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# Follicular Dendritic Cells Carry MHC Class II-Expressing Microvesicles at Their Surface<sup>1</sup>

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Follicular dendritic cells (FDCs) present in lymphoid follicles play a critical role in germinal center reactions. They trap native Ags in the form of immune complexes providing a source for continuous stimulation of specific B lymphocytes. FDCs have been reported to express MHC class II molecules, suggesting an additional role in the presentation of not only native, but also processed Ag in the form of peptide-loaded MHC class II. Adoptive bone marrow transfer experiments have shown that MHC class II molecules are only passively acquired. Up to now the origin of these MHC class II molecules was not clear. Here we show by cryoimmunogold electron microscopy that MHC class II molecules are not present at the plasma membrane of FDCs. In contrast, microvesicles attached to the FDC surface contain MHC class II and other surface proteins not expressed by FDCs themselves. The size and marker profiles of these microvesicles resemble exosomes. Exosomes, which are secreted internal vesicles from multivesicular endosomes, have been shown earlier to stimulate proliferation of specific T lymphocytes *in vitro*, but their target *in vivo* remained a matter of speculation. We demonstrate here that isolated exosomes *in vitro* bind specifically to FDCs and not to other cell types, suggesting that FDCs might be a physiological target for exosomes. *The Journal of Immunology*, 2000, 165: 1259–1265.

Follicular dendritic cells (FDCs)<sup>3</sup> are accessory cells of the immune system essential for the germinal center reaction (1–4). During germinal center reactions, FDCs present Ags to B lymphocytes and T lymphocytes (5). B cells recognize native Ags held in immune complexes at the surface of FDCs. To this aim, FDCs express a set of several different complement receptors (6, 7). The binding of B lymphocytes to these Ags is highly competitive and an essential selection step for memory B lymphocytes. A prerequisite for stimulation of T helper cells is MHC class II expression, which has been reported for FDCs in several studies using light microscopy (summarized in Ref. 8). However, the suggested role in presentation of processed Ag in the form of peptide-loaded MHC class II is not in line with the fact that FDCs characteristically do not have phagocytic activity (9). Furthermore, FDCs do not synthesize MHC class II molecules themselves (5). This was demonstrated in bone marrow transplantation studies of Gray et al. (5), who showed that murine FDCs passively acquire peptide-loaded MHC class II molecules from the donor. The mechanistic basis of this phenomenon remained an enigma up to now.

In B cells, MHC class II molecules predominantly reside in MHC class II compartments (MIICs) (10), which are the subcellular site of peptide loading onto MHC class II molecules (11). MIICs are part of the endosomal/lysosomal pathway in APC and often have a multivesicular phenotype (12, 13). The internal vesicles of multivesicular MIICs are probably formed by inward budding from the limiting membrane (14), a process that seems to require phosphatidylinositol 3-kinase activity (15). Multivesicular MIICs can fuse with the plasma membrane in an exocytic manner, thereby releasing their internal vesicles, which have been shown to carry MHC class II molecules at their surface and to be capable of specifically stimulating CD4-positive T cells *in vitro*. The released internal vesicles of MIICs have been called exosomes (16), a term that was introduced for exocytosed vesicles in reticulocytes (17). Exocytosis of MIICs or multivesicular bodies has also been found in other APC, such as dendritic cells (18, 19), tonsil B cells (M.J.K. and H.J.G., unpublished data), and other hemopoietic cells, such as mast cells (20) and platelets (21).

We have previously shown that the internal vesicles of MIICs and exosomes are enriched in members of the tetraspan protein superfamily, such as CD37, CD53, CD63, CD81, and CD82 (22). Tetraspan proteins have been shown to be involved in Ag presentation, T cell signaling, T cell activation, motility, and adhesion. They are able to form oligomeric complexes with other tetraspan proteins (23–25), HLA-DR (23, 26), HLA-DM and -DO (26) and interact with integrins (25, 27) and the T cell coreceptors CD4 and CD8 (28). By immunoelectron microscopy (IEM), tetraspan proteins are confined to the internal vesicles of MIICs and exosomes, whereas other MIIC markers, such as Lamp-1, Lamp-2, and HLA-DM, reside primarily in the MIIC-limiting membrane (22) and are absent from exosomes.

In this paper, we present an IEM analysis of the microvesicles attached to the FDC surface *in situ* and show that these vesicles represent exosomes, possibly derived from B cells. The specific accumulation of exosomes on FDCs might play a role in fine tuning of the immune response.

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<sup>3</sup> Abbreviations used in this paper: FDC, follicular dendritic cell; IEM, immunoelectron microscopy; LBPA, lysobisphosphatidic acid; MIIC, MHC class II-enriched compartment.

## Materials and Methods

### Cells and Abs

The EBV-transformed B cell line RN was maintained as described (16). Commercial mouse mAbs were anti-CD37 (S-B3; Sanofi, Montpellier, France), anti-CD107b/Lamp-2 (H4B4; PharMingen, San Diego, CA), anti-CD63/Lamp-3 (CLB-gran1/2, 435), anti-CD53 (MEM-53), and anti-LFA-1 (CLB-LFA-1/1, CD18) from CLB (Amsterdam, The Netherlands). The mAbs against CD81 (M38) and CD82 (C33) were a gift of Dr. O. Yoshie (Osaka, Japan); mAb 5C1 (HLA-DM) was obtained from Dr. J. Trowsdale (Cambridge, U.K.). The mAb 6C4 (against lysobisphosphatidic acid (LBPA)) was provided by Jean Gruenberg (Geneva, Switzerland). The rabbit polyclonal Abs used in this study were: anti-MHC class II from Dr. H. L. Ploegh (Massachusetts Institute of Technology, Cambridge, MA), anti-biotin (Rockland, Gilbertsville, PA), anti-CD35/CR1 (3D9) obtained from J. A. Schifferli (Basel, Switzerland) (29), polyclonal anti-Ii (ICN-1 and ICC5) from Dr. P. A. Morton (Monsanto Co., St. Louis, MO) (30), and anti-mouse IgG from Dako (Glostrup, Denmark).

### Coisolation of FDCs with *in vivo* activated B lymphocytes

Fresh human tonsils were obtained from patients undergoing routine tonsillectomy. B cells were purified as previously described (31, 32). In short, T cells were removed by two cycles of rosetting with neuraminidase-treated sheep erythrocytes. The nonrosetting cells were further depleted of adherent cells by incubation in tissue culture flasks in HEPES-buffered RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with 50% heat-inactivated FCS for 1 h at 37°C. The nonadherent cells were recovered and subjected to centrifugation through a discontinuous 65, 52.5, and 42.5% Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden). At the interface of 42.5%–52.5% Percoll, the low density fraction was harvested which contained *in vivo* activated B cells and several FDCs. Cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer and processed for ultracytometry as described below.

### Isolation of FDC-B cell clusters

FDCs were isolated from tonsils as described by Parmentier et al. (33). Tonsils were cut into pieces and treated with a collagenase (200 U/ml collagenase IV, Worthington Biochemical, Lakewood, NJ)-DNase (10 U/ml DNase I, Boehringer Mannheim, Mannheim, Germany) solution in IMDM (Life Technologies), followed by density sedimentation on a cold discontinuous BSA gradient (Path-o-cyte 4, bovine albumin, Instruchemie, The Netherlands) in HBSS consisting of layers of 1.5, 2.5, and 5%. The cells at the 2.5%–5% interface were harvested and washed in HBSS and IMDM. After incubating the cells with a mAb against human LFA-1 for 30 min at 4°C, LFA-1 expressing B cells were depleted by sheep anti-mouse Ig-coated Dynabeads (Dynal, Oslo, Norway). The resulting FDC-enriched cell suspension was cultured for 12–14 h at 37°C in IMDM supplemented with 10% FCS. After culture, it contained FDC/B cell clusters and other cell types as small lymphocytes, plasma cells, macrophages, dendritic cells, erythrocytes, and exceptionally mast cells and basophils.

### Isolation of exosomes

Exosomes were isolated by differential centrifugation as previously described (16). Briefly, RN cells were washed by centrifugation and recultured in fresh medium for 18 h. Cell culture media (35 ml) containing about  $5 \times 10^7$  cells were centrifuged once for 10 min at  $200 \times g$  (pellet P<sub>1</sub>), twice for 10 min at  $500 \times g$  (pellet P<sub>2</sub>), twice for 15 min at  $2,000 \times g$  (pellet P<sub>3</sub>), once for 30 min at  $10,000 \times g$  (pellet P<sub>4</sub>), and finally once for 60 min at  $70,000 \times g$  (pellet P<sub>5</sub>), using a rotor SW27 (Beckman Instruments, Fullerton, CA). To remove the excess of serum proteins, P<sub>5</sub> was washed once in 35 ml PBS and centrifuged again for 60 min at  $70,000 \times g$ . For *in vitro* binding studies with isolated human FDCs, this pellet was resuspended in 1 ml PBS and biotinylated by adding EZ-Link sulfo-NHS-biotin (Pierce, Rockford, IL) at a final concentration of 1 mg/ml for 30 min at room temperature. The reaction was blocked by resuspension in 34 ml PBS/15 mM ammonium chloride and sedimented for 60 min at  $70,000 \times g$ . The pellet was washed once in 35 ml PBS and resuspended in 100  $\mu$ l PBS, 1% BSA, 5 $\times$  penicillin/streptomycin. The resulting homogenous suspension of small vesicles contained only small amounts of plasma membrane and is referred to as biotinylated exosomes. Biotinylation efficiency was >95% as checked by whole mount immunoelectron microscopic analysis (22).

### *In vitro* attachment of exosomes

Isolated FDC/B cell clusters were allowed to attach overnight in a 35-mm petri dish. After the cells were washed three times with 1 ml HBSS at 37°C, the removal of cells in suspension was checked microscopically and 900  $\mu$ l

HBSS, 10% FCS was added. Parallel plates were incubated for 1 h at 37°C with 100  $\mu$ l PBS, 1% BSA, 5 $\times$  penicillin/streptomycin with or without biotinylated exosomes. Cells were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer and processed as described below.

### Immunoelectron microscopy

Cells were processed for ultrathin cryosectioning and immunolabeling as described previously (34–37). Briefly, cells were fixed in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in phosphate buffer. After being washed with PBS-glycine and PBS, cell pellets were embedded in 10 or 12% gelatin, cut into small blocks, which were infiltrated with 2.3 M sucrose overnight at 4°C. Finally, the blocks were frozen in liquid nitrogen for cryosectioning. Ultrathin cryosections were picked up from the knife with a mixture of 2.3 M sucrose and 2% methylcellulose. The sections were indirectly immunolabeled with 10-nm protein A-gold particles in single labelings or 10- and 15-nm particles in double labelings. Finally, the ultrathin sections were embedded in a mixture of 2% methylcellulose and 0.4% uranyl.

### Quantitative analysis

Ultrathin cryosections of cells from the *in vitro* binding experiment were immunolabeled by use of a polyclonal rabbit Ab against biotin and subsequently visualized by 10 nm protein A-gold. Electron micrographs were randomly taken from FDC-B cell clusters and various other cell types at magnifications of  $\times 10,000$  and  $\times 5,000$ – $10,000$ , respectively. The plasma membrane length of FDCs forming a cluster and solitary other cells was determined according to the method of Weibel (38). The number of biotin-positive vesicles attached to it was determined by counting vesicles with a distance of <40 nm from the plasma membrane. Finally, a Student *t* test was performed.

## Results

### FDCs display microvesicles at their surface

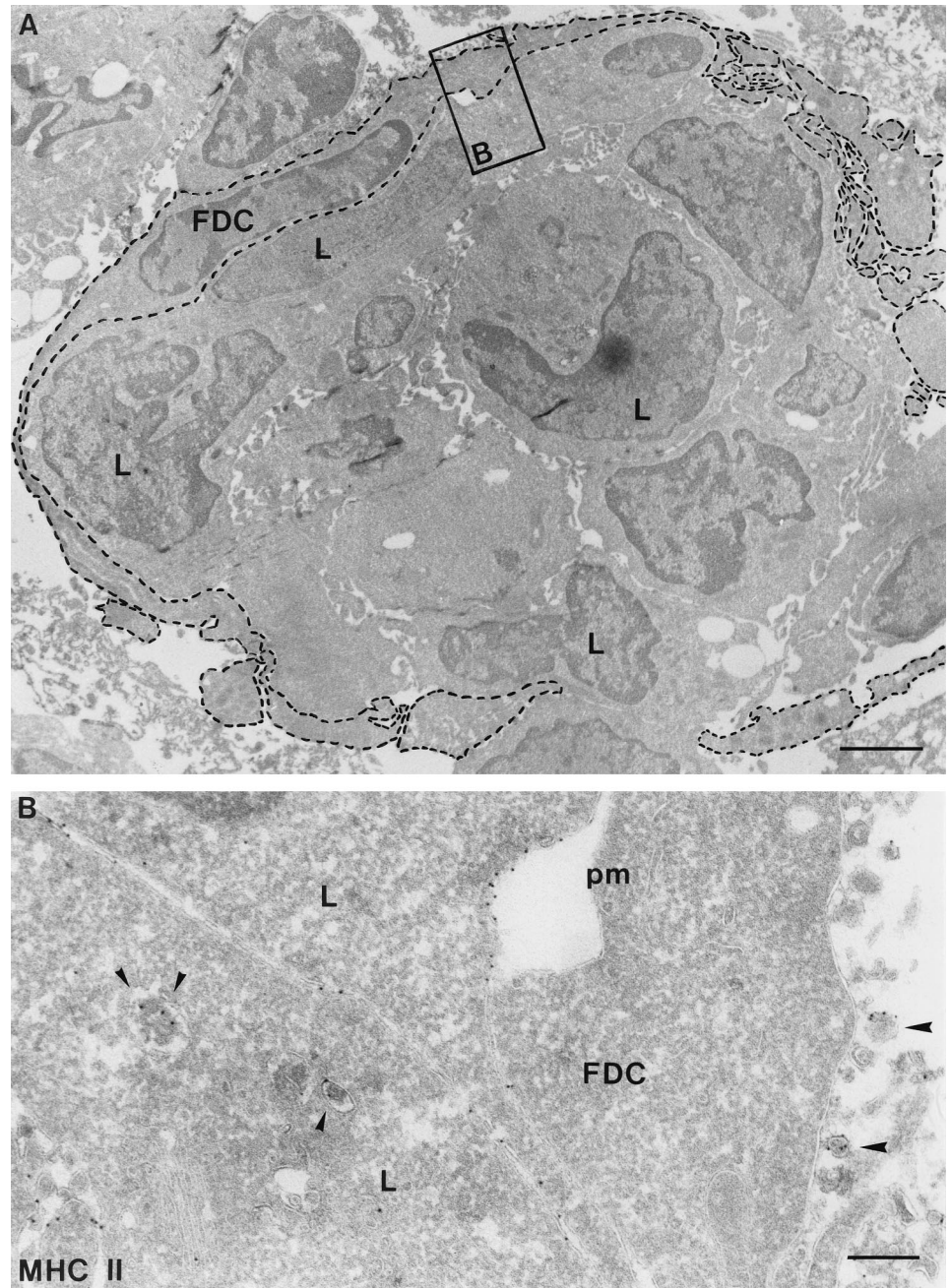
FDCs coisolated with *in vivo* activated B lymphocytes were morphologically characterized by the presence of long and branched dendrites with desmosomes at contact sites between neighboring dendrites. Immunogold labeling showed that FDCs were heavily labeled for CD35 (complement receptor 1), whereas different Abs against other FDC-specific markers such as the long isoform of CR2/CD21 (39) failed to react in IEM. Interestingly, numerous 40- to 70-nm membrane vesicles were seen attached to the surface of FDCs (Fig. 1), but not those of B cells. These microvesicles were different from the iccosomes described by Szakal et al. (40), which are  $\sim 5$  times larger. Microvesicles were found on FDCs from several donors and with three different isolation procedures.

### Microvesicles attached to FDCs, but not FDCs themselves, express MHC class II

FDCs have been shown to exhibit MHC class II at their cell surface (8) but are unable to synthesize it themselves (5). Therefore cells must acquire MHC class II passively from other sources (5), a process that has not yet been clarified. As shown in Fig. 1B, MHC class II molecules at the FDC surface are confined to the microvesicles attached to their plasma membrane. In accordance with the functional data of Gray et al. (5), the FDC plasma membrane as well as the intracellular compartments were devoid of MHC class II by IEM. These observations suggest that the microvesicles represent MHC class II carrier vesicles derived from other cells that provide the FDCs with exogenous MHC class II at their surface.

### The composition of microvesicles resembles that of exosomes

The size of the microvesicles, as well as their labeling for MHC class II, prompted us to investigate whether these structures represent exosomes. We have previously shown that B cells secrete  $\sim 50$ -nm MHC class II-expressing exosomes during exocytosis of multivesicular endosomes or MIICs (16). B cell-derived exosomes are enriched in members of the tetraspan family of proteins, expose



**FIGURE 1.** FDCs carry MHC class II positive-microvesicles. *A*, The overview shows a FDC engulfing several B lymphocytes (L). The circumference of the FDC is highlighted by a dotted line. A rectangle depicts the area that is shown at a higher magnification in *B*. *B*, The FDC plasma membrane (pm) is not labeled for MHC class II, in contrast to MIICs (small arrowheads) and plasma membrane of underlying B lymphocytes (L). Microvesicles labeled for MHC class II are attached to the FDC surface (large arrowheads). Bar: *A*, 2  $\mu\text{m}$ ; *B*, 250 nm.

MHC class I and II at their surface, contain the costimulatory molecule CD86, but are poor in the lysosomal membrane proteins Lamp-1 and Lamp-2, as well as HLA-DM (22). Microvesicles attached to FDCs exhibit a remarkably similar marker profile as characterized by IEM (Table I). They contain MHC class I and class II molecules. In addition, they are positive for the tetraspan proteins CD37, CD53, CD63, CD81, and CD82 (Fig. 2), but are poor or negative for Lamp-1 and Lamp-2 and proteins associated with Ag presentation such as Ii and HLA-DM. Importantly, the microvesicles contained lysobisphosphatidic acid (Fig. 3A), a lipid of the internal vesicles of multivesicular endosomes (Fig. 3B) (41). Taken together, these marker patterns strongly suggest that the microvesicles are exosomes secreted by APC and that they adhere but not fuse with the plasma membrane of FDCs.

#### *Exosomes bind specifically to FDCs*

Because FDC/B cell clusters isolated with an enzymatic treatment (as described in *Materials and Methods*) did not show many FDC-

attached microvesicles, we decided to use these FDC-enriched cell populations for an *in vitro* binding assay with exogenous biotinylated exosomes derived from human B cells. Exosomes were isolated as described before (16). Binding of exosomes to the various cell types present in the FDC-enriched cell population was expressed as the number of biotin-labeled exosomes per unit plasma membrane length (Table II). Biotin-positive microvesicles were found attached to the FDC surface (Fig. 4). On average, the FDC plasma membrane showed 39 attached biotin-positive vesicles per 100- $\mu\text{m}$  plasma membrane. This was in marked contrast to other cell types present in the FDC preparation like lymphocytes, plasma cells, macrophages, dendritic cells, erythrocytes, and occasional mast cells and basophils. For these cells, which served as internal controls, the number of biotinylated microvesicles attached to their surface did not exceed 7 per 100- $\mu\text{m}$  plasma membrane. We conclude that B cell-derived exosomes exhibit a high preference to bind to FDCs.

Table I. Markers present on FDCs, attached microvesicles, B lymphocyte MIICs, and B lymphocyte-derived exosomes<sup>a</sup>

Markers	FDC	Attached Microvesicles	B Lymphocyte MIICs	Exosomes
MHC class II	–	+	+	+
MHC class I	–	+	+	+
CD37	–	+	+	+
CD53	–	+	+	+
CD63	+	+	+	+
CD81	–	+	+	+
CD82	–	+	+	+
LBPA	–	+	+	+
Ii	–	–	+	–
HLA-DM	–	–	+	–
Lamp-1	+	–	+	–
Lamp-2	+	–	+	–

<sup>a</sup> Ultrathin cryosections of FDCs coisolated with activated B lymphocytes were immunolabeled for different marker proteins for B lymphocyte-derived exosomes or MIICs. Nonreactivity on FDCs for MHC class I is probably due to expression below the detection level.

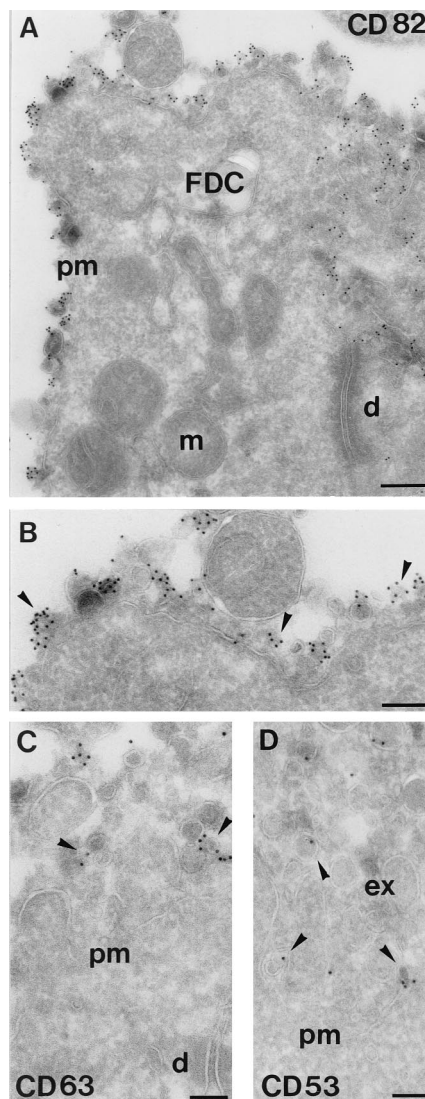
#### Exosomes are displayed by FDCs in situ

To exclude the possibility that binding of exosomes to FDCs is merely an *in vitro* artifact, we analyzed FDCs *in situ* in germinal centers of human tonsils by cryoimmunogold electron microscopy. In their physiological state, FDCs form a follicular reticulum together with B lymphocytes. A typical example of such a binucleated FDC surrounded by several B lymphocytes is shown in Fig. 5A. We frequently observed microvesicles labeled for the exosomal marker CD82 and MHC class II attached to the plasma membrane of FDCs *in situ* (Fig. 5B). This labeling pattern could be followed along the branching processes of FDCs (not shown). These observations indicate that exosome binding to the surface of FDCs is not due to experimental manipulations, but is a physiological phenomenon.

#### Discussion

In this study, we have shown for the first time the occurrence of numerous microvesicles attached to the FDC surface. They may have been observed earlier by Parmentier et al. (33), but these authors referred to them as peripheral villous extensions of FDCs rather than free vesicles as we found. The microvesicles we describe here contain MHC class II molecules as well as other proteins not expressed by FDCs. The striking differences in protein composition between the microvesicles and the FDC plasma membrane make it very unlikely that the microvesicles originate from FDCs, but rather resemble exosomes (22). In an *in vitro* binding assay, we show that isolated B cell exosomes specifically bind to FDCs.

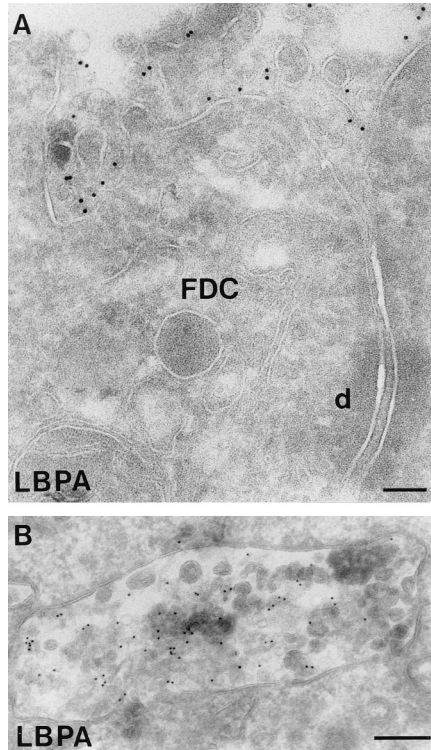
FDCs have been reported to passively acquire MHC class II molecules (5), and the authors suggested that shed material from surrounding B cells (42) might bind to the FDC surface. However, the morphological basis for this phenomenon was not further investigated. We now show that FDCs bear MHC class II-containing microvesicles with a size of 40–70 nm at their surface. These microvesicles were different from FDC-derived iccosomes (40). Iccosomes are pinched off from the dendritic processes of FDCs during the early phase of a germinal center reaction and therefore should contain the same proteins as the FDC plasma membrane. This is not the case for the microvesicles. Furthermore, the diameter of iccosomes is 0.25–0.4  $\mu\text{m}$ , which is  $\sim 5$  times larger than



**FIGURE 2.** Microvesicles on FDCs contain various proteins of the tetraspan family. Ultrathin cryosection of FDCs coisolated with activated B lymphocytes from human tonsils were immunolabeled for different members of the tetraspan family (arrowheads). A, Overview of a FDC showing a fuzzy cell surface (pm) and a characteristic desmosome (d). m, mitochondrion. B, Higher magnification of the upper left part of A, demonstrating microvesicles attached to the FDC surface, which are heavily labeled for CD82. CD63 (C) and CD53 (D) are also present on the microvesicles. d, desmosome; ex, extracellular space. Bar: A, 250 nm; B, 150 nm; C, 100 nm; D, 100 nm.

the microvesicles attached to FDCs described in this study. The presence of MHC class II molecules at the cell surface of mouse FDCs has been demonstrated before at the ultrastructural level using the immunoperoxidase technique (43). This technique resulted in a diffuse staining at the FDC surface and did not allow the discrimination between peroxidase deposits on the FDC plasma membrane and adjacent vesicles. On the basis of our findings that microvesicles attached to the FDC surface contain MHC class II, we suggest that these earlier data show staining of exosomes rather than surface staining of FDCs. This interpretation is more consistent with bone marrow transplantation experiments published by Gray et al. (5), showing that FDCs passively acquire MHC class II molecules from the donor.

B cell apoptosis is an abundant event in lymphoid follicles and may result in membrane fragments that in some way or another



**FIGURE 3.** LBPA is present in microvesicles attached to FDCs. Microvesicles attached to a FDC (A), and internal vesicles of a multivesicular MIIC from a RN cell (B) are labeled for LBPA. d, desmosome. Bar: A, 100 nm; B, 250 nm.

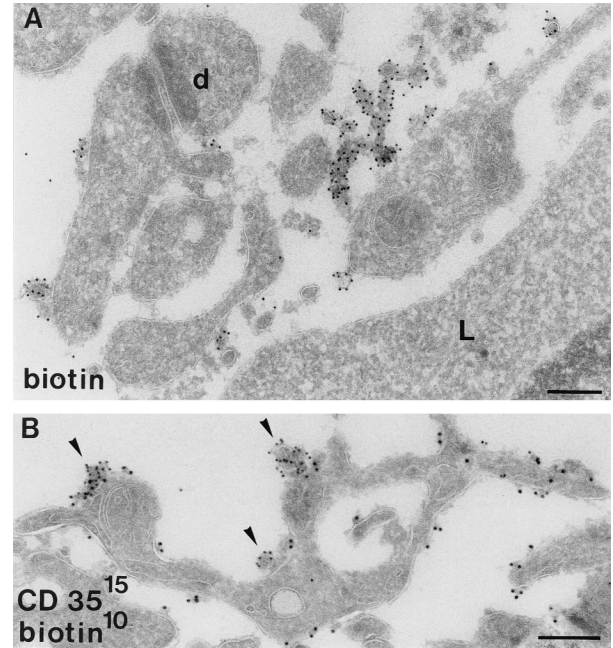
might bind to FDCs. However, the FDC attached microvesicles do not represent B cell remnants. They are of rather uniform size and are enriched in lysosomal markers and LBPA, a lipid highly enriched in internal membranes of multivesicular endosomes (41).

We have previously demonstrated that B cells secrete microvesicles called exosomes (16) that are characterized by the presence of MHC class II, the costimulatory molecule CD86, and several tetraspan proteins (CD37, CD53, CD63, CD81, CD82) (22). The protein composition of microvesicles attached to FDCs turned out to be identical with that of B cell-derived exosomes (22) (Table I). All tetraspan proteins present on exosomes were also found on the microvesicles. Together, these data strongly suggest that the microvesicles on FDCs represent exosomes. Because

Table II. B cell-derived biotinylated exosomes bind specifically to FDCs in vitro<sup>a</sup>

Cell Type	No. of Cells	Plasma Membrane (μm)	No. of Biotin <sup>+</sup> Vesicles/100-μm Membrane
FDC	7	1515	39 ± 15.2
Lymphocyte	11	374	7 ± 7.4*
Plasma cell	5	238	5 ± 3.3*
Macrophage/dendritic cell	5	393	1 ± 1.7*
Basophil	1	85	2
Mast cell	1	86	1
Erythrocyte	6	112	0 ± 0.0*

<sup>a</sup> Isolated FDC-B cell clusters were incubated with biotinylated exosomes from cultured B lymphocytes and processed for cryoimmunogold labeling. Ultrathin cryosections were immunolabeled for biotin. Plasma membranes of the various cell types present in the preparation were counted, and the number of biotin-positive vesicles attached to it expressed per 100 μm membrane ± S.D. In comparison with FDCs, other hemopoietic cells showed significantly less attached microvesicles as established by Student's *t* test (\*).

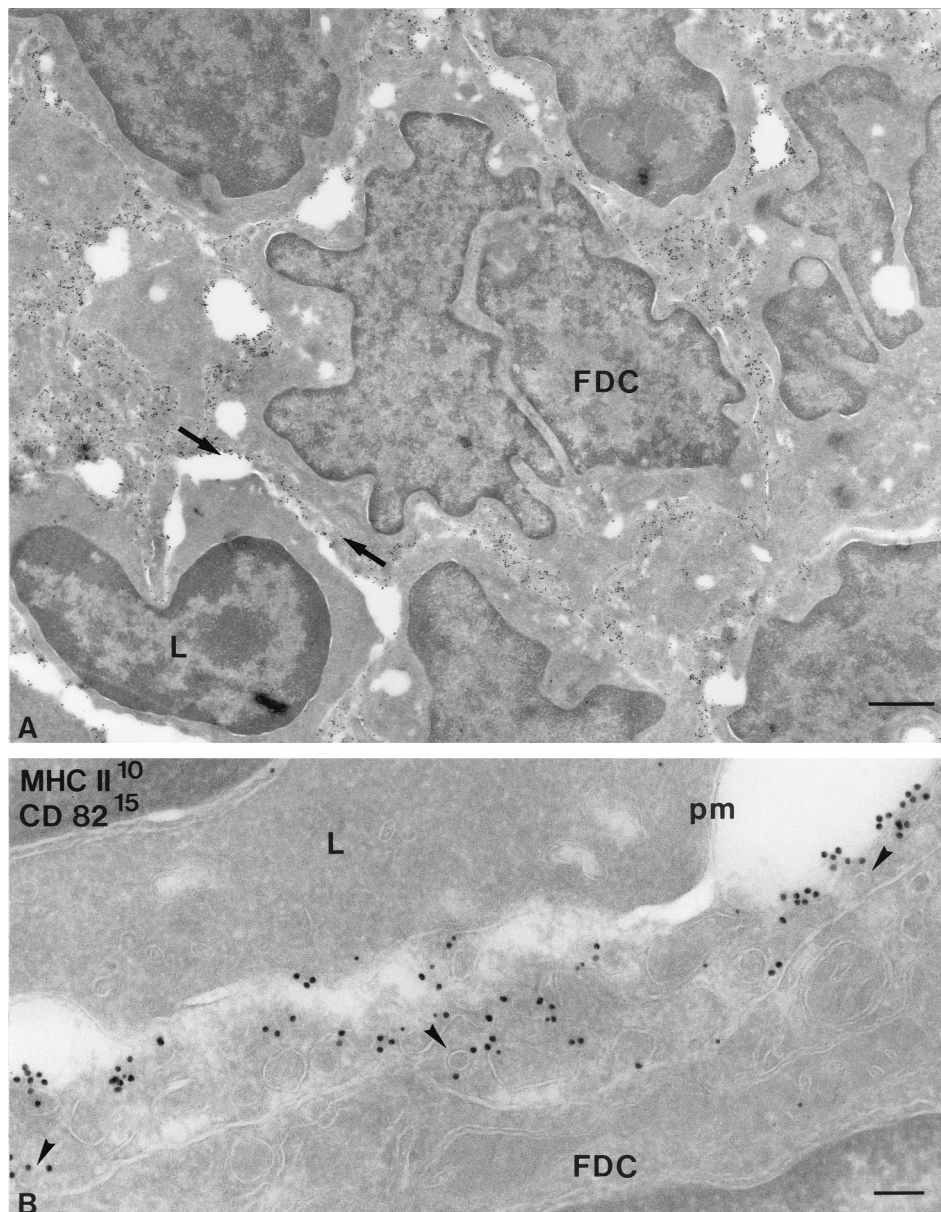


**FIGURE 4.** Biotinylated B cell derived exosomes bind preferentially to FDCs. A, Solitary or clusters of biotin-labeled microvesicles attached to the FDC surface. L, B lymphocyte. B, Microvesicles labeled for biotin with 10-nm gold particles (arrowheads) are attached to CD35-positive dendritic FDC extensions (15-nm gold particles). d, desmosome. Bar, 250 nm.

FDCs are in close contact with B cells, these latter are likely candidates for exosome delivery to FDCs. Although the molecular basis of this interaction has not yet been elucidated, we found that exosomes bind specifically to FDCs as shown in vitro (Table II). Tetraspan proteins are enriched on exosomes and microvesicles attached to FDCs. Their precise function is largely unknown, and it is tempting to speculate that they might play a role in the attachment of microvesicles to FDCs. Tetraspan proteins are able to form oligomeric complexes with other tetraspan proteins (23–25), HLA-DR (23, 26) and to interact with integrins (25, 27) and the T cell coreceptors CD4 and CD8 (28). CD81 is part of the CD19/CD21/CD81-signaling complex, which is proposed to lower the threshold for B cell responses (44). CD81 acts as a costimulatory molecule, which is important for the optimal stimulation of Ab production (45). CD82 and CD63 have been reported to form complexes with MHC class II, HLA-DM, and HLA-DO, which reside in MIICs, suggesting an important role for these tetraspan proteins in peptide loading of MHC class II (26). HLA-DM is restricted to the limiting membrane of MIICs and is not present on the internal vesicles of MIICs and their secreted equivalent, the exosomes (22, 26). The same holds true for the lysosomal membrane proteins Lamp-1 and Lamp-2. How internal vesicles of MIICs are formed and how proteins are sorted into or excluded from the vesicles is not known, but recent data showed that multivesicular body morphogenesis seems to require phosphatidylinositol 3-kinase activity (15).

Exosomes have been discussed earlier to have a costimulatory function in vitro (16), and in previous studies we have shown that the MHC class II molecules on exosomes are in a SDS-stable configuration, implying that they are peptide loaded (16). Indeed, they can specifically stimulate CD4-positive T cells in vitro (16), but their physiological target has remained undetermined. We have shown that FDCs display microvesicles at their surface, which strongly resemble exosomes. What could be the function of exosomes attached to FDCs? Ready-to-use peptide-loaded MHC class II molecules on exosomes might play a role in Th cell recruitment

**FIGURE 5.** Exosomes are displayed by FDCs in tonsil tissue. *A*, Ultrathin cryosection of a tonsillar germinal center showing a typical binucleated FDC surrounded by several B lymphocytes (L). Arrows indicate the area enlarged in *B*. *B*, Exosomes labeled for CD82 (arrowheads) and MHC class II. They are bound at the FDC surface, but not the plasma membrane of a neighboring B lymphocyte (L). Bar: *A*, 1  $\mu\text{m}$ ; *B*, 100 nm.



into the apical light zone of the germinal center. In this microenvironment, the last check for Ag specificity of B cells during germinal center reaction takes place, namely, the isotype switching and differentiation into either plasma cells or memory B cells (4), which also involves T cell help. It has been demonstrated that CD4-positive memory T cells are present in the outer zone of germinal centers (46) and that coculture of these T cells with germinal center B cells rapidly induces a memory phenotype in B cells (47). However, it is unclear how Ag-specific T cells manage to find the selected candidate memory B lymphocytes in the three-dimensional structure of the germinal center. We hypothesize that exosomes, which display peptide-loaded MHC class II molecules, attach to the highly mobile processes of FDCs, which could provide guidance for Ag-specific T cells from the outer zone of the follicle into the apical light zone. These exosomes could be derived from B cells in the germinal center, but might also originate from DCs (18, 19). If exosomes on FDCs have the function to guide Ag-specific T cells for costimulation of B cell differentiation to the place of action, one should assume that this step must be tightly regulated to avoid constant stimulation. Clearance of exosomes

and their half-life in vivo are therefore interesting subjects of further investigation. According to observations in our in vitro binding experiment, macrophages might play a role in removing exosomes, because we found biotin-labeled microvesicles throughout their endocytic system (not shown). Due to the fact that half-life determination in humans is impossible, further functional experiments are crucially dependent on the availability of a murine B cell line that generates sufficient exosomes. Future experiments in the mouse system will probably give more insight in the role of exosomes in vivo.

### Acknowledgments

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## References

- Liu, Y. J., G. Grouard, O. de Bouteiller, and J. Banchereau. 1996. Follicular dendritic cells and germinal centers. *Int. Rev. Cytol.* 166:139.
- Tew, J. G., G. F. Burton, L. I. Kupp, and A. Szakal. 1993. Follicular dendritic cells in germinal center reactions. *Adv. Exp. Med. Biol.* 329:461.
- Tew, J. G., G. F. Burton, and A. Szakal. 1997. Accessory cells in antibody responses. In *Weirs Handbook of Experimental Immunology*. L. A. Herzenberg, D. M. Weir, and C. Blackwell, eds. Blackwell, Malden, p. 90.1.
- Lindhout, E., G. Koopman, S. T. Pals, and C. de Groot. 1997. Triple check for antigen specificity of B cells during germinal centre reactions. *Immunol. Today* 18:573.
- Gray, D., M. Kosco, and B. Stockinger. 1991. Novel pathways of antigen presentation for the maintenance of memory. *Int. Immunol.* 3:141.
- Liu, Y. J., D. E. Joshua, G. T. Williams, C. A. Smith, J. Gordon, and I. C. MacLennan. 1989. Mechanism of antigen-driven selection in germinal centres. *Nature* 342:929.
- Lindhout, E., M. L. Mevissen, J. Kwekkeboom, J. M. Tager, and C. de Groot. 1993. Direct evidence that human follicular dendritic cells (FDC) rescue germinal centre B cells from death by apoptosis. *Clin. Exp. Immunol.* 91:330.
- Schriever, F., and L. M. Nadler. 1992. The central role of follicular dendritic cells in lymphoid tissues. *Adv. Immunol.* 51:243.
- Tew, J. G., G. J. Thorbecke, and R. M. Steinman. 1982. Dendritic cells in the immune response: characteristics and recommended nomenclature (a report from the Reticuloendothelial Society Committee on Nomenclature). *J. Reticuloendothel. Soc.* 31:371.
- Peters, P. J., J. J. Neefjes, V. Oorschot, H. L. Ploegh, and H. J. Geuze. 1991. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 349:669.
- Harding, C. V., and H. J. Geuze. 1993. Immunogenic peptides bind to class II MHC molecules in an early lysosomal compartment. *J. Immunol.* 151:3988.
- Kleijmeer, M. J., S. Morkowski, J. M. Griffith, A. Y. Rudensky, and H. J. Geuze. 1997. Major histocompatibility complex class II compartments in human and mouse B lymphoblasts represent conventional endocytic compartments. *J. Cell Biol.* 139:639.
- Geuze, H. J. 1998. The role of endosomes and lysosomes in MHC class II functioning. *Immunol. Today* 19:282.
- Riberdy, J. M., R. R. Avva, H. J. Geuze, and P. Cresswell. 1994. Transport and intracellular distribution of MHC class II molecules and associated invariant chain in normal and antigen-processing mutant cell lines. *J. Cell Biol.* 125:1225.
- Fernandez-Borja, M., R. Wubbolts, J. Calafat, H. Janssen, N. Divecha, S. Dusseljee, and J. Neefjes. 1999. Multivesicular body morphogenesis requires phosphatidylinositol 3-kinase activity. *Curr. Biol.* 9:55.
- Raposo, G., H. W. Nijman, W. Stoorvogel, R. Leijendekker, C. V. Harding, C. J. Melief, and H. J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* 183:1161.
- Johnstone, R. M., M. Adam, J. R. Hammond, L. Orr, and C. Turbide. 1987. Vesicle formation during reticulocyte maturation: association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* 262:9412.
- Zitvogel, L., A. Regnault, A. Lozier, J. Wolfers, C. Flament, D. Tenza, P. Ricciardi-Castagnoli, G. Raposo, and S. Amigorena. 1998. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat. Med.* 4:594.
- Thery, C., A. Regnault, J. Garin, J. Wolfers, L. Zitvogel, P. Ricciardi-Castagnoli, G. Raposo, and S. Amigorena. 1999. Molecular characterization of dendritic cell-derived exosomes: selective accumulation of the heat shock protein hsc73. *J. Cell Biol.* 147:599.
- Raposo, G., D. Tenza, S. Mecheri, R. Peronet, C. Bonnerot, and C. Desaynard. 1997. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol. Biol. Cell* 8:2631.
- Heijnen, H. F., A. E. Schiel, R. Fijnheer, H. J. Geuze, and J. J. Sixma. 1999. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis derived from exocytosis of multivesicular bodies and  $\alpha$ -granules. *Blood* 94:3191.
- Escola, J. M., M. J. Kleijmeer, W. Stoorvogel, J. M. Griffith, O. Yoshie, and H. J. Geuze. 1998. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* 273:20121.
- Angelisova, P., I. Hilgert, and V. Horejsi. 1994. Association of four antigens of the tetraspan family (CD37, CD53, TAPA-1, and R2/C33) with MHC class II glycoproteins. *Immunogenetics* 39:249.
- Berdichevski, F., K. F. Toliass, K. Wong, C. L. Carpenter, and M. E. Hemler. 1997. A novel link between integrins, transmembrane-4 superfamily proteins (CD63 and CD81), and phosphatidylinositol 4-kinase. *J. Biol. Chem.* 272:2595.
- Rubinstein, E., F. Le Naour, C. Lagaudriere-Gesbert, M. Billard, H. Conjeaud, and C. Boucheix. 1996. CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. *Eur. J. Immunol.* 26:2657.
- Hammond, C., L. K. Denzin, M. Pan, J. M. Griffith, H. J. Geuze, and P. Cresswell. 1998. The tetraspan protein CD82 is a resident of MHC class II compartments where it associates with HLA-DR, -DM, and -DO molecules. *J. Immunol.* 161:3282.
- Fitter, S., P. M. Sincoc, C. N. Jolliffe, and L. K. Ashman. 1999. Transmembrane 4 superfamily protein CD151 (PETA-3) associates with beta1 and  $\alpha$ <sub>IIb</sub> $\beta$ <sub>3</sub> integrins in haemopoietic cell lines and modulates cell-cell adhesion. *Biochem. J.* 338:61.
- Imai, T., M. Kakizaki, M. Nishimura, and O. Yoshie. 1995. Molecular analyses of the association of CD4 with two members of the transmembrane 4 superfamily, CD81 and CD82. *J. Immunol.* 154:1229.
- O'Shea, J. J., E. J. Brown, B. E. Seligmann, J. A. Metcalf, M. M. Frank, and J. I. Gallin. 1985. Evidence for distinct intracellular pools of receptors for C3b and C3bi in human neutrophils. *J. Immunol.* 134:2580.
- Morton, P. A., M. L. Zacheis, K. S. Giacoleto, J. A. Manning, and B. D. Schwartz. 1995. Delivery of nascent MHC class II-invariant chain complexes to lysosomal compartments and proteolysis of invariant chain by cysteine proteases precedes peptide binding in B-lymphoblastoid cells. *J. Immunol.* 154:137.
- Aman, P., B. Ehlin-Henriksson, and G. Klein. 1984. Epstein-Barr virus susceptibility of normal human B lymphocyte populations. *J. Exp. Med.* 159:208.
- Wikén, M., P. Björck, B. Axelsson, and P. Perlmann. 1988. Induction of CD43 expression during activation and terminal differentiation of human B cells. *Scand J. Immunol.* 28:457.
- Parmentier, H. K., J. A. van der Linden, J. Krijnen, D. F. van Wichen, L. H. Rademakers, A. C. Bloem, and H. J. Schuurman. 1991. Human follicular dendritic cells: isolation and characteristics in situ and in suspension. *Scand. J. Immunol.* 33:441.
- Geuze, H. J., J. W. Slot, P. A. van der Ley, and R. C. Scheffer. 1981. Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen tissue sections. *J. Cell Biol.* 89:653.
- Slot, J. W., H. J. Geuze, and A. J. Weerkamp. 1988. Localization of macromolecular components by application of the immunogold technique on cryosectioned bacteria. *Methods Microbiol.* 21:211.
- Raposo, G., M. J. Kleijmeer, G. Posthuma, J. W. Slot, and H. J. Geuze. 1997. Immunogold labeling of ultrathin cryosections: application in immunology. In *Weirs Handbook of Experimental Immunology*. L. A. Herzenberg, D. M. Weir, and C. Blackwell, eds. Blackwell, Malden, p. 208.1.
- Liou, W., H. J. Geuze, and J. W. Slot. 1996. Improving structural integrity of cryosections for immunogold labeling. *Histochem. Cell Biol.* 106:41.
- Weibel, E. R. 1979. *Stereological Methods, Vol. 1*. Academic Press, New York.
- Liu, Y. J., J. Xu, O. de Bouteiller, C. L. Parham, G. Grouard, O. Djossou, B. de Saint Vis, S. Lebecque, J. Banchereau, and K. W. Moore. 1997. Follicular dendritic cells specifically express the long CR2/CD21 isoform. *J. Exp. Med.* 185:165.
- Szakal, A. K., M. H. Kosco, and J. G. Tew. 1988. A novel in vivo follicular dendritic cell-dependent icosome-mediated mechanism for delivery of antigen to antigen-processing cells. *J. Immunol.* 140:341.
- Kobayashi, T., E. Stang, K. S. Fang, P. de Moerloose, R. G. Parton, and J. Gruenberg. 1998. A lipid associated with the antiphospholipid syndrome regulates endosome structure and function. *Nature* 392:193.
- Emerson, S. G., and R. E. Cone. 1981. I-Kk and H-2Kk antigens are shed as supramolecular particles in association with membrane lipids. *J. Immunol.* 127:482.
- Kosco, M. H., J. G. Tew, and A. K. Szakal. 1986. Antigenic phenotyping of isolated and in situ rodent follicular dendritic cells (FDC) with emphasis on the ultrastructural demonstration of Ia antigens. *Anat. Rec.* 215:201.
- Carter, R. H., and D. T. Fearon. 1992. CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* 256:105.
- Maecker, H. T., and S. Levy. 1997. Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J. Exp. Med.* 185:1505.
- Casamayor-Palleja, M., M. Khan, and I. C. MacLennan. 1995. A subset of CD4<sup>+</sup> memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. *J. Exp. Med.* 181:1293.
- Casamayor-Palleja, M., J. Feuillard, J. Ball, M. Drew, and I. C. MacLennan. 1996. Centrocytes rapidly adopt a memory B cell phenotype on co-culture with autologous germinal centre T cell-enriched preparations. *Int. Immunol.* 8:737.