as a consequence of activation [35]. Though the type of Ly-6 antigen was not characterized, expression of Ly-6 determinants on MΦ is consistent with our demonstration of the Monts-1 antigen in granulomas and its induction by IFN-γ. However, we propose that Ly-6 expression, at least expression of Ly-6C, is not solely a consequence of activation, but is regulated in a complex manner *in vivo* during MO/MΦ differentiation and activation. This is based on the observations that (a) Ly-6C is expressed at high levels on young, unactivated MO; (b) differentiated resting resident MΦ bear only low to undetectable levels; (c) the antigen is readily induced by IFN-γ on MO and MO-derived inflammatory MΦ, but (d) resident tissue MΦ appear refractory to the Ly-6C-inducing effects of IFN-γ.

Immunoperoxidase staining of acetone fixed sections of a variety of tissues showed that Ly-6C was also expressed on a subpopulation of small vessel endothelium. The expression of Ly-6C by endothelial cells was equal to or perhaps greater than the expression by MO as evaluated by immunohistology. The endothelial cell antigen defined by Monts-1 was not characterized, but the reactivity of Al-21 and 6C3 with the same endothelial cells strongly suggests that these endothelial cells do indeed express Ly-6C. Interestingly, the expression of Ly-6C on endothelial cells was constitutive and not appreciably altered by the inflammatory events studied. The expression of Ly-6C on endothelial cells suggests the possibility of using Ly-6 allotypic systems in studies of endothelial cells. For example, Ly-6-congenic mice could be used to follow the contribution of donor endothelium in the vascularization of transplanted allografts, or to assess the potential of transplanted endothelial cells to differentiate into different endothelial phenotypes.

In conclusion, we have described an antibody, Monts-1, that defines a MO/MΦ and endothelial cell differentiation antigen encoded by the Ly-6 loci. The high level of the Ly-6C antigen on MO and endothelial cells, the induction of this antigen on MO by products of immune cells, and the potential relevance of this antigen for cellular activation suggest important functions for Ly-6 antigens beyond their previously identified role in T cell activation.

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