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Heterogeneity of Mouse Spleen Dendritic Cells: In Vivo Phagocytic Activity, Expression of Macrophage Markers, and Subpopulation Turnover

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In the normal mouse spleen, two distinct populations of dendritic cells (DC) are present that differ in microanatomical location. The major population of marginal DC is found in the “marginal zone bridging channels” and extends into the red pulp. The interdigitating cells (IDC) are localized in the T cell areas in the white pulp. The aim of the present study was to characterize these two splenic DC populations with regard to their phenotype, in vivo phagocytic function, and turnover. Both marginal DC and IDC are CD11c+ and CD13+, but only IDC are NLDC-145+ and CD8α+. Notably, both populations, when freshly isolated, express the macrophage markers F4/80, BM8, and Mac-1. To study the phagocytic capacity of these cells, we employed the macrophage “suicide” technique by injecting liposomes loaded with clodronate i.v. Marginal DC, but not IDC, were eliminated by this treatment. Phagocytosis of DiI-labeled liposomes by DC confirmed this finding. The two DC populations differed significantly with regard to their turnover rates, as studied in a transgenic mouse model of conditional depletion of DC populations with high turnover. In these mice, marginal DC were completely eliminated, but the IDC population remained virtually intact. From these data we conclude that the marginal DC population has a high turnover, in contrast to the IDC population. Taken together, the present results indicate that marginal DC and IDC represent two essentially distinct populations of DC in the mouse spleen. They differ not only in location, but also in phenotype, phagocytic ability, and turnover. The Journal of Immunology, 1998, 160: 2166–2173.
found this enzyme to be a marker for APC, and recently it was implicated in the extracellular trimming of MHC-bound peptides (18, 19). In addition to the classic IDC in the white pulp, a population of nonlymphocytic cells located in patches at the border of marginal zone and red pulp was recognized by ER-BMDM1 (17). On the basis of their expression of genuine macrophage makers such as F4/80 and BMS, detected by immunofluorescence double labeling, we then suggested the designation of the latter cells as "marginal red pulp macrophages." In the present study, we compared splenic DC and CD13+/ER-BMDM1+ marginal red pulp macrophages with respect to their phenotype and phagocytic function. We also compared their turnover, using a model for conditional depletion of DC, based on the selective expression in DC of herpes simplex thymidine kinase in a transgenic mouse (20, 21). Treatment of these HIV-1 long terminal repeat-herpes simplex virus type 1 thymidine kinase (LTR-TK) transgenic mice, or mice made chimeric after engraftment of transgenic BM, with ganciclovir (GCV) specifically induces killing of DC, which actively synthesizes DNA, but not of resting DC.

We show that the CD13+/ER-BMDM1+ marginal red pulp macrophages are actually identical to the previously identified marginal DC. These cells are essentially distinct from the splenic white pulp IDC. Marginal DC are able to phagocytose particulates in vivo, express markers characteristic of both DC and macrophages, and have a high turnover. In contrast, IDC are not phagocytic in vivo and have slow turnover.

Materials and Methods

Mice

C57BL/6 mice used between 6 and 20 wk of age. In some DC isolation experiments, C57BL/10 mice (20 wk of age) were used with similar results. Mice were kept under clean conditions (specific pathogen free, grade 5). Animals were killed by CO2 exposure and spleens were removed for cell isolation or histology.

Derivation of HIV1-LTR/HSV1-TK transgenic mice (abbreviated as LTR-TK mice) has been described (20). These mice, originally (C57BL/6J × DBA/2JF1), were back-crossed to a C57BL/6J genetic background. LTR-TK transgenic mice were generated by lethal irradiation (1200 rad) of grade 5. Animals were killed by CO2 exposure and spleens were removed for cell isolation or histology.

Preparation and staining of cryostat sections for single markers were performed essentially as described before (28). Briefly, 5-μm spleen sections were cut from OCT compound-embedded blocks, air dried, and stored at –20°C until use. After thawing, sections were fixed with p-rosaniline and subsequently incubated with mAb and optimally diluted peroxidase-labeled secondary Ab supplemented with 2% normal mouse serum. Sections were washed between steps with PBS supplemented with 0.05% Tween20. Ab binding was visualized using NiSO4-supplemented dianinobenzidine as substrate, yielding a black reaction product. Sections were counterstained with nuclear fast red, dehydrated, and embedded in Entellan (Merck, Darmstadt, Germany).

Adaptation for immunohistochemical double labeling was as follows: sections were sequentially incubated with N418 hamster anti-mouse mAb, peroxidase-conjugated anti-hamster Ig, rat anti-mouse mAb, and finally goat anti-rat Ig-alkaline phosphatase (Southern Biotechnology, Birmingham, AL). Alkaline phosphatase activity was visualized first in a 30-min incubation in the dark using a substrate solution of alkaline ASMX phosphate (Merek) and Fast Blue BB base (Merek) (final concentration of both, 0.025% in 200 mM Tris-HCl, pH 8.5) as substrate and complexing agent, respectively. Levamisole (0.024%) was added to the reaction mixture to block endogenous alkaline phosphatase activity. After washing the sections in tap water and PBS-Tween, 3-amino-9-ethylcarbazole (0.05% in 100 mM acetate buffer, pH 4.6, supplemented with 0.03% H2O2) was used in a 30-min incubation to detect peroxidase activity. Next, the sections were rinsed with PBS-Tween, embedded in Kaisers Glycergelatinel (Merek), and coverslipped. In these preparations, alkaline phosphatase activity yields a blue reaction product, whereas peroxidase activity appears red.

Spleen cell and DC isolation and culture

Spleens were cut into small pieces and incubated for 1 h at 37°C with 130 U/ml collagenase III (Worthington Biochemical, Freehold, NJ) and 0.1 mg/ml DNase I (Boehringer Mannheim) in RPMI/25 mM HEPES/penicillin/streptomycin (RPMI-HA). We have chosen for this procedure, instead of mechanical disruption in the cold, as it provides a significantly higher yield of splenic DC and macrophages. Control experiments have indicated that DC isolated by either procedure are similar. The resulting digested tissue suspension was teased through a 100-μm sieve and centrifuged. Next, erythrocytes were lysed by resuspending the cell pellet in 17 mM Tris-HCl, pH 7.2, containing 144 mM NH4Cl and incubating this for 10 min on ice. Subsequently, the cell suspension was washed with RPMI-HA supplemented with 10% FCS (RPMI-HA-FCS) and resuspended. Part of this suspension was used for flow cytometric analysis and referred to as "fresh total spleen cells." The rest of the cells were cultured overnight at 37°C and 5% CO2 in plastic tissue culture flasks. Next, the nonadherent cell population, collected, centrifuged, and resuspended, was used to give a final density of 1 to 2 × 107 cells per ml (referred to as "overnight total spleen cells"). For DC enrichment, aliquots of 2 ml of this cell suspension were layered on top of 2 ml of Nycodenz (Nycomed Pharma AS, Oslo, Norway) (14.5% in RPMI-HA-FCS) and centrifuged for 20 min at 530 × g. The interface was collected and routinely contained 60 to 80% N418mAb cells.

Immunofluorescence labeling and flow cytometric analysis

Labeling and subsequent phenotypic analysis of spleen cells by flow cytometry were performed essentially as described before (29). Data were collected using a FACScan (Becton Dickinson, Sunnyvale, CA) and CellQuest software. Resulting data files were further processed for presentation using WinMDI (accessible at http://facs.scripps.edu/).

Conditional elimination of DC in LTR-TK BM chimeric mice

To deplete LTR-TK-expressing DC in BM chimeric mice (see above), these mice received 50 mg of GCV/kg body weight/day using a miniosmotic Alzet pump (Alza Corp., Palo Alto, CA) as described (20). Control mice were sham treated. After 7 days, the mice were killed and spleens were isolated. Efficacy of GCV on LTR-TK transgenic BM was verified by inhibition of granulocyte macrophage-CSF-induced DC generation in BM cultures.
Results

Mouse spleen contains two CD13+ populations of DC in anatomically distinct locations

Previously, we have found that the CD13 mAb ER-BMDM1 detects two major populations of nonlymphocytic cells in mouse spleen: IDC in the T cell areas and “marginal red pulp macrophages” in patches at the periphery of the splenic marginal zone (17). To investigate a possible identity of these marginal red pulp macrophages with the marginal DC population identified by Agger et al. (10), we labeled serial spleen sections with ER-BMDM1/CD13 and N418/CD11c. Figure 1, a and b, shows that indeed both mAb detect the same populations. Immunohistochemical double labeling further advanced the notion that ER-BMDM1 and N418 recognize the same DC populations, i.e., both IDC in the T cell zone and the marginal DC, which are located adjacent to the marginal zone and extend into the red pulp (data not shown). Also, a minor population of cells scattered in the red pulp is labeled by both mAb. In contrast, NLDC-145 only recognizes the population of IDC in the white pulp (Fig. 6c). Double labeling of spleen sections with N418 and various anti-macrophage mAb indicated that the marginal DC accumulate at sites where the continuous rim of marginal zone and metallophilic macrophages is interrupted by the so-called marginal zone bridging channels (Fig. 2a). These channels, which are more apparent in rat than in mouse spleen, are also visible as slight accumulations of T cells and interruptions of the marginal zone B cell rim (Fig. 2, c and d). Double labeling with BM8 (Fig. 2b) showed that red pulp macrophages and marginal DC are essentially distinct populations: only few N418+ marginal DC show traces of BM8 labeling.

FIGURE 1. Splenic DC comprise two populations, both expressing CD13 and CD11c Ags. Serial spleen sections stained with (a) ER-BMDM1/CD13 and (b) N418/CD11c show that both mAb detect the same DC populations: a smaller population of IDC in the T cell zone of the white pulp (wp) and a larger population of marginal DC extending from the marginal zone into the red pulp area. Original magnification, ×135.

FIGURE 2. Marginal DC are located in the marginal zone bridging channels, interrupting the marginal zone and the rim of marginal metallophilic macrophages. Immunohistochemical double labeling of near-serial sections shows the spatial relationship between: in red (N418), marginal DC; and in blue (a), marginal metallophilic macrophages (MOMA-1); (b) red pulp macrophages (BM8); (c) T cells (KT3); (d) B cells (RA3 6B2). Marginal DC are primarily located at the sites where the marginal zone is interrupted by the marginal zone bridging channels, indicated by the discontinued rim of metallophilic macrophages and marginal zone B cells, and slight accumulation of T cells. Original magnification: ×135.
Thus, CD13 mAb ER-BMDM1 detects both CD11c\(^+\) DC populations in spleen: IDC in T cell areas and marginal DC, which accumulate in the marginal zone bridging channels and extend into the red pulp area.

**Splenic DC express both DC and macrophage markers**

The conclusion reached above, that the previously defined marginal red pulp macrophages are in fact DC, is in seeming conflict with the observation, made by immunofluorescence double labeling, that these cells express multiple markers characteristic of macrophages (17). To respond to this controversy, we analyzed the phenotype of splenic DC by flow cytometric double labeling in fresh whole spleen cell suspensions, after overnight culture, and after subsequent DC enrichment (Fig. 3). DC were identified by N418 labeling. In accordance with previous reports (30–32), freshly isolated splenic DC were found to express high levels of MHC class II, which further increased during overnight culture. Also, CD13/ER-BMDM1 was uniformly present on fresh and cultured cells. In contrast, splenic DC showed heterogeneous labeling for NLDC-145 and CD8\(^\alpha\): for both markers, only about 25% of freshly isolated cells was positive. Overnight culture increased NLDC-145 labeling of both populations, thus retaining their distinction. Similarly, both populations remained distinct upon culture with respect to the expression of CD8\(^\alpha\). These results confirm previous findings and show that our procedures generate similar populations to those obtained by others. Remarkably, with regard to macrophage markers, freshly isolated spleen DC were found to be uniformly positive for F4/80 and BM8, as well as for Mac-1. Both F4/80 and BM8 expression decreased upon culture, whereas Mac-1 expression was retained. Taken together, these findings indicate that freshly isolated splenic DC express markers characteristic of DC as well as macrophage markers. Upon culture, expression of some of the latter decreases, whereas expression of some typical DC markers is enhanced.

**Marginal DC are phagocytic in vivo**

The finding that freshly isolated spleen DC express genuine macrophage markers raises the question of whether these cells also display macrophage functions in vivo, especially phagocytosis of particulate matter. To test this, we injected mice i.v. with liposomes loaded with clodronate. These liposomes are endocytosed by phagocytic cells only, and, when applied i.v., eliminate splenic red pulp macrophages, marginal zone macrophages, and marginal metallophils (27). Figure 4 confirms that red pulp macrophages, detected by BM8 labeling, are indeed eliminated 2 days after liposome application (Fig. 4b). In addition, marginal zone macrophages and metallophils were depleted (not shown). Using N418 and ER-BMDM1 to detect DC in these sections, we found that marginal DC and red pulp DC are eliminated due to phagocytosis of clodronate-liposomes. Alternatively, the severe damage caused by depletion of red pulp and marginal zone macrophages may cause migration of DC. To check this possibility, we i.v. injected liposomes, now labeled with the fluorescent dye DiI instead of loaded with clodronate. After approximately 18 h, total spleen cell suspensions were prepared and analyzed by flow cytometry to identify cells that had phagocytosed DiI-liposomes. Figure 5 shows that, on average, almost half of the CD11c\(^+\) DC was DiI positive, indicative of their phagocytosis of liposomes. Interestingly, the intensity of fluorescence of DC was only three- to fourfold lower compared with that of the genuine F4/80\(^{high}\) macrophages. From these
results, we conclude that splenic marginal DC, in addition to their expression of macrophage markers, also display actual phagocytic activity in vivo. In support of this, in a 60-min in vitro phagocytosis assay using FITC-labeled *Listeria*, we found that about 30% of ER-BMDM1 DC in a freshly isolated spleen cell suspension were able to internalize bacteria (data not shown). IDC probably are not phagocytic, as they are not affected in the clodronate-liposome-treated mice. It should be considered, however, that the microcirculation in the splenic white pulp allows only limited access of the IDC to i.v. injected liposomes.

Marginal DC are selectively eliminated upon GCV treatment of LTR-TK BM chimeric mice

To delineate further the presumed distinction between the two splenic DC populations, we asked whether these subsets differed significantly in turnover. We approached this question in a transgenic mouse model for conditional DC depletion (20). Previously, in these LTR-TK transgenic mice, a severe depletion of DC was observed in various organs after only 7 days of treatment with GCV. Such a brief depletion time is indicative of a high population turnover, as only TK-expressing cycling cells are affected by this treatment. In the current study, we used an improved model, realized by generating chimeras with LTR-TK transgenic BM (21). From Figure 6 it is evident that N418<sup>ER-BMDM1</sup> marginal DC are completely eliminated by 7-day GCV treatment of LTR-TK chimeras (Fig. 6, a and b). In contrast, the NLDC-145<sup>IDC</sup> population in these mice is hardly affected (Fig. 6, c and d). In GCV-treated mice, red pulp macrophages as well as marginal zone macrophages and marginal metallophils are still present, thus supporting the selective elimination of marginal DC on the basis of their expression of TK and high turnover (data not shown). Thus, from these experiments we conclude that marginal DC have a significantly higher turnover compared with IDC.

Discussion

In the present study, we have investigated the characteristics of two populations of DC in the mouse spleen: the classic IDC in the white pulp T cell area, and the marginal DC, located in the marginal zone bridging channels and extending from the marginal zone into the red pulp. Both populations express aminopeptidase N/CD13, recognized by ER-BMDM1 mAb, as well as CD11c. Thus, the previously identified CD13<sup>+</sup> marginal red pulp macrophages (17) are identical to the marginal DC. Further phenotypic
analysis of splenic DC has shown that freshly isolated marginal DC, as well as IDC, express significant levels of typical macrophage markers. In addition, the marginal DC population is phagocytic in vivo, as indicated by their depletion by clodronate-liposomes and the ability to accumulate fluorescent dye from Dil-labeled liposomes. For the IDC population, we found no indications that point to in vivo phagocytic activity. A further distinction between the two DC populations is substantiated by the finding that marginal DC, but not IDC, are depleted by GCV in LTR-TK BM chimeric mice, indicative of the high turnover rate of only the marginal DC population.

Our findings on the splenic DC marker profiles confirm and extend previous reports on splenic DC phenotypes (30–33). Not only marginal DC but also IDC, when freshly isolated, express markers thought to be characteristic of macrophages, such as F4/80, BM8, and Mac-1/CD11b when Ab binding is assessed by flow cytometry. Using less sensitive immunohistology, however, a clear distinction can be detected between the red pulp macrophages, which express high levels of F4/80 and BM8, and the DC, which express lower levels of these markers. This is in agreement with previous findings (12). Upon overnight culture, DC undergo a maturation step and show decreased binding of F4/80 and BM8 mAb, whereas the DC markers, MHC class II and NLDC-145, are increasingly expressed. With respect to the latter, now cells originating from the marginal DC population also become NLDC-145\textsuperscript{+}, but the two populations still are recognized as separate and discrete. In accordance with recent findings, we observe CD8\textsuperscript{a} expression by only a subpopulation of DC (3, 31, 32). This subset corresponds quantitatively to the NLDC-145\textsuperscript{+} population over a range of frequencies (compare this study with Refs. 3 and 31), and in freshly purified splenic DC it was recently shown that NLDC-145 and CD8\textsuperscript{a} are expressed by the same cells (32). Based on this, IDC...
are the most likely candidates for the CD8α⁺ subset of DC (31, 32),
leaving the marginal DC as the CD8α⁻ subset. The latter was indeed
confirmed by immunohistology (our unpublished observations). It
cannot be excluded, however, that some cells with a marginal DC
phenotype are located in the white pulp also under steady-state con-
ditions, as was observed after LPS administration (34).

Expression of a number of genuine macrophage markers by
splenic DC prompted us to investigate the ability of these cells to
phagocytose particles as a typical function of macrophages. We
have chosen to use relatively large multilamellar phosphatidylchlo-
line liposomes as model particles since numerous previous studies
have shown that these agents have a high selectivity for macro-
phages in vivo and, when loaded with clodronate, can be used for
selective elimination of phagocytic cells (27, 35, 36). Based on the
actual depletion of marginal DC by clodronate-liposomes and the
observed labeling of isolated DC after application of Dil-carrying
liposomes, we conclude that at least a subset of DC is phagocytic
in vivo. In support of this finding, recent studies in the rat have
shown that i.v. applied particulates can be phagocytosed in vivo by
DC (16). These cells were detected in hepatic lymph, but the site
of actual phagocytosis remained unclear. At least part of the
phagocytic DC was suggested to have migrated from the spleen to
the liver into hepatic lymph, and in line with this, we propose that
the marginal DC are the most likely candidates. Interestingly, mi-
gration studies have shown preferential homing of the majority of
isolated splenic DC to the liver (37).

The elimination of marginal DC after phagocytosis of clod-
ronate-liposomes occurs in all likelihood by apoptosis of these
cells. Recent studies have shown that accumulation of clodronate
encapsulated in multilamellar liposomes is a trigger for the apo-
ptic pathway in activated human monocytes and mouse macro-
phages (36, 38, 39). Not all cell types, however, that incorporate
liposomes seem to die. In vitro application showed that, in addition
to monocytes, polymorphonuclear cells and endothelial cells also
take up liposomes, but these cells do not undergo apoptosis (39).
Elimination is only accomplished in cells 1) in which a sufficiently
high threshold of clodronate is reached, and 2) that possess the
lysosomal machinery to cleave the liposome membranes and so
release their content. These requirements are only met in activated
monocytes, macrophages, and macrophage cell lines. Our finding
that marginal DC are also depleted by uptake of clodronate-lipo-
somes implies that these cells not only phagocytose at a sufficiently
high level, but also possess the required lysosomal activity to break
down the liposomes. Thus, in addition to the phenotypic resem-
bance, these features support the view that the marginal DC are
closely related to the macrophage lineage.

The capacity of marginal DC to phagocytose is likely indicative
of the relative immaturity of these cells. In vitro-proliferating BM
progenitors of DC have been shown to phagocytose latex and bact-
eria, but lose this capacity upon maturation (15). In addition, the
in vivo phagocytic DC, detected in liver and hepatic lymph after i.v.
application of particulates, are derived from recently divided
precursor cells (16). Furthermore, the mouse precursor DC cell
lines XS52 and FSDC show avid pinocytosis and phagocytosis,
which is suppressed when the cells are stimulated to mature (40,
41). Maturation of DC is induced by cytokines such as IFN-γ,
IL-1, granulocyte macrophage-CSF, and TNF, which in vivo are
likely produced by T cells and macrophages, communicating with
the immature DC (41–43). Thus, phagocytosis by marginal DC,
like endocytosis by DC in general, is most likely an immature
trait (13).

Immaturity of the marginal DC is underlined by our finding that
these cells are depleted in GCV-treated chimeric LTR-TK mice.
Depletion in this system is based upon the termination of elongat-
ing DNA by a phosphorylated form of the nucleoside analogue
GCV (44). Initial phosphorylation occurs by the Herpes simplex
thymidine kinase. Thus, actual depletion is only observed in those
cells that synthesize DNA and express the TK transgene, i.e., do-
nor BM-derived DC. So, either the marginal DC themselves or
their immediate precursors multiply, while these cells are retained
in the marginal location only for a short period of time. Remark-
ably, IDC were hardly affected in these mice, indicating that these
cells have a much slower turnover rate. Such a dichotomy between
DC subsets has been suggested before (7), although limited exper-
imental evidence has been provided.

The question then arises whether the “immature” marginal DC
are the precursors of “mature” IDC, as has been suggested (10).
Culture of freshly isolated DC, i.e., primarily marginal DC, indeed
induces expression of NLDC-145 and M342 Ags, typical for IDC
(Fig. 3 and Refs. 10 and 32). In addition, isolated splenic DC can
home to the white pulp T cell zones when injected i.v. (45). Fur-
thermore, in vivo administration of LPS induces maturation of
marginal DC, concomitant with their migration into the T cell ar-
eas (34). Some observations, however, challenge a putative pre-
cursor-endstage relationship between marginal DC and IDC under
steady-state conditions. As demonstrated in our results, a clear
phenotypic distinction remains between the two populations of DC
upon culture, despite the induction of NLDC-145 Ag expression in
marginal DC. And, as mentioned before, the majority of isolated
splenic DC homes to the liver, whereas the minority homing to the
splenic T cell areas might represent the population originally iso-
lated from this location. In addition, CD8α also seems to be a
stable determinant discriminating between the two populations, as
it is present only on IDC. It has been proposed that CD8α is a
marker for DC of lymphoid origin, rather than of myeloid deriv-
a (3, 31, 32). In contrast, the marginal DC bear all characteris-
tics of a myeloid origin and close relationship to macrophages, as
is apparent from their elimination by clodronate-liposomes. A dif-
ferent lineage derivation of marginal DC and IDC, although for-
mally not proven, is clearly incompatible with a precursor-end-
stage relationship between these cells.

Both DC populations seem to play fundamentally distinct roles
in the regulation of T cell responses. CD8α⁺, presumed lymphoid,
DC have recently been shown to kill Ag-specific CD4⁺ T cells via
Fas-Fas ligand interaction. These cells can stimulate a prolonged
CD8⁺ T cell response only if exogenous IL-2 is present (8, 9). In
contrast, CD8α⁻, presumed myeloid, DC are the classic profes-
sionals of both CD4⁺ and CD8⁺ T cell responses. In this
respect, it is an intriguing notion that the stimulating, myeloid DC
are located outside the classic splenic T cell area, yet are in the
migration pathway of homing T cells, namely in the marginal zone
bridging channels (46, 47). In line with this, activated T cells ex-
pressing CD40L have been found in immunized spleen only near
the terminal arteriole, compatible with the location of the marginal
DC, but not in the T cell area of the white pulp (48). A hypothe-
sical scenario thus might be that circulating T cells are activated outside
the splenic white pulp upon recognition of cognate Ag presented
by myeloid DC, and then move into the periarteriolar lymphoid
sheath, either or not accompanied by the maturing myeloid DC. In
the T cell areas then, the T cell response is finalized, tightly bal-
anced by the regulatory, lymphoid DC.

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