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Follicular Dendritic Cell (FDC) Precursors in Primary Lymphoid Tissues

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The origin of follicular dendritic cells (FDC) is unresolved, and as such, remains controversial. Based on the migration of Ag-transporting cells (ATC) into lymphoid follicles and the phenotypic similarity between FDC and ATC, one hypothesis is that ATC may represent emigrating FDC precursors. This contrasts with the view that FDC originate from local stromal cells in the secondary lymphoid tissues. Mice homozygous for the severe combined immunodeficiency (prkdcscid) mutation (scid) lack FDC. Thus, they provide a powerful tool for assessing de novo generation of FDC. To test whether FDC precursors could be found in bone marrow or fetal liver, scid/scid mice were reconstituted with either: 1) bone marrow cells from (BALB/c × C57BL/6)F1 donors, 2) bone marrow cells from ROSA BL/6 F1 (lacZ-transfected) mice, 3) rat bone marrow cells, or 4) rat fetal liver cells. Six to eight weeks after reconstitution with F1 bone marrow cells, recipients did not express FDC-M1, the mAb used to detect FDC. In contrast, mice reconstituted with lacZ-transfected bone marrow cells, these cells were also positive for the lacZ gene product. Furthermore, in spleens of animals reconstituted with either rat bone marrow or rat fetal liver, FDC were identified using the specifically labeling mAb, ED5. In all cases, host FDC were also present, indicating that scid/scid mice have FDC precursors that will mature in the presence of allogeneic or xenogeneic lymphoid cells. In summary, FDC can be derived from progenitor cells present in primary lymphoid tissues. The Journal of Immunology, 1998, 160: 1078–1084.

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5 Abbreviations used in this paper: FDC, follicular dendritic cell; ATC, antigen-transporting cell; FDG, fluorescein-di-β-D-galactopyranoside; PE, phycoerythrin; H-2L, heavy plus light chain.

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Severe combined immunodeficiency (prkdc<sup>scid</sup>) mice, hereafter termed SCID mice, lack functional B and T cells (13) and have been shown to also lack FDC (14). As such, the SCID mouse appears to provide an optimal system to study the origin of FDC, since the problem of eliminating radioresistant mature FDC could be bypassed. Using the SCID mouse model, the objective of the present study was to determine whether primary lymphoid tissues contained FDC precursors. We report in this work that FDC of the donor phenotype can be found in SCID mice after reconstitution with primary lymphoid tissue from either mice or rats. These data indicate that FDC can be derived from precursors in primary lymphoid tissues that migrate into secondary lymphoid tissues, and these results raise questions about FDC being derived from local stromal cells in the secondary lymphoid tissue.

Materials and Methods

Animals

Pregnant females (for newborn mice) or 6- to 8-wk-old homozygous mutant C.B-17-scid/scid (H-2<sup>d</sup>, SCID), C57BL/6J-scid/scid (H-2<sup>b</sup>, SCID), and F<sub>1</sub> (BALB/cBy × C57BL/6J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Lewis rats (day 16 gestation) were purchased from Harlan Laboratories (Indianapolis, IN). ROSA BL/6 F<sub>1</sub> (H-2<sup>b</sup>) were a generous gift of Herzenberg Laboratory at Stanford University (Palo Alto, CA). The animals were housed in a specific pathogen-free environment and given food and water ad libitum.

Cell transfers for reconstitution

Before cell transfer, C.B-17-SCID mice were irradiated with 300 rad to facilitate reconstitution (15). Bone marrow cells were obtained from femurs and tibias of F<sub>1</sub> mice, ROSA BL/6 F<sub>1</sub> mice, and Lewis rats. Rat fetal liver cells were obtained by homogenizing the fetal livers between frosted glass slides. For reconstitutions, 6- to 8-wk-old SCID mice received i.v. 2 × 10<sup>6</sup> murine bone marrow or 5 × 10<sup>6</sup> rat fetal liver cells suspended in 200 µl of HBSS supplemented with HEPES (25 mM) and gentamicin (50 µg/ml). Newborn C.B-17-SCID mice received 10<sup>7</sup> rat bone marrow or rat fetal liver cells i.p. Newborn C57BL/6J-SCID mice received 5 × 10<sup>6</sup> ROSA BL/6 F<sub>1</sub> bone marrow cells i.p.

Immunizations

Mice received an initial 0.1 ml injection consisting of 200 µg/ml alum-precipitated human serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO) plus 5 × 10<sup>8</sup> heat-killed Bordetella pertussis s.c. behind the neck. Two to ten weeks later, the mice were given a booster immunization (0.05 ml) on the dorsum of each footpad with the same immunogen. Lymph nodes and spleens were obtained from these mice 5 days after the secondary challenge.

Immunohistochemistry

The mouse anti-rat FDC-specific mAb 6D5 (17) (a generous gift from Dr. C. D. Dijkstra, Free University, Amsterdam, The Netherlands) was used to detect rat FDC. Mouse FDC were detected by unconjugated or biotinylated rat anti-mouse FDC-reactive mAb, FDC-M1, followed by washing and incubation with streptavidin-phycoerythrin (PE) or FITC. The mouse anti-rat FDC-specific mAb ED5 (17) (a generous gift from Dr. Schnitzlein et al. (18) and modified by Kosco et al. (11, 19) was used to isolate FDC. The low density, non-adherent cell fraction was obtained from enzymatically digested lymph nodes of immune mice 2 days after whole body irradiation (600 rad; cesium source). The gentle isolation procedure is essential for isolating FDC, and radiation treatment is important to isolate FDC with far fewer contaminating lymphocytes. The association of B cells interwoven within the FDC dendritic processes makes standard depletion techniques difficult (19). In brief, the draining lymph nodes from immunized mice were placed in small tissue culture plates containing an enzyme mixture of collagenase (2.5 mg/ml;CLS 4, number 4188; Worthington, Biochemical Corp., Freehold, NJ) and deoxyribonuclease I (1/5%;DN25; Sigma Chemical Co.) in 2 ml HBSS. Each lymph node capsule was opened using two 26-gauge needles, and the preparation was placed in an incubator at 37°C. 5% CO<sub>2</sub>. After 20 to 30 min, the partially digested stroma was gently pipetted. The released cells within the supernatants were then collected in tubes containing HBSS plus 5% FCS and placed on ice. A fresh aliquot of enzyme mixture was then added to the remaining tissues and returned to the incubator. After another 30 min, the tissue was again pipetted, this time to the point that nearly all of the cells were released from the stroma. The few remaining large pieces of tissue were discarded, and all supernatant fluids were pooled. The cells were then washed by centrifugation in HBSS and resuspended to 1 × 10<sup>8</sup> cells/ml, and 2 ml was layered over each continuous Percoll gradient. The gradients were then centrifuged at 400 × g for 30 min, and the 1.060 to 1.065 g/ml low density band was removed. After washing this fraction two or three times in HBSS, the cells were resuspended in complete medium (HBSS plus 10% FCS, penicillin/streptomycin, and 2-ME). The suspension was placed in a small tissue culture dish (Costar, 3035, Cambridge, MA) and incubated at 37°C for 1 h to deplete adherent populations.

Flow cytometric analysis

To elucidate the expression of lacZ, the FDC-enriched cell preparation was stained with a fluorogenic substrate for β-galactosidase, fluorescein-di-β-d-galactopyranoside (FDG) (Molecular Probes, Eugene, OR), as described in detail elsewhere (20). Briefly, cells were loaded with FDG substrate by a hypotonic shock at 37°C for 1 min. FDC were identified by incubating the cells with biotinylated rat anti-mouse FDC-reactive mAb, FDC-M1, followed by washing and incubation with streptavidin-phycocerythrin (PE) (Southern Biotechnology). FDC-enriched cell preparations from BALB/cBy mice served as a negative control for lacZ expression. Cells (1 × 10<sup>6</sup>) were analyzed on a FACSScan (Becton Dickinson, San Jose, CA) flow cytometer. Dead cells were excluded from analysis using propidium iodide uptake.

Results

Reconstitution of SCID mice with bone marrow from F<sub>1</sub> donors

Four to six months after transferring F<sub>1</sub> bone marrow cells into adult SCID mice, spleen and lymph nodes were harvested, sectioned, and analyzed for FDC of both the donor and host phenotype. In many follicles of the F<sub>1</sub> bone marrow reconstituted SCID mice, it appeared that FDC expressed only the host class I molecules in germinal centers (Fig. 1f). However, in other follicles, numerous FDC bore donor class I molecules, as indicated by double labeling for donor class I and FDC-M1 (Fig. 1c).

Reconstitution of SCID mice with bone marrow from lacZ-transfected ROSA BL/6 F<sub>1</sub> mice

Confirmation of FDC precursors in murine bone marrow was obtained using the lacZ mouse model. ROSA BL/6 F<sub>1</sub> mice transfected with the lacZ gene express the gene product, β-galactosidase, in all cells (16). Through the action of this gene product, the fluorescein conjugated substrate, FDG (20), is cleaved and fluorescein is released into the cytoplasm. As a result, the cell becomes fluorescent and detectable by flow cytometry. Using the same protocol for the construction of chimeras as above, newborn SCID mice received bone marrow cell transfers from ROSA BL/6 F<sub>1</sub> mice. We reasoned that if SCID mice received ROSA BL/6 F<sub>1</sub>-derived bone marrow FDC precursors, then donor FDC could be identified by
the presence of the lacZ gene product. Newborn mice were chosen to minimize the potential for host FDC precursors to establish themselves before the donor FDC precursors. As indicated in Figure 2D, more than one-half of the FDC-M1− cells obtained from these chimeric mice also labeled with FDG, indicating the presence of the lacZ gene product representing donor-derived FDC. Incubating these preparations without the fluoresceinated substrate or streptavidin-PE provided the background level of labeling (Fig. 2A). An FDC-enriched preparation obtained from normal BALB/cBy mice treated with the FDC-M1 mAb and the fluoresceinated substrate (Fig. 2B) is included for comparison, and finally, the FDC-enriched preparation from chimeric mice incubated without the FDC-M1 mAb is shown in Figure 2C.

Lymphocytes are often intimately associated with FDC, and thus possibly contributing to the lacZ-positive population detected by flow cytometry. Irradiation eliminates most lymphocytes and minimizes this problem. However, to rule out the possibility that the lacZ-positive lymphocytes were responsible for the labeling, FDC in the preparations were identified also by morphology using a microscope equipped with Nomarski optics. The results shown in Figure 3, a–h, confirmed the presence of the lacZ gene product in cells with FDC morphology. FDC isolated from BALB/cBy control mice did not show green fluorescence (control; data not shown).

Reconstitution of SCID mice with bone marrow or fetal liver from rats

Both donor and host FDC appear to develop in the recipients of a murine bone marrow transfer. The mAb FDC-M1 does not distinguish between donor vs host FDC. We therefore sought to confirm the presence of donor FDC using a second system in which the mAb will only label donor FDC. SCID mice can be reconstituted with rat cells (21). We therefore reconstituted newborn SCID mice with bone marrow or fetal liver cells derived from rats, and 4 to 6 mo later, spleen and lymph nodes were analyzed for the presence of FDC of either rat (ED5-positive) or murine (FDC-M1-positive) origin. Of seven rat bone marrow reconstituted SCID mice, three mice clearly showed the presence of ED5+ FDC networks in lymph nodes and spleens (Fig. 4, a and c). Similarly, one of five recipients of rat fetal liver contained rat FDC in their lymphoid organs (Fig. 4f). We also noted that a given follicle tended to have either rat- or mouse-derived FDC predominating, although there were follicles with both cell types (Figs. 4, d and g). It should be noted that FDC-M1+ cells were not reactive with ED5, indicating that the mAb ED5, as reported (17), does not cross-react with mouse FDC. Similarly, the ED5+ cells did not cross-react with the mAb FDC-M1 (Fig. 4, c and d). Clearly, reconstituting mice with rat bone marrow or fetal liver was sufficient to elicit the maturation
of murine FDC in both the lymph nodes and the spleens (Figs. 4, d and g). In some rat bone marrow or fetal liver reconstituted SCID mice that lacked rat FDC, it appears that the rat cells had simply not engrafted. In others, there were sites in which a few ED5-positive cells appeared to be present in follicles, but the typical large FDC reticulum was not apparent and the animals were scored as negative.

**Discussion**

This study demonstrates that bone marrow contains FDC precursors, and this conclusion is supported by results obtained in three different systems: 1) donor class I molecules on FDC in F1 bone marrow reconstituted SCID mice, 2) lacZ-positive FDC in ROSA BL/6 F1 bone marrow reconstituted SCID mice, and 3) the presence of ED5-positive FDC in rat bone marrow (or rat fetal liver) reconstituted SCID mice. These results strongly support the concept that FDC can be derived from primary lymphoid tissue and need not develop from cells that originate in the local secondary lymphoid tissue.

The radioresistant nature of FDC makes it difficult to eliminate these cells, and it is possible that in radiation chimeras, the persistence of host FDC reported in previous studies (8) inhibited or markedly diluted the donor source of FDC in the tissue. It should be noted that FDC bear immunogen for many months to years, suggesting that the FDC are not turning over, and thus, FDC in secondary lymphoid tissue might be expected to persist for months to years (1). SCID mice lack FDC (14), and therefore, the problem of eliminating host FDC at the outset can be bypassed. Recently, we showed that transferring B and T cells can induce host FDC development in SCID mice, suggesting the presence of FDC precursors in these mice (14). The fact that rat bone marrow cells would support the development of murine FDC, noted in the present study, further supports the concept that FDC precursors are present in SCID mice. Previous reports showed that FDC are first found in lymphoid tissues at about 3 wk after birth (2, 4, 22). We reasoned that pre-FDC may migrate from the bone marrow and may be dispersed in tissues throughout the body. We also believe that these dispersed precursors play an important role in Ag transport and repopulation of FDC networks in recipients, whether these are SCID mice or radiation chimeras. Thus, even if FDC precursors in the bone marrow are less radioresistant than mature FDC, the pool of intermediate-type pre-FDC could be large.
enough to supply precursors for months, resulting in the development of new FDC networks. By injecting the donor bone marrow cells containing FDC precursors into newborn SCID mice, we sought to minimize the competition from host FDC and their precursors. The injected donor bone marrow cells are known to home to the host bone marrow (23), and we reason that both donor and host FDC precursor cells are dispersed to various connective tissue compartments via the blood. The mobilization stimulus for these donor and host pre-FDC (ATC) (10) may be a cytokine or a lymphokine, as suggested by the B and T cell requirements for FDC development (14). The absence of FDC networks in TNF-\(\alpha^{--}\) (24) or LT-\(\alpha^{--}\) (25) mice suggests these lymphokines may play a role in FDC development. The formation of immune complexes that bind the pre-FDC may lead to the migration of donor and host ATC into the draining lymph nodes. Thus, the presence of both donor and host FDC in the lymph nodes and spleen of reconstituted newborn SCID mice might be predicted.

Recently, Yoshida et al. (26) showed the presence of host FDC in the spleen of SCID mice after transferring allogeneic lymphocytes. This is in agreement with our data showing the development of mouse FDC in SCID mice reconstituted with rat bone marrow and fetal liver cells. It appears that FDC precursors in SCID mice

FIGURE 3. Nomarski differential interference contrast and fluorescence micrographs of FDC-enriched cell populations isolated from SCID mice reconstituted with ROSA BL/6 F\(_1\) bone marrow cells. \(a, c, e, \) and \(g\), Nomarski micrographs, show four different FDC that are positive for the \( \text{lacZ} \) gene product shown in \(b, d, f, \) and \(h\), fluorescence micrographs, respectively. Magnification: \( \times 1200\)
can develop in presence of syngeneic, allogeneic, or even xenogeneic lymphocytes, as shown in this study. Additionally, they also noted the presence of FDC-M1-positive cells that did not appear to express the host phenotype. These authors indicate that FDC-M1 reacts with two FDC populations of different origin (26). It is possible that heterogenous populations of FDC may have different

**FIGURE 4.** a–h, Micrographs of lymph node and spleen FDC reticula from SCID mice reconstituted with rat bone marrow or fetal liver. a, ED5-positive rat FDC reticulum (arrows) in the spleen of a SCID mouse reconstituted with rat bone marrow; central arteriole (arrowhead). Magnification: ×175. b, Adjacent serial section to the one depicted in a, labeled only with the secondary Ab. Note the absence of any labeling of FDC reticulum; central arteriole (arrowhead). Magnification: ×175. c, ED5-positive rat FDC reticulum (arrows) in a lymph node of a SCID mouse reconstituted with rat bone marrow. Magnification: ×175. d, Adjacent serial section to the one depicted in c, labeled with biotinylated mouse FDC-specific mAb, FDC-M1. Note that there are very few FDC-M1-positive cells, and these do not overlap the ED5-labeled cells shown in c. Magnification: ×175. e, Section adjacent to those depicted in c and d, labeled only with the secondary Ab. Note the absence of any labeling. Magnification: ×175. f, ED5-positive rat FDC reticulum (arrows) in the lymph node of a SCID mouse reconstituted with rat fetal liver cells. Magnification: ×175. g, FDC-M1-positive mouse FDC reticulum (arrows) in a splenic follicle of a SCID mouse reconstituted with rat fetal liver cells; central arteriole (arrowhead). Magnification: ×175. h, Adjacent serial section to the one depicted in g, labeled with the rat FDC-specific mAb ED5. Note the absence of any ED5 labeling, indicating that ED5 does not cross-react with mouse FDC; central arteriole (arrowhead). (The double, FITC/Texas Red filter of the fluorescent microscope gives a reddish-brown color to unstained tissues.) Magnification: ×175.
origin. However, since they used 6-wk-old SCID mice, in contrast to newborn SCID mice, for reconstitution and splenic cells enriched for B lymphocytes in contrast to bone marrow cells, it may have minimized the development of FDC of donor origin in their study. Moreover, these authors examined the spleens 5 to 6 wk after reconstitution in contrast to 3 to 6 mo in most of our experiments. We believe that due to slow turnover of FDC (1), long-term reconstitution (3–6 mo) allows time for donor precursors to be recruited in the formation of FDC networks. This time frame contrasts with our previous study, in which FDC were found 2 to 3 wk after reconstitution with B and T cells (14). We reason that numerous FDC precursors are present in adult SCID mice, and that they only require lymphocyte stimulation to complete development.

In the present study, we deliberately used newborn SCID mice so that both donor and recipient precursors are at an early stage of development, and we tried to inject enough bone marrow cells so that the number of host FDC precursors did not overwhelm the small number of donor FDC precursors injected. The presence of similar numbers of host and donor FDC in the adult mice suggests that we were successful (Fig. 2).

In previous studies, a one-to-one relationship between the number of follicles trapping immune complex and the number of germinal centers that subsequently developed was described (27, 28).

This suggests the Ag-bearing FDC elicited the germinal center and that the FDC is critical to the process. The fact that TNF-α−/− (24) and LT-α−/− (25) mice lack FDC and also lack germinal centers further supports the concept that FDC are critical to the process of normal germinal center development. Interestingly, Cr2−/− mice also lack normal germinal center development (29), and this may also relate to a deficit in FDC-B cell communication. FDC bear immune complexes and C3 fragments that are known ligands for CR2, and this prompted the hypothesis that CR2 ligand on the FDC binds CR2 receptor on the B cell (30). Recent data from our laboratory support this hypothesis and indicate that Ab responses are depressed dramatically when FDC-B cell communication via CR2 ligand/CR2 is blocked (Qin et al., unpublished).

The chief issue raised and unresolved by the present study is whether bone marrow FDC precursors are derived from hemopoietic or stromal cells. Experiments are underway in which we are looking at SCID mice reconstituted with various kinds of bone marrow cells, including leukocyte precursors. In preliminary studies, we have identified cells in the bone marrow reacting with the mouse FDC mAb FDC-M1, and these cells are also reactive with F4/80. It is possible that the F4/80-FDC-M1 reactivity may be a coincidence or a true indication of a hemopoietic/myeloid lineage for FDC. The fact that Parwaresch et al. (12) demonstrated that the FDC-specific mAb KiM4 reacts with a mononuclear cell in human blood, together with the fact that the mAb was originally raised against the U-937 cell line, which is closely related to monocytes, further suggests the idea that FDC may be of a hemopoietic/myeloid lineage.

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