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The monoclonal antibody ER-BMDM1 recognizes a macrophage and dendritic cell differentiation antigen with aminopeptidase activity*

Here we describe the reactivity of monoclonal antibody (mAb) ER-BMDM1, directed against a 160-kDa cell membrane-associated antigen (Ag) with aminopeptidase activity. The aminopeptidase recognized by ER-BMDM1 is present on various mouse macrophage (MΦ) and dendritic cell (DC) subpopulations as well as on microvillous epithelia. Analysis of ER-BMDM1 Ag expression in *in vitro* models of MΦ maturation revealed that the Ag is expressed at increasing levels upon maturation of MΦ. *In vivo*, high level expression of the ER-BMDM1 Ag occurs after the monocytic stage of maturation, since bone marrow cells and peripheral blood monocytes are essentially ER-BMDM1 negative. Analysis of isolated-resident and elicited MΦ populations showed that ER-BMDM1 recognizes a specific subpopulation of mature MΦ: only some resident peritoneal and alveolar MΦ are ER-BMDM1 positive, whereas virtually all thioglycollate-elicited peritoneal exudate MΦ bind the mAb. In lymphoid organs, a subpopulation of MΦ is recognized as well as interdigitating cells (IDC) located in T cell areas. Phenotypic analysis of isolated DC – the *in vitro* equivalents of IDC – from spleen and lymph nodes confirmed that the majority of this important antigen-presenting cell population expresses the ER-BMDM1 aminopeptidase. The molecular characteristics of the ER-BMDM1 Ag suggest that it may represent the mouse homolog of human CD13.

1 Introduction

Mature mononuclear phagocytes comprise a very heterogeneous BM-derived cell population. The various representatives perform divergent functions, such as Ag presentation, phagocytosis of senescent cells, and killing of harmful microorganisms and tumor cells (reviewed in [1–3]). Most of these functions have been studied using *in vitro* assays on isolated populations and/or cultured cells. It remains difficult, however, to ascribe these functions to specific MΦ subpopulations located in different tissue compartments. Therefore, a detailed characterization of MΦ heterogeneity *in situ* is needed, using markers that are specifically indicative of particular MΦ functions.

mAb directed against MΦ Ag greatly facilitate defining different MΦ subpopulations. For instance, ER-TR9 and MOMA-1 recognize two MΦ subpopulations in the spleen, located at the marginal zone and at the inner border of the marginal sinus, respectively [4, 5]. Other mAb, such as F4/80, BM8 or MOMA-2, show broader specificity, yet fail to detect particular MΦ subpopulations [6–9].

In this report we define a new subpopulation of mature mouse MΦ, characterized by the binding of mAb ER-

BMDM1. A significant subpopulation of dendrocytes – *i.e.* Langerhans cells, veiled cells, interdigitating cells (IDC) and their *in vitro* equivalents, dendritic cells (DC) [10] – is also ER-BMDM1 positive. Biochemical analysis of the ER-BMDM1 Ag demonstrates that it is a 160-kDa protein which exhibits aminopeptidase activity.

2 Materials and methods

2.1 Animals, mAb and conjugates

Male and female BALB/c mice were bred at our facility under clean, routine conditions and killed at 10–20 weeks of age. A female F₁ (Louvain × Lewis) rat was immunized (see below) and killed as donor of immune spleen cells.

As sources of mAb we used undiluted hybridoma culture supernatants, optimally diluted ascites fluid, or reconstituted purified mAb (BMA Biomedicals AG, Augst, Switzerland). In addition to the ER-BMDM1 mAb, described below, we used BM8 (anti-MΦ; [8]), ER-MP20 (anti-MΦ precursor; [11]), ER-TR9 (anti-MΦ; [4]), F4/80 (anti-MΦ; [6]), H129.19 (anti-CD4; [12]), M3/38 (anti-Mac-2 MΦ Ag; [13]), MOMA-1 (anti-MΦ; [5]), MOMA-2 (anti-MΦ; [9]), NLDC-145 (anti-IDC/DC; [14]), and RA3 6B2 (anti-B-220 B cell Ag; [15]).

The hybridoma producing mAb ER-BMDM1 (Erasmus University Rotterdam-BM-derived MΦ; IgG_{2a} subclass) was generated essentially as described before [11], using MΦ obtained after 7 days from M-CSF stimulated BM culture [16] as the immunogen and rat Y3-Ag1.2.3 myeloma cells as the fusion partner.

The conjugates used in this study were anti-rat Ig coupled to β-galactosidase (Radiochemical Centre, Amersham, GB or

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Abbreviations: BMDM: BM-derived mononuclear phagocyte
DC: Dendritic cell IDC: Interdigitating cell

Zymed Laboratories, San Francisco, CA; used in ELISA), anti-rat Ig coupled to horseradish peroxidase (Dako, Copenhagen, Denmark; used in immunohistology), and FITC-coupled F(ab')₂ fragments of rabbit anti-rat IgG (Cappel, Malvern, PA; used in FCM).

2.2 Target cells for hybridoma screening

mAb were screened in three previously described models of MΦ maturation for the recognition of maturation-stage related Ag: (a) a panel of MΦ cell lines, ordered in a linear maturation sequence [17]; (b) immature and mature BMDM, isolated from macrophage (M)-CSF-stimulated BM cultures after 4 and 7 days, respectively [11, 16]; (c) induction of maturation of the MΦ precursor hybrid R0C2 using mouse post-LPS serum + IFN-γ [11, 16].

In addition, ER-BMDM1 mAb was characterized by binding to several freshly isolated cell populations. BM cell suspensions were prepared in HBSS + 5% FCS by grinding femora and tibiae using a mortar. Peripheral blood nucleated cells were obtained after lysis of E by a 10-min incubation at 4°C in 17 mM Tris-HCl, pH 7.2 containing 144 mM NH₄Cl. We obtained resident peritoneal and alveolar cells by lavage, using serum-free DMEM and PBS + 0.6 mM EDTA, respectively. PEC were isolated at 18 h and 4 days after i.p. injection of 1 ml Brewer's thioglycollate medium (Difco, Detroit, MI). These populations were highly enriched for polymorphonuclear cells (PMN) and MΦ, respectively. DC were isolated from LN and spleen as described previously [18].

2.3 ELISA, immunofluorescence labeling and FCM analysis

Expression of ER-BMDM1 Ag by MΦ cell lines and BMDM was quantified by a fluorescence micro-ELISA in Terasaki trays as described earlier [16].

Labeling procedures for immunofluorescence and FCM analysis were performed essentially as described [11, 19]. mAb binding to cells was quantified by comparison of the obtained fluorescence values with those from quantitative fluorescent microbead standards (Becton Dickinson, Sunnyvale, CA). Resident peritoneal MΦ were identified in the lavage mixture on the basis of their forward- and perpendicular light-scatter characteristics. Other isolated cell populations were analyzed as total populations.

2.4 Immunoperoxidase staining of cell suspensions and tissue sections

ER-BMDM1 Ag expression by isolated DC was assessed at the single-cell level using an immunoperoxidase assay on cytocentrifuge preparations [20]. Immunoperoxidase staining of cryostat tissue sections was performed essentially as described by de Jong et al. [21]. A hexazotized pararosaniline solution was used for tissue fixation [21]. mAb binding was detected using either a routine diaminobenzidine (DAB) visualization of peroxidase [21], or a modified protocol involving NiSO₄-supplemented DAB [22]. In the

latter case sections were counterstained with nuclear fast red [22], whereas sections developed according to the regular DAB method were counterstained with hematoxylin.

2.5 Biochemical characterization of the Ag

The molecular mass of the ER-BMDM1 Ag was determined by surface radio-iodination of cells, followed by specific immunoprecipitation and SDS-PAGE analysis, essentially as described by Pont et al. [23].

Amino-peptidase activity was demonstrated in lysates from P388D1 MΦ and small intestine cells, which were obtained by scraping. After isolation, the cells were washed twice in serum-free DMEM and lysed in 1 or 2 ml 30 mM TrisHCl, pH 8.0, 1% Triton X-100, 150 mM NaCl for 30 min on ice. The lysates were cleared by centrifugation for 15 min at 15 000 × g. Antigens were specifically immunoprecipitated using mAb coupled to anti-rat κ chain (MARK-1)-coated Sepharose-4B beads [23]. Prior to immunoprecipitation, the lysates were incubated for 2 h with plain MARK-1 beads to remove non-specifically bound proteins. The beads were washed three times in lysate buffer and incubated for 90 min with 0.21 mM leucyl-β-naphthylamide as amino-peptidase substrate in 200 mM phosphate buffer, pH 8.0, supplemented with 0.2% Triton X-100. The reaction was stopped by adding 400 μl 1 M Na₂CO₃ to 10 μl reaction mixture. Generation of the product β-naphthylamine was monitored on a fluorometer, using an excitation wavelength of 340 nm and an emission wavelength of 410 nm.

Table 1. Precipitation of aminopeptidase activity by ER-BMDM1

	Cells	Sample	APA ^{a)}
Exp. 1	P388D1	Untreated lysate (10 ⁵ cells)	231
		ER-BMDM1 precipitate ^{b)}	175
		H129.19 precipitate ^{b,c)}	4
Exp. 2	Small intestine	Untreated lysate (10 ⁵ cells)	153
		ER-BMDM1 precipitate ^{b)}	111
		NLDC-145 precipitate ^{b,c)}	0
Exp. 3	P388D1	Untreated lysate (10 ⁵ cells)	161
		Lysate + ER-BMDM1 ^{d)}	163
		Lysate + ER-MP20 ^{c,d)}	164

- a) APA = aminopeptidase activity determined as generation of fluorescent β-naphthylamine from L-leucyl-β-naphthylamide; fluorescence is expressed in arbitrary units.
 b) MARK-1-coupled Sepharose-4B beads (40 μl wet volume), to which rat mAb was linked, were incubated with excess cell lysate, washed and assayed for precipitated aminopeptidase activity.
 c) H129.19, NLDC-145 and ER-MP20 served as IgG_{2a} isotype-matched control mAb.
 d) mAb was added as 5 μl ascites fluid (1:1 diluted in PBS) per 50 μl lysate (corresponding to 10⁶ cells); after 30 min of incubation on ice, an aliquot from this mixture corresponding to 10⁵ cells was taken for determination of aminopeptidase activity.

3 Results

3.1 Biochemical characterization of the ER-BMDM1 Ag

The aim of the present study was the development of mAb against MΦ which would help define functional and phenotypic heterogeneity of these cells. mAb ER-BMDM1 was found to recognize an Ag on various MΦ subpopulations as well as on microvillous brush borders. The latter finding prompted us to investigate a putative proteolytic function of the Ag.

Immunoprecipitation of the ER-BMDM1 Ag from WR19M.1 MΦ tumor cells and subsequent SDS-PAGE showed that the Ag is a single polypeptide chain of approximately 160 kDa apparent molecular mass (data not shown). Using substrates for enzymes within this size range, we investigated whether immunopurified ER-BMDM1 Ag possessed any proteolytic activity. Table 1 shows that the ER-BMDM1 mAb precipitated an Ag, from both the P388D1 MΦ cell line and small intestine cells, that exhibits leucine aminopeptidase activity. The mAb is, most likely, directed against an epitope distinct from the catalytic site of the enzyme, since addition of the mAb did not influence the observed aminopeptidase activity.

3.2 Distribution of ER-BMDM1 aminopeptidase *in situ*

3.2.1 Lymphoid organs

In the spleen strongly ER-BMDM1-positive cells, presumably MΦ [24], surround the central arteriole (Fig. 1a). In the T cell area (periarteriolar lymphocyte sheath; PALS), IDC and PALS MΦ are stained. Scattered irregularly shaped cells, distinct from the marginal metallophilic MΦ (Fig. 1c), are present at the inner border of the marginal sinus. Immunofluorescence double labeling (not shown) using the IDC marker NLDC-145 and the marginal metallophilic MΦ marker MOMA-1 confirmed these observations. In the splenic red pulp, two ER-BMDM1-positive cell types are present. First, patches of irregularly shaped cells are seen at the periphery of the marginal zone (Fig. 1a). In addition to ER-BMDM1 Ag, these cells express BM8, F4/80, MOMA-2, and Mac-2 Ag (de Jong and Leenen, manuscript in preparation). Based on the cellular morphology, phenotype and localization, we propose to designate these cells as “marginal red pulp MΦ”. The second population of ER-BMDM1-positive cells in the red pulp is a small subpopulation of “classical” red pulp MΦ, located deeper in the red pulp.

In the mesenteric LN stretched MΦ, lining the inside of the LN capsule are clearly ER-BMDM1 positive (Fig. 1e). ER-BMDM1-positive IDC are easily detected in the paracortex (T cell area). In the LN medulla, positive MΦ are located primarily within the medullary cords. These cells are clearly distinct from the ER-TR9-positive sinus MΦ (cf. Fig. 1g and h).

In the thymus, cells, probably MΦ [24], on the inside of the thymic capsule and lining the connective tissue septa show a variable staining pattern (Fig. 2). In the cortex and corticomedullary junction, most but not all acid phosphatase-positive MΦ are recognized by ER-BMDM1. Furthermore,

mAb reactivity occurs with medullary IDC and with cells associated with cortical and medullary capillaries.

3.2.2 Non-lymphoid organs

Villous epithelial cells in the small intestine are heavily labeled, especially at the brush border (Fig. 3a). The lamina propria in the villi contains only a few scattered positive cells. In contrast, in the large intestine the epithelial cells are negative, whereas the numerous MΦ in the lamina propria are clearly positive (Fig. 3b).

Strong ER-BMDM1 staining in the liver appears to be associated with the microvillous brush borders of bile capillaries (Fig. 3c). The acid phosphatase positive Kupffer cells are negative.

In the lung, there are two ER-BMDM1-positive cell types (Fig. 3d). Stretched cells surround blood vessels, and, at lower density, bronchi. Furthermore, type-II pneumocytes that express microvilli are also positive. Most alveolar MΦ are negative.

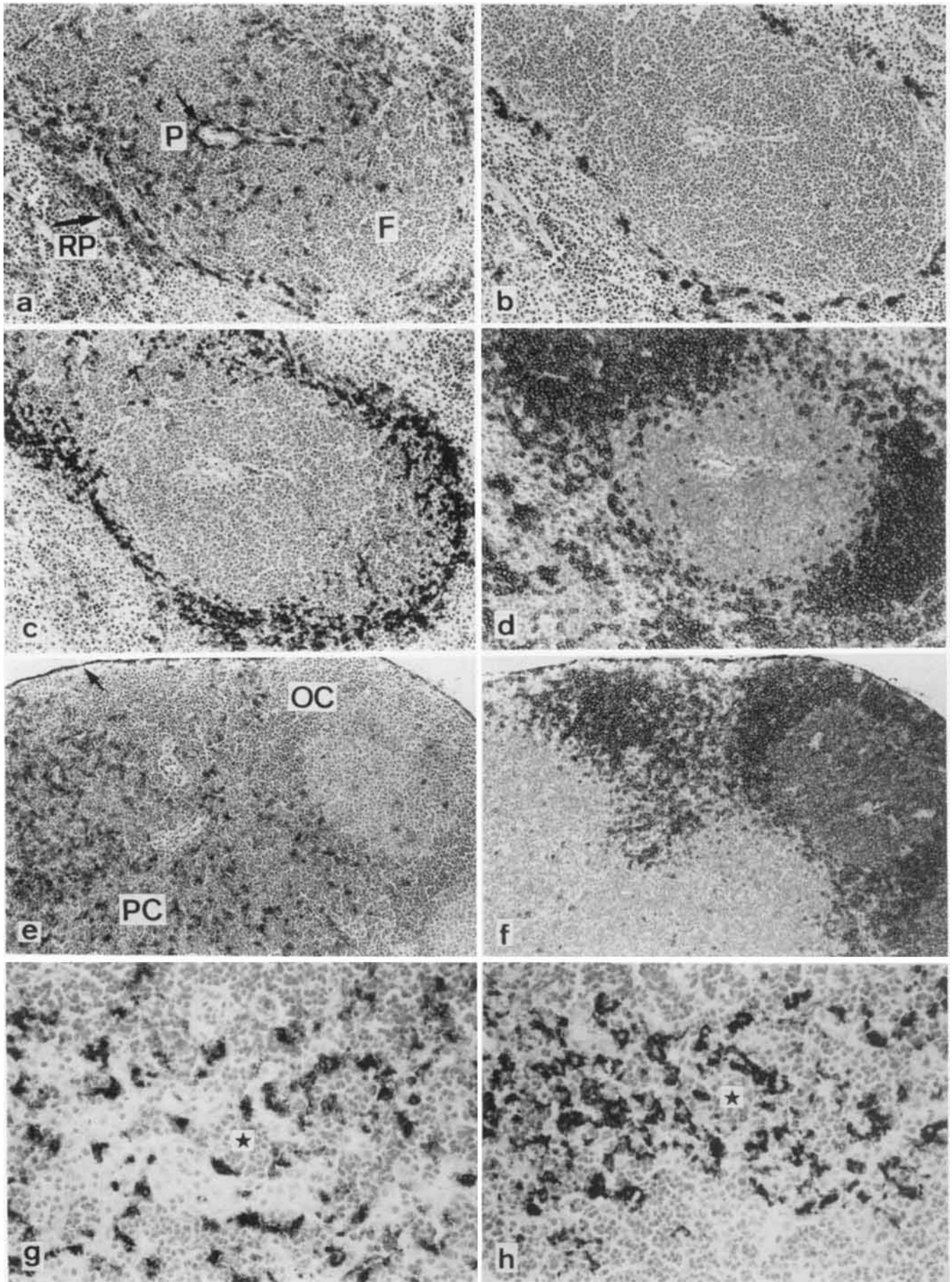
3.3 ER-BMDM1 aminopeptidase expression by isolated DC and MΦ populations

To confirm the notion that both MΦ and IDC in lymphoid organs are recognized by ER-BMDM1, we stained DC – the *in vitro* equivalents of IDC – as well as various isolated MΦ populations. LN DC, identified by positive NLDC-145 staining and acid phosphatase activity located in a cytoplasmic spot, were ER-BMDM1 positive (data not shown). Virtually all LN DC are positive, whereas approximately 50 % of the NLDC-145-positive splenic DC are recognized by ER-BMDM1.

BM MΦ precursors and peripheral blood monocytes express little ER-BMDM1 Ag (Fig. 4A). In contrast, approximately 60 % and 30 % of the MΦ in the resident peritoneal and alveolar cell populations, respectively, readily express this marker. Remarkably, virtually all thioglycollate-elicited peritoneal exudate MΦ are strongly ER-BMDM1 positive. The small positive population present in the granulocytic exudate, isolated 18 h after thioglycollate injection, most likely represents the contribution of remaining resident peritoneal MΦ. Together, these data indicate that ER-BMDM1 recognizes a particular subpopulation of DC and MΦ.

3.4 ER-BMDM1 aminopeptidase expression in the course of MT maturation

The observation that BM MΦ precursors and peripheral blood monocytes expressed little ER-BMDM1 Ag prompted us to investigate the onset of Ag expression during MΦ maturation in more detail. Screening of ER-BMDM1 reactivity on a panel of MΦ cell lines, arrested in distinct, characterized stages of maturation [17], revealed that only cell lines with mature MΦ characteristics were recognized (Fig. 4B). In M-CSF-stimulated BM cultures, mAb binding was hardly detectable on nonadherent MΦ precursors isolated after 4 days, whereas mature MΦ



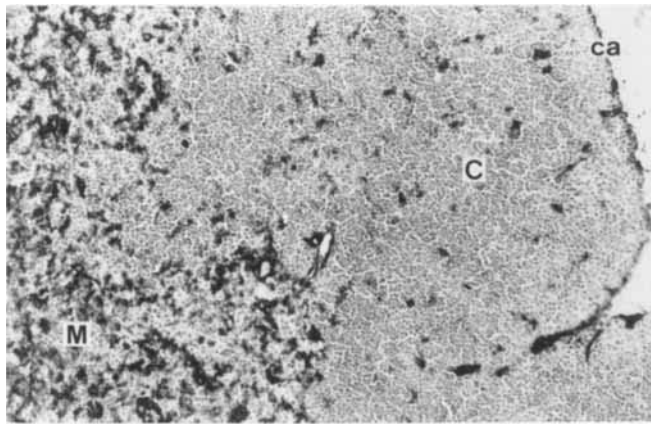


Figure 2. ER-BMDM1-positive MΦ and IDC in the thymus. ca = capsule; C = cortex; M = medulla. Original magnification × 140.

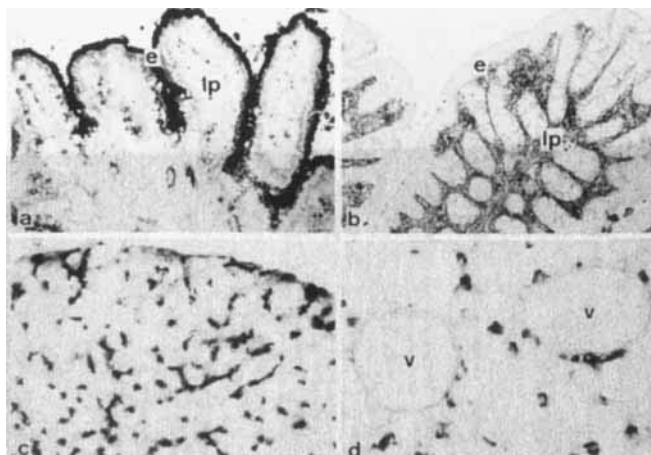


Figure 3. ER-BMDM1 reactivity on frozen sections of non-lymphoid organs. (a) small intestine; (b) large intestine; (c) liver; (d) lung; e = epithelium; lp = lamina propria; v = venule. Original magnifications × 125 (a,b) and × 310 (c,d).

isolated after 7 days strongly expressed the ER-BMDM1 Ag (Fig. 4B). Similarly, the MΦ precursor cell line R0C2 [16] expressed little Ag, but induction of maturation simultaneously induced expression of the ER-BMDM1 Ag

◀ **Figure 1.** ER-BMDM1 reactivity on frozen sections of spleen and mesenteric LN. For comparison, serial spleen sections (a–d) were stained with ER-BMDM1 (a), ER-TR9 (b; marginal zone MΦ), MOMA-1 (c; marginal metallophilic MΦ), and RA3 6B2 (d; B-cells). ER-BMDM1 stains IDC and PALS MΦ in the splenic white pulp and “marginal red pulp MΦ” (indicated with the large arrow) in the red pulp (RP). The small arrow indicates the central arteriole; P = periarteriolar lymphocyte sheath; F = follicle. Serial LN sections (e,f and g,h) were stained with ER-BMDM1 (e,g), RA3 6B2 (f; B cells) and ER-TR9 (h; medullary sinus MΦ). In the LN cortex, ER-BMDM1-positive MΦ occur in the capsule (indicated with the arrow) and mainly outside the B cell area (OC = outer cortex). IDC are easily distinguished in the paracortex (PC). In the medulla, ER-BMDM1-positive MΦ are associated mainly with the medullary cords and clearly distinct from the medullary sinus MΦ detected by ER-TR9. Asterisks in (g) and (h) indicate the same locations in serial sections. Original magnifications × 125 (a–d), × 95 (e,f) and × 245 (g,h).

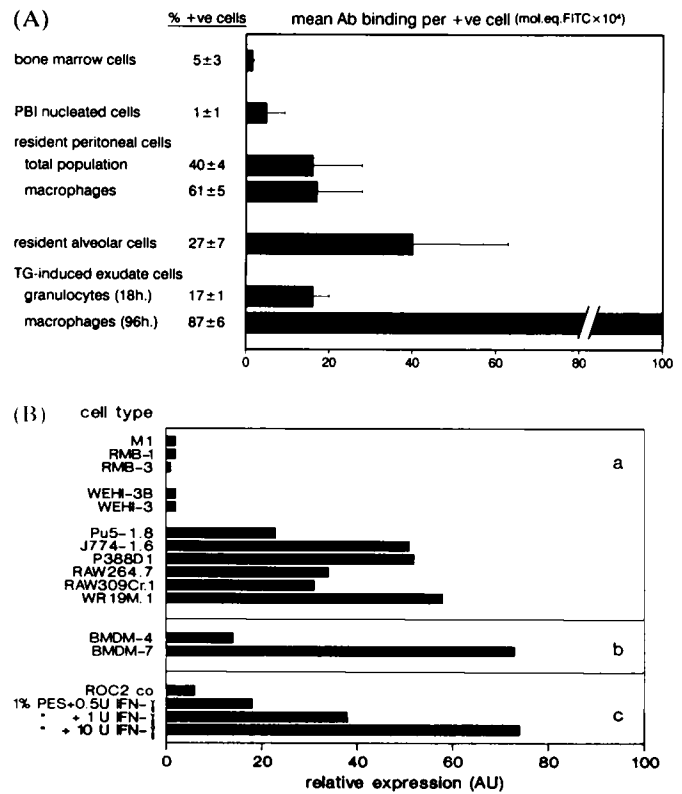


Figure 4. (A) ER-BMDM1 Ag expression by freshly isolated cell populations as determined by FCM. The percent of positive cells in these populations and the net mAb binding per positive cell represent the mean of at least three experiments ± SD. mAb binding is expressed in soluble FITC molecular equivalents (mol.eq.FITC). PBI = peripheral blood; TG = thioglycollate. (B) ER-BMDM1 Ag expression during MΦ maturation as determined using three maturation models. (a) A panel of MΦ cell lines ordered in a linear differentiation sequence [17]: MΦ precursor (M1, RMB-1, RMB-3), immature MΦ (WEHI-3B, WEHI-3) and mature MΦ cell lines (Pu5-1.8, J774-1.6, P388D1, RAW264.7, RAW309Cr.1, WR19M.1); (b) Immature nonadherent MΦ precursor cells, isolated after 4 days of M-CSF stimulated BM culture (BMDM-4) are compared to mature adherent BM-derived MΦ, isolated after 7 days of culture (BMDM-7); (c) Induction of maturation in the MΦ precursor hybrid R0C2 [16] by culture for 5 days with 1% post-LPS serum (PES) supplemented with 0.5 U, 1 U or 10 U IFN-γ/ml. The level of mAb binding is determined by ELISA and expressed in arbitrary units (AU), relative to an internal standard [16]. Data show one of three independent experiments with similar results.

(Fig. 4B). Taking these results together, we conclude that the ER-BMDM1 aminopeptidase is expressed increasingly beyond the monocytic stage in a strict maturation-related manner.

4 Discussion

The data presented in this report indicate that mAb ER-BMDM1 binds to an aminopeptidase at the cell surface of mouse MΦ, DC and microvillous epithelia. Moreover, the ER-BMDM1 Ag is expressed in increasing amounts as MΦ mature beyond the monocytic stage. Our finding that – within the hemopoietic lineage – only DC/IDC and a MΦ subpopulation express the ER-BMDM1 Ag, contributes to the evidence for a close phenotypic relationship between

these cell types [9, 20, 25]. Recently published data indicate that a developmental relationship exists as well, since monocytes can be stimulated to differentiate into cells with morphological, phenotypical and functional features of DC [26, 27]. In addition, stimuli which induce M Φ differentiation also induce development of DC from BM precursors [28, 29]. The observation that BM-derived DC likely pass through the monocytic stage during their development [29] is in line with our finding that ER-BMDM1 Ag expression significantly increases after the monocytic stage. Hence, our data support the view that DC/IDC belong to the mononuclear phagocyte system, rather than constituting a separate cell lineage as suggested by others [10, 30].

The specific reactivity of ER-BMDM1 mAb with microvilli in non-lymphoid organs correlates with the demonstrated enzymatic activity of the recognized Ag. Most likely, the ER-BMDM1 Ag is identical to leucine aminopeptidase [31] and aminopeptidase N [32]. We base this identification on the similar linkage between M Φ maturation stage and expression of leucine aminopeptidase [33] as well as on the similarity in tissue distribution of aminopeptidase N and the ER-BMDM1 Ag [32]. Recently it was recognized that the human CD13 Ag exhibits aminopeptidase N activity [34, 35]. The similarity in both molecular mass and enzyme activity suggests that the Ag recognized by ER-BMDM1 is the mouse homolog of human CD13. However, CD13 Ag is expressed by immature human myeloid cells and granulocytes [36], in contrast to the mouse ER-BMDM1 Ag. Additional studies are now in progress to investigate the putative homology between the ER-BMDM1 aminopeptidase and the CD13 Ag.

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