Release and Intercellular Transfer of Cell Surface CD81 Via Microparticles[1]

Benedikt Fritzsching,[2]* Björn Schwer,[2]* Jürgen Kartenbeck,[†] Angelika Pedal,* Vaclav Horejsi,[‡] and Melanie Ott[3]*

The human tetraspan molecule CD81 is a coreceptor in B and T cell activation and a candidate receptor for hepatitis C virus infection. We examined the surface expression of CD81 on B and T lymphocytes by quantitative flow cytometry. Upon cellular activation, CD81 surface levels were rapidly reduced. This reduction occurred as early as 1 h after activation and was linked to the release of CD81-positive microparticles into the cell culture medium. CD81 mRNA levels were not affected early after activation, but the release of CD81-positive microparticles was rapidly enhanced. In addition, intercellular transfer of CD81 was observed upon coculture of CD81-positive donor cells (Jurkat T cell line) with CD81-negative acceptor cells (U937 promonocytic cell line). This transfer was rapidly increased upon T cell activation, coinciding with enhanced CD81 release from activated Jurkat cells. We propose that the release and intercellular trafficking of CD81-positive microparticles regulate the expression of CD81 surface receptors in lymphocytes and play a role in the immune response during infections. *The Journal of Immunology,* **2002, 169: 5531–5537.**

C D81, a 26-kDa surface protein, belongs to the superfamily of tetraspanin proteins. Tetraspanins are composed of four transmembrane and two extracellular domains. They are characterized by short intracellular amino and C termini without any predictable signaling function. Nevertheless, tetraspanins have been linked to the regulation of cell proliferation (1, 2), cell adhesion and migration (3), human T cell leukemia virus (HTLV-1)[4]-induced syncytium formation (4), and cell signaling (5). Some tetraspanins such as CD81 are nearly ubiquitously expressed in the body. However, most tetraspanins were identified as leukocyte Ags, and functional studies have been mainly focused on the immune system.

CD81 is a nonglycosylated component of large multiprotein complexes that include other tetraspanins, β_1 integrins, MHC class II molecules, CD19/CD21 on B cells, and CD4/CD8 on T cells (6). Recently, a novel subgroup of transmembrane proteins of the Ig superfamily have been identified as CD81 interacting partners (7–10). In mice the expression of CD81 appears critical for the regulation of B and T cell proliferation (11, 12). In the absence of CD81 expression, proliferation of B cells was severely impaired, and the proliferative response of T cells was enhanced (12). In humans, CD81 (TAPA-1) was originally identified as a target of the antiproliferative Ab 5A6 in a B cell line (1). Others have re-

ported a costimulatory effect of anti-CD81 mAbs on human thymocyte differentiation (13). It remains unclear whether treatment of cells with anti-CD81 mAbs disengages CD81 on the surface and inhibits its function or increases its association with other proteins and mimics its natural function (6).

Although its natural ligand has not been identified, CD81 has recently gained attention as a potential receptor for the hepatitis C virus (HCV). HCV is a positive strand, enveloped RNA virus of the flaviviridae family (14) that infects hepatocytes and lymphocytes. The HCV envelope protein E2 binds to human, but not murine, CD81 in accordance with the species specificity of HCV infection (15). Binding of E2 was mapped to the large extracellular domain of CD81 (EC2), which is also recognized by most antiCD81 mAbs. Indeed, like the 5A6 mAb, soluble E2 protein added to the culture medium inhibited B cell proliferation (16), indicating that E2 binding to surface CD81 triggered downstream signaling events similar to those induced by the Ab.

In our effort to characterize the role of CD81 in B and T cell activation during HCV infection we studied CD81 expression in human B and T lymphocytes. We found that CD81 surface levels were rapidly down-regulated upon cellular activation. Interestingly, we also found that CD81 was released into the cell culture medium of Jurkat T cells, and that this release was enhanced after T cell activation. Previous work describing the selective enrichment of tetraspan proteins on microparticles called exosomes secreted from B cells (17) led us to test the hypothesis that CD81 surface expression is regulated via microparticle release. The term exosome originally described small membrane vesicles (50–90 nm in diameter) released by terminally differentiating reticulocytes (18, 19). Subsequently, exosomal release has been described in several hemopoietic cell cultures, including platelets, cytotoxic T cells, dendritic cells, B cells, monocytes, and macrophages (20), and very recently also in Jurkat T cells (21, 22).

Materials and Methods

Cell lines and primary lymphocyte purification

All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and maintained according to standard cell culture conditions. Primary human T cells were purified from PBMCs isolated from healthy donors with commercially available human T cell enrichment columns (R&D Systems, Minneapolis, MN). More than 95% of the purified

---

Divisions of *Applied Tumorvirology and [†]Cell Biology, Deutsches Krebsforschungszentrum, Heidelberg, Germany; and [‡]Academy of the Sciences of the Czech Republic, Prague, Czech Republic

[2] B.F. and B.S. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Melanie Ott, Gladstone Institute of Virology and Immunology, 365 Vermont Street, San Francisco, CA 94103. E-mail address: mott@gladstone.ucsf.edu

[4] Abbreviations used in this paper: HTLV, human T cell leukemia virus; E2, envelope protein 2; EC, extracellular domain; HCV, hepatitis C virus; MVB, multivesicular bodies; P1–5, pellets 1–5; PI3-kinase, phosphatidylinositol 3-kinase; TAPA, target of antiproliferative Ab.

---

cells were CD3 positive as determined by flow cytometry. Primary B cells were isolated from freshly removed human tonsils by immunomagnetic isolation of CD19-positive cells (Dynal Biotech, Oslo, Norway) as previously described (23). Purified cultures contained >98% CD19-positive cells, as determined by flow cytometry.

Antibodies and Western blot analysis

Anti-CD81 mAb (provided by V. Horejsi with permission of O. Yoshi, clone M38) (4) was purified from ascites using Gammabind Plus Sepharose (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions. We also used PE-conjugated anti-CD81 mAbs (clone JS81 (BD Biosciences, Franklin Lakes, NJ) and clone 1.3.3.22 (Ancell, Bayport, MN)) and unconjugated anti-CD81 mAb (clone 1.3.3.22; Ancell). Mouse anti-Lamp-1 mAb (BD Biosciences); mouse anti-actin mAb (ICN, Costa Mesa, CA); goat anti-CD71 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-elongation factor 1α mAb (Upstate Biotechnologies, Waltham, MA); mouse anti-Lamp-3 mAb, rabbit anti-CD4, goat anti-CD3ε, and rabbit anti-14-3-3β (all from Santa Cruz Biotechnology) Abs; and all fluorescence-conjugated mAbs (BD Biosciences) were obtained from commercial sources. Rabbit anti-CD59 Abs were provided by V. Horejsi. Anti-MHC class I mAbs (clone W6/32, American Type Culture Collection HB95) were a gift from G. Devitt and M. Zoeller (Heidelberg, Germany). Western blot analysis with anti-CD81 and anti-CD59 Abs was performed under nonreducing conditions.

Lymphocyte activation

Primary T cells and Jurkat T cells were activated with plate-bound anti-CD3 (3 μg/ml; clone 454.3.21; N. Chiorazzi, Manhasset, NY) and soluble anti-CD28 (3 μg/ml; clone 28.2; D. Olive, Marseilles, France) mAbs as previously reported (24). B cells were activated with 15 μg/ml anti-μ/γ-F(ab)$_2$ mAbs (Jackson ImmunoResearch, West Grove, PA) and 500 U/ml IL-4 (Strathmann Biotech, Hamburg, Germany).

RNase protection

The CD81 construct was generated by amplification of the complete CD81 open reading frame from a cDNA derived from Jurkat T cells (gift from H. Poepperl, Heidelberg, Germany) with primers containing additional HindIII restriction sites and cloning into the pGEM vector (Promega, Madison, WI). Inserts were oriented and fully sequenced. Linearization of CD81/pGEM for RNase protection assay was performed with EcoRI (sense) and AflIII (antisense), and sense and antisense probes were prepared as previously described (25). The GAPDH antisense probe (Amersham Pharmacia Biotech) was prepared accordingly. The RNase protection assay was performed with cellular lysates from nonactivated and activated Jurkat T cells as previously reported (24).

Quantitative flow cytometry

The surface expression of CD81 was quantitated with QuantiBRITE beads (BD Biosciences). These precalibrated beads, conjugated with four known levels of PE, were analyzed with the same instrument settings as anti-CD81-PE-stained cells. The linear regression obtained from the analysis of the QuantiBRITE beads was used to convert the relative FL-2 intensity of the PE-stained cell population into the number of PE molecules bound per cell as previously reported (26).

Electron microscopy

For internalization studies Jurkat T cells were seeded on Con A-coated glass coverslips for 30 min and incubated with the M38 Ab (1/100) for 4 min and then with anti-mouse IgG+IgM Abs coupled to 10-nm gold particles (Amersham Life Sciences, Little Chalfont, U.K.) for 15 min at 37°C. After a short wash in PBS cells were fixed with formaldehyde (2.5% in PBS) for 30 min, washed with 50 mmol/l NH$_4$Cl, and processed for electron microscopy as previously described (27). To visualize exosomal vesicles, Jurkat T cells, attached to coverslips, were immediately fixed with formaldehyde before application of the M38 mAb (1/100) and the secondary Ab, each for 60 min. As a control, the primary Ab was replaced by an Ab recognizing the desmoplakin protein that is localized in the cytoplasm (DP 2.15/17/20; Progen Biotechnik, Heidelberg, Germany). For negative staining, resuspended P5 pellets were diluted 1/100 to 1/1000 with PBS. One drop was placed on a carbon-coated glow discharged copper grid for 1 min. The removed drop was replaced by a drop of M38 Ab solution (1/100) for 20 min, followed by incubation with anti-mouse IgG+IgM Abs coupled to 5-nm gold particles (Amersham) and treatment with 2% aqueous uranyl acetate.

Microparticle isolation and sucrose equilibration centrifugation

Microparticles were isolated by differential centrifugation as previously described (28). Cell culture medium containing 7×10^7 Jurkat T cells was centrifuged once at $200 \times g$ for 10 min to obtain the cell pellet (P1). The resulting supernatant was centrifuged twice at $500 \times g$ for 10 min (P2), twice at $1,500 \times g$ for 15 min, and once at $10,000 \times g$ for 30 min (P4). Small microparticles in the size of exosomes (P5) were then pelleted by centrifugation for 1 h at $70,000 \times g$. Pellets from each centrifugation step were resuspended in nonreducing Laemmli sample buffer for CD81 and CD59 Western blot analysis. For all other blots, DTT was added. P5 fractions were further analyzed by sucrose equilibrium centrifugation as previously described (28, 29). Briefly, P5 pellets were resuspended in 2 ml of 66% (w/w) sucrose in PBS and transferred to the bottom of a SW41 centrifugation tube (Beckman Coulter, Fullerton, CA). A 10–55% (w/w) discontinuous sucrose density gradient was layered on top, and the gradient was centrifuged at $100,000 \times g$ for 15 h. Fractions were collected from the bottom of the tube, and the density of each fraction was determined with a refractometer (Schmidt & Haensch, Berlin, Germany). Fractions were diluted to 5 ml with PBS and were centrifuged at $200,000 \times g$ for 1.5 h. The pellets from each fraction were solubilized for 60 min at room temperature in nonreducing Laemmli buffer, heated to 100°C for 5 min, and analyzed by immunoblotting.

Results

Early reduction of CD81 surface levels upon B and T cell activation

First, we examined CD81 surface expression on hemopoietic cells. We performed quantitative flow cytometric analysis of CD81 on different lymphoid cell lines and PBMCs using PE-conjugated anti-CD81 mAbs and standardized PE-conjugated beads. CD81 surface expression varied markedly between transformed cell lines and primary cells (Fig. 1A). Highest CD81 expression was found on B cell lines (3×10^5 molecules/cell), while expression on T cell lines was similar to levels found on PBMCs (10^4 molecules/cell). No CD81 was found on the promonocytic cell line U937 as previously reported (6).

CD81 levels were markedly reduced on freshly isolated human T cells upon activation with anti-CD3 and anti-CD28 mAbs (Fig. 1B, upper panel). A similar reduction was observed on human tonsillar B cells after activation with anti-IgM mAbs and IL-4 (Fig. 1B, lower panel). Flow cytometric analysis with two different anti-CD81 mAbs (clones JS81 and 1.3.3.22) yielded similar results. Activation was confirmed by costaining for CD25 on T cells and CD69 on B cells. Both markers were strongly up-regulated in activated cell cultures (Fig. 1B, right panels).

Activation-induced reduction of CD81 surface expression levels was also observed in the CD4-positive Jurkat T cell line. We chose Jurkat T cells because of their known responsiveness to anti-CD3 and anti-CD28 mAbs and CD81 surface levels in the same range as primary cells (Fig. 1A). The reduction of surface CD81 occurred as early as 1 h after activation and proceeded rapidly during the first 8 h after T cell activation (Fig. 1C). Further reduction occurred at later time points with slower kinetics, until half of CD81 surface molecules were lost at 24 h after activation (Fig. 1D).

Activation-induced loss of total CD81 protein and CD81 mRNA

Next, we analyzed CD81 levels in cellular lysates of activated and nonactivated Jurkat T cells. The cells were directly lysed in Laemmli buffer without any additional lysis or clearing steps to avoid reduction of CD81 levels through activation-induced association of the molecule with the cytoskeleton. Upon treatment with anti-CD3 and anti-CD28 mAbs for 2 h, CD81 became nearly undetectable by Western blot analysis (Fig. 2A). In the same lysates actin levels remained unchanged, confirming equal protein loading in activated and nonactivated samples. This experiment demonstrated that the early reduction of CD81 surface levels coincided with a reduction of total CD81 protein in the cell.

FIGURE 1. Rapid reduction of surface CD81 upon B and T cell activation. *A,* Surface levels of CD81 on PBMCs and various lymphoid cell lines were quantified by flow cytometric analysis using standardized Quanti-BRITE calibration beads. *B*, Freshly isolated human T cells (T), either nonactivated (−) or activated with anti-CD3 and anti-CD28 mAbs for 24 h (+), were analyzed by flow cytometry for CD81 and CD25 surface expression. CD81 and CD69 expression was examined on tonsillar B cells (B), either untreated (−) or treated with anti-IgM mAbs and IL-4 for 60 h (+). The isotype control is indicated in each panel by a hatched line. *C*, Time-course analysis of CD81 surface expression on Jurkat T cells after activation with anti-CD3 and anti-CD28 mAbs. *D*, Quantitation of surface CD81 (MFI, mean fluorescence intensity) after activation of Jurkat T cells with anti-CD3 and anti-CD28 mAbs. Values are expressed as percentage of nonactivated cells (100%). The average of three independent experiments (mean ± SD) for each time point is shown.
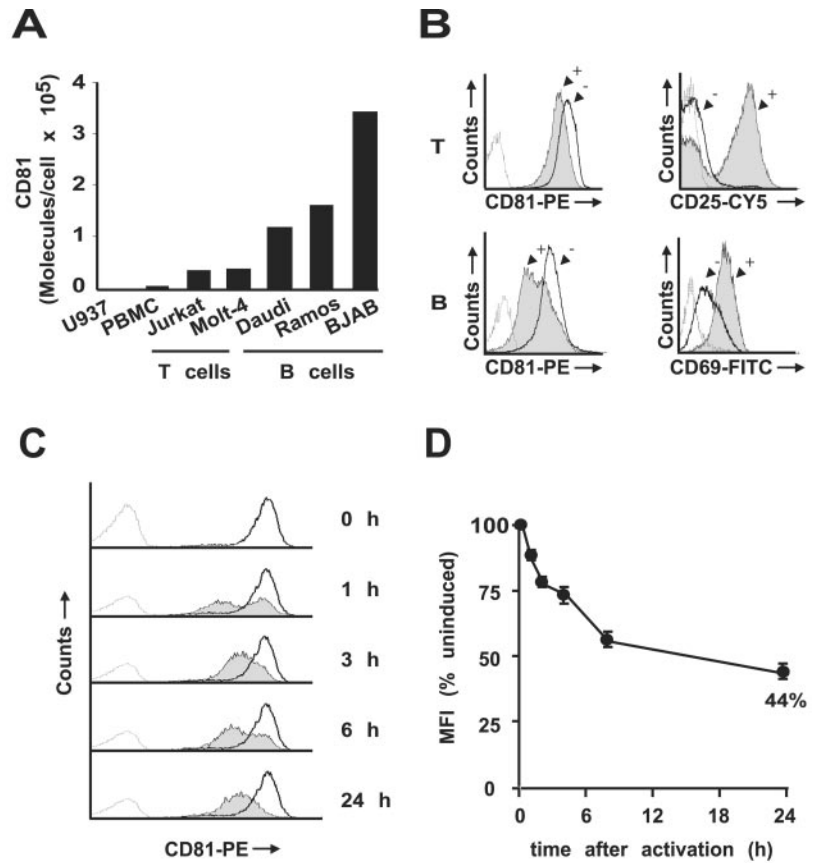
To examine CD81 transcription upon T cell activation, RNase protection analysis was performed in Jurkat T cells. Using a full-length antisense CD81-specific riboprobe, we observed a reduction of CD81 mRNA at 9 h after activation (Fig. 2*B*). This time point coincided with the late phase of activation-induced CD81 reduction seen in Fig. 1*D*. However, during the first 4 h after activation, CD81 mRNA levels remained unchanged, indicating that the early phase of activation-induced CD81 reduction was not caused by a transcriptional effect. All extracts were analyzed with an antisense probe for the GAPDH gene, and no down-regulation of steady state RNA was observed (Fig. 2*B*).

Internalization of cell surface CD81 into endosomes and multivesicular bodies

To investigate CD81 internalization, we performed immunogold electron microscopy. Living Jurkat cells were incubated with the primary anti-CD81 mAb and then with a gold-labeled secondary

Ab at 37°C before fixation and processing for electron microscopy. Gold particles were detected at the cell surface in structures resembling coated pits (Fig. 3, *A–C*), but were also imported into endosomal (Fig. 3*D*) and endolysosomal vacuoles (Fig. 3*E*). Gold particles were also found in characteristic vacuoles displaying intraluminal vesicles (Fig. 3*F*). These multivesicular bodies (MVBs) are part of the endosomal system and the origin of small exocytosed vesicles named exosomes (20). Interestingly, gold particles in coated pits were occasionally also associated with small vesicular structures (Fig. 3*C*). No association with cell surface structures or cellular uptake of gold particles was detected when cells were incubated with a primary mAb against the desmoplakin protein, which localizes to the cytoplasm (data not shown).

Release of CD81 from Jurkat T cells via microparticles

The import of surface CD81 into MVBs and the previously reported enrichment of CD81 on exosomes led us to determine
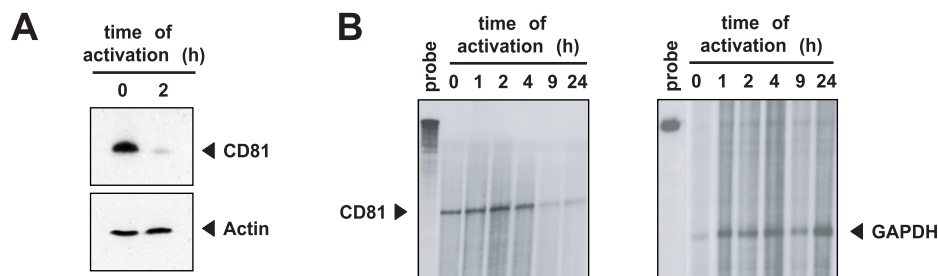

FIGURE 2. Activation-induced decrease in total CD81 protein and mRNA. *A,* Western blot analysis of CD81 expression in Jurkat T cells after treatment with anti-CD3 and anti-CD28 mAbs for 2 h. The samples were lysed directly in Laemmli buffer and were also analyzed for actin expression. *B*, RNase protection analysis of CD81 expression in Jurkat T cells after activation with anti-CD3 and anti-CD28 mAbs. Cellular lysates were incubated with specific antisense probes corresponding to the full-length CD81 mRNA and the GAPDH promoter.

FIGURE 3. Internalization of surface CD81 via coated pits and the endosomal/lysosomal compartment. Immunogold electron microscopic analysis of surface CD81 internalization in nonfixed Jurkat T cells using a primary anti-CD81 mAb (M38) and a secondary Ab labeled with gold particles (10 nm). *A–C,* Gold particles are localized at coated pits (CP); *D,* in vesicles of the endosomal (E) or *E,* endosomal/lysosomal (E/L) compartment; and *F,* in MVB. Arrows in *C* and arrowheads in *F* indicate small vesicular structures. GA, Golgi apparatus; PM, plasma membrane. Scale bar in *A–C* and *F,* 0.2 μm.
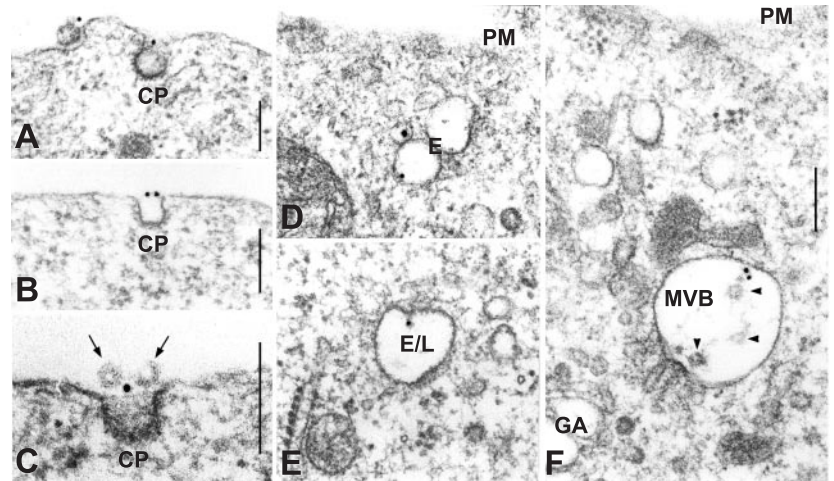
whether CD81 was released into Jurkat supernatant. Sequential differential centrifugation (28) yielded five pellets (P1–P5). P1 corresponds to the cell pellet, and P2–P5 were recovered from the cell supernatant. P2 and P3 result from sequential centrifugation of the cell supernatant at 500 and 1,500 $\times$ *g*, respectively. During this step, larger particles, such as cell debris, are cleared. P4 and P5 are obtained by ultracentrifugation of the precleared supernatant at 10,000 $\times$ *g* (P4) and then at 70,000 $\times$ *g* (P5). Both fractions contain microparticles. P4 contains mainly larger membrane-derived particles ($>$0.1 μm in diameter), which bleb directly from the plasma membrane and are called microvesicles (30). P5 contains mainly smaller particles (50–90 nm in diameter), which, in the case of exosomes, originate from multivesicular bodies after endocytosis from the plasma membrane (28). CD81 was recovered in P4 and P5 (Fig. 4*A*), indicating that it was released from Jurkat cells in microparticles. In contrary, the lysosomal protein Lamp-1 was not present in any supernatant fraction, as previously reported (17).

To further study the release of CD81, we focused our efforts on P5. We studied several molecules that are recruited to microparticles in other cell types (Fig. 4*B*). CD71, the transferrin receptor, which is highly enriched in exosomes of maturing reticulocytes (18, 19), was abundantly present in P5 of Jurkat cells. In addition, we recovered 14-3-3 proteins, which were recently described in murine dendritic cell-derived exosomes (31). The glycosylphosphaditylinositol-anchored protein CD59, present on reticulocyte-derived exosomes (32), was expressed in cell lysates of Jurkat cells (P1), but only at very low levels in P5. We did not find actin or the elongation factor 1α in P5. This result supported the conclusion that Jurkat cells constitutively release microparticles that resemble exosomes described in other cell types.

Interestingly, T cell-specific membrane proteins, such as CD3ε and CD4, were not found in substantial amounts in released microparticles (Fig. 4*C*). Since CD4 levels were very low in Jurkat cell lysates (P1), we tested supernatants from 293 cells transfected with a CD4 expression construct and did not find any secreted CD4 (data not shown). To confirm the vesicular nature of the P5 fraction, CD81-positive P5 pellets were further analyzed by sucrose gradient velocity centrifugation (Fig. 4*D*). A peak migration density of 1.15–1.19 g/ml was obtained, which lies in the range of the density observed with B cell-derived exosomes (17).

Jurkat-derived microparticles resemble exosomes

When we performed immunogold electron microscopy of fixed Jurkat cells, gold particles were directly associated with the plasma

membrane and were present on groups of small vesicular structures closely associated with the plasma membrane (Fig. 5, *A–F*). In addition, a strong accumulation of gold particles was found on microvilli (Fig. 5, *E* and *G–H*). CD81-positive microparticles were, on the average, ~60 nm in diameter (ranging from 27–74 nm), in accordance with the exosomal nature of these vesicles. Vesicles of the same size were recovered from the P5 fraction after immunolabeling with CD81 mAbs and negative staining (Fig. 5*H*, inset). No direct budding of CD81-positive microvesicles from the plasma membrane was observed. However, domains of microvilli formation were strongly labeled with gold particles (Fig. 5*G*).
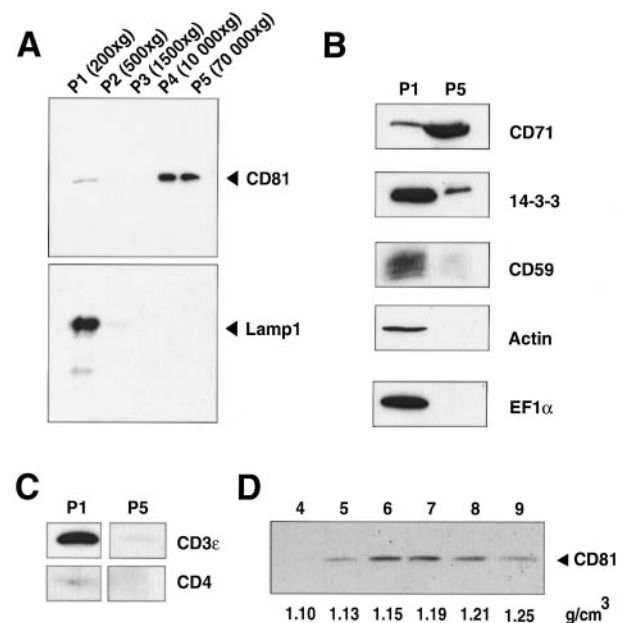

FIGURE 4. Secretion of CD81 from Jurkat T cells via microparticles. *A,* Western blot analysis of pellets obtained from Jurkat T cells (P1) and from Jurkat T cell supernatant (P2–P5) following sequential centrifugation. *B* and *C,* Western blot analysis of known exosomal proteins or T cell-specific surface receptors in cellular (P1) and supernatant (P5) fractions. *D,* Velocity sucrose gradient centrifugation of Jurkat-derived microparticles (P5). Individual fractions (above the graph) were measured by refractometry (density below the graph) and analyzed for CD81 expression by Western blot analysis.

FIGURE 5. Accumulation of CD81 on microparticles and microvilli. Immunogold electron microscopy of surface CD81 in Jurkat T cells using a primary CD81-specific mAb (M38) and a secondary Ab labeled with gold particles (10 nm). Gold particles were associated with single or clustered vesicular structures 27–74 nm in diameter, close to or loosely associated with the plasma membrane of the cell body (*A–D* and *F*) or with microvilli (*E*). Plasma membrane areas of microvilli (*E* and *H*) or small membrane protrusions at the cell surface (*G*), representing membrane domains of microvilli formation, were also strongly labeled. Arrows in *H* indicate microfilaments. The *inset* in *H* shows a negatively stained single vesicle (obtained from fraction P5) after immunoreaction with M38 Ab and a secondary Ab, labeled with gold particles (5 nm). Scale bar in *A* and *C–H*, 0.2 μm; *B*, 0.1 μm; *H* inset, 0.05 μm.
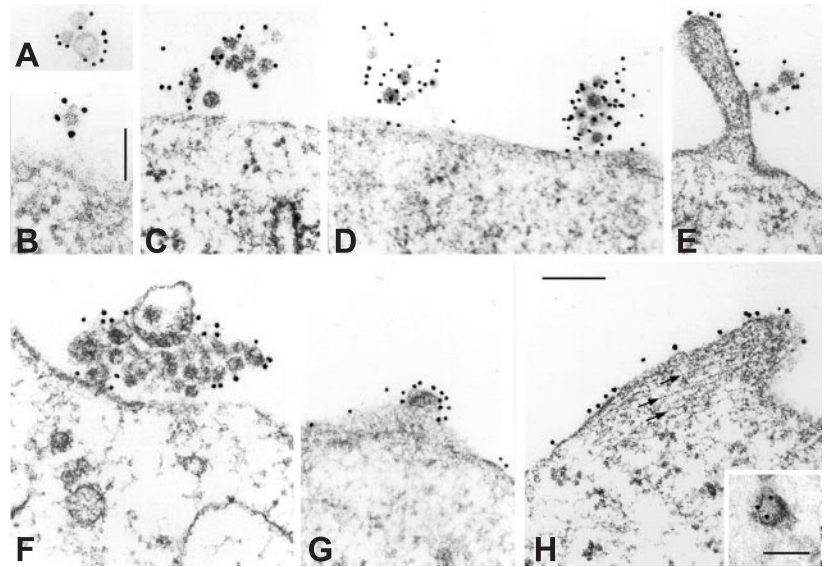
Activation-induced enhancement of CD81 release

Next, we examined CD81 release after T cell activation. CD81 in P5 was strongly up-regulated in Jurkat supernatant 2 h after activation (Fig. 6*A*), paralleling the rapid decline in CD81 surface expression observed in Fig. 1. Interestingly, CD81 levels in P4 remained the same regardless of whether Jurkat cells were activated. P5 fractions derived from activated Jurkat cells were also strongly enriched in CD59, while CD71 and 14-3-3 protein levels were unchanged (Fig. 6*B*). Interestingly, CD3ε levels in P5 were also markedly increased, indicating the release of TCR components upon T cell activation (Fig. 6*C*). MHC class I and CD63 (Lamp-3), known exosomal markers, were also enriched on acti-

vated Jurkat-derived microparticles, supporting the conclusion that these particles might be exosomes (Fig. 6*C*). These results show that the release of CD81 molecules from T cells was strongly up-regulated early after T cell activation. The effect of T cell activation on CD81 release appeared selective, since the release of other proteins was unaffected.

Intercellular transfer of CD81

Intercellular shuttling of surface molecules via secreted microparticles has been reported in endothelial cells (33) and follicular dendritic cells (34). To determine whether CD81 can be transferred from cell to cell, nonactivated Jurkat T cells (CD81$^+$) and U937
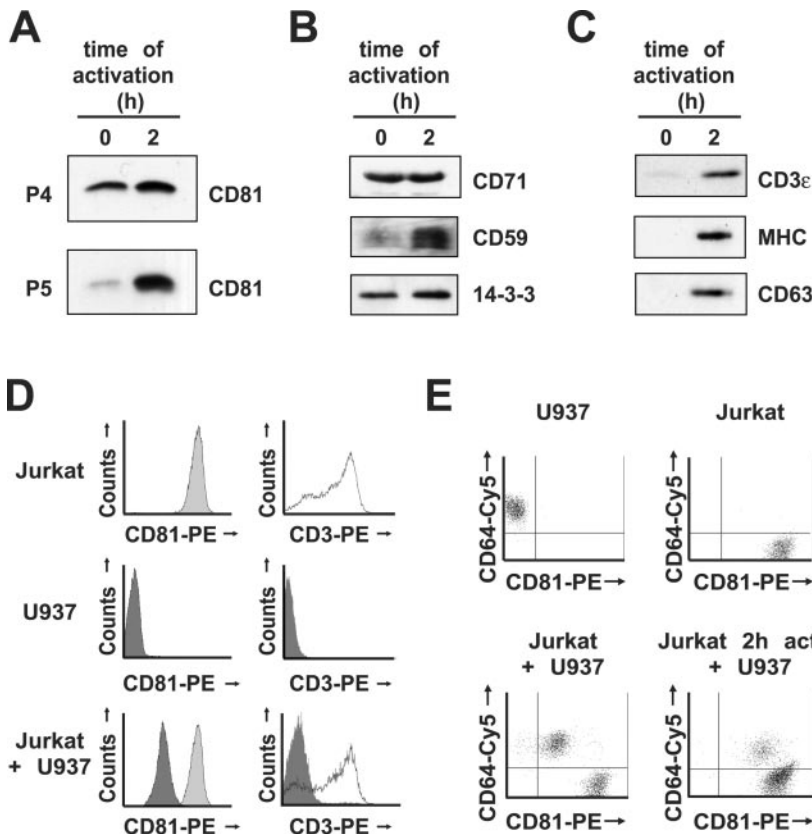

FIGURE 6. Enhanced release and intercellular transfer of CD81 following T cell activation. *A*, CD81 Western blot in supernatant fractions P4 and P5 of nonactivated or 2 h activated Jurkat cells. *B* and *C*, P5 fractions were also analyzed for other proteins found in microparticles or T cell surface proteins. *D*, Flow cytometric analysis of surface CD81 or CD3 on nonactivated Jurkat and U937 cells, which were either cultured separately or were cocultured for 10 min. *E*, Costaining of Jurkat and U937 cells with anti-CD81 (specific for Jurkat) and anti-CD64 mAbs (specific for U937). Cocultured Jurkat cells were nonactivated or activated for 2 h. MHC, MHC class I.

promonocytic cells (CD81⁻) were cocultivated for 10 min at a 1:1 ratio and examined by flow cytometry. The mean fluorescence intensity for CD81 on U937 cells was increased, while no transfer of CD3 molecules was observed (Fig. 6D). We chose CD3, as, unlike CD81, CD3 is not released from nonactivated Jurkat cells and is not expressed on U937 cells.

To further distinguish between U937 and Jurkat cells following cocultivation, we costained both cell types with anti-CD81 mAbs (specific for Jurkat T cells) and anti-CD64 mAbs (specific for U937 cells) and analyzed the surface expression by flow cytometry (Fig. 6E). Again, following cocultivation with Jurkat cells, U937 cells acquired surface CD81. Interestingly, Jurkat T cells remained unchanged, and no reverse transfer of CD64 from U937 to Jurkat T cells was observed. When Jurkat cells were activated for 2 h before cocultivation, the transfer of CD81 to U937 cells was enhanced. Concomitantly, Jurkat CD81 surface levels were decreased in the activated cultures, confirming the loss of CD81 from the plasma membrane. We conclude that the CD81 surface receptor can be released from T cells in an activation-dependent manner and that it transfers to the surface of neighboring cells.

Discussion

This study shows that surface CD81 is reduced upon T and B cell activation. This is in contrast to murine CD81, which is up-regulated upon activation (35). We observed two distinct mechanisms accounting for the down-regulation of human CD81: an early release of CD81-positive microparticles and a late decrease of CD81 mRNA levels. Possible mechanisms for the late decrease in CD81 mRNA include a transcriptional block of the CD81 promoter or enhanced mRNA decay. The latter possibility is supported by the fact that CD81 cDNA contains a long 3′-untranslated region (36). The activation-induced down-regulation is consistent with the idea that cell surface CD81 functions as a coreceptor in T cell activation. However, the presence of CD81 on microparticles and its up-regulation after T cell activation point to an additional role of CD81 in the paracrine regulation of the immune response.

Regulation of cell surface receptor expression through exocytosis was originally described in reticulocytes (18, 19). During its maturation into an erythrocyte, the reticulocyte loses several obsolete membrane-associated molecules such as the transferrin receptor (CD71) via release into the extracellular medium. This process includes initial internalization of the surface molecules into multivesicular endosomes, subsequent packaging into exosomes, and secretion into the extracellular environment.

We observed a similar internalization process for CD81 expressed on T cells. Immunogold electron microscopic analysis of internalized CD81 showed that CD81 is continuously recruited to multivesicular bodies after internalization from the plasma membrane. However, it remains unclear whether these internalized CD81 molecules are secreted into the supernatant. Although the microparticles that we describe in this study resemble exosomes derived from other cell types or exosomes that have previously been described in Jurkat cells (21, 22), we cannot exclude that cell surface CD81 might be recruited to microvesicles and might bleb directly from the plasma membrane. Microvesicles identified in platelets are bigger and are mainly recovered in the P4 fraction (30). However, T cell-derived microvesicles might be smaller and might therefore contaminate the exosomal P5 fraction. This is specifically of concern after T cell activation, which induces membrane blebbing and release of apoptotic microvesicles. However, Blanchard and colleagues (22) showed that microparticles released from activated Jurkat cells were not apoptotic blebs, since they were equally retrieved from cells treated with caspase inhibitors.

The timely coincidence of CD81 surface down-regulation and enhanced release via microparticles suggests that the two processes are directly linked. One possibility is that internalized cell surface CD81 is directly routed to MVBs and released via exosomes from the activated T cell. Alternatively, T cell activation could target internalized cell surface CD81 to lysosomal degradation and route newly synthesized molecules exclusively to exosomal processing. It has been proposed that clustering of proteins might be a general sorting signal for exosomal processing (37). Multimolecular aggregation caused by lymphocyte activation might therefore favor CD81 recruitment to the exosomal pathway. In contrast, it has been also proposed that tetraspanins themselves trigger the formation of large protein complexes and therefore facilitate the incorporation of proteins into exosomes (20). Alternatively, T cell activation could enhance vesicle formation through activation of the phosphatidylinositol 3-kinase (PI3-kinase). Treatment of cells with a PI3-kinase inhibitor prevented budding of internal vesicles from the limiting membrane into MVBs (38). The engagement of PI3-kinase during T cell activation (39) could therefore enhance vesicle formation or budding in MVBs.

It is noteworthy that not all surface molecules were enriched in the activated P5 fraction, suggesting a selective influence of activation signals on vesicle formation. Membrane molecules might be routed to microparticles via individual activation-sensitive or -insensitive pathways. Alternatively, distinct subpopulations of vesicles might exist that respond to T cell activation differently. One subpopulation might coexpress CD81 and CD59, and its formation or release might be enhanced by T cell activation. New vesicle subtypes, coexpressing CD3, MHC class I, and CD63, might be formed upon T cell activation. In fact, some molecules, such as CD71 and 14-3-3, might be specifically excluded from microparticles after T cell activation.

To gain more insight into the function of CD81-containing microparticles, their physiological targets must be identified. B cell-derived exosomes contain peptide-loaded MHC molecules that activate T lymphocytes (28). Tumor-derived exosomes transfer tumor Ags to dendritic cells and prime anti-tumor cytotoxic T cells (40). In contrast, tumor-derived exosomes, expressing Fas ligand on their surface, kill Fas-expressing anti-tumor lymphocytes (41). Interestingly, B cell-derived exosomes are abundantly present on the cell surface of follicular dendritic cells and transfer MHC class II molecules that are not synthesized by follicular dendritic cells to their cell surface (34).

There are several potential ways for microparticles to interact with target cells: binding to the cell surface, fusion with the plasma membrane, and endocytic uptake and recycling to the membrane. In the immunoelectron microscopy of cell surface CD81 we observed that endocytosed gold particles were occasionally associated with small vesicles (Fig. 3C). This indicates endocytic uptake of CD81-positive microparticles and potential recycling to the membrane of target cells. It also shows that donor cells themselves are not resistant to binding and uptake of CD81-positive vesicles. CD81 on microparticles might therefore affect cells in an autocrine and paracrine way.

The transfer of CD81 to U937 was stable through cell sorting, consistent with the integration of the transferred receptor into the plasma membrane (data not shown). However, when we treated sorted CD81-positive U937 cells (sorted via their CD64 content) with anti-CD81 mAbs, we did not observe any morphological changes characteristic of CD81 activation. In addition, intercellular transfer occurred almost immediately upon coculture and was partially resistant to exposure to 4°C (not shown). It, therefore, seems likely that the acquired CD81 content at the surface of U937

cells remained exosomal. Indeed, Jurkat donor cells themselves are densely covered with CD81-positive vesicles (Fig. 5*A*).

Since CD81 is ubiquitously expressed, it might be present on microparticles secreted from various cell types. However, the fact that T cell activation enriches CD81 levels together with TCR components and MHC class I on secreted microparticles suggests a specific costimulatory function for secreted CD81 during the immune response. We have successfully isolated CD81-positive microparticles from the blood of healthy individuals, confirming their existence in vivo (data not shown). Further studies will focus on the identification of targets cells and the characterization of the response that secreted CD81 elicits in these target cells. Furthermore, we will test whether circulating microparticles bind HCV particles via their CD81 content and represent a reservoir for virions in HCV-infected individuals.

Acknowledgments

References

1. Oren, R., S. Takahashi, C. Doss, R. Levy, and S. Levy. 1990. TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. *Mol. Cell. Biol. 10:4007.*

2. Kallin, B., R. de Martin, T. Etzold, V. Sorrentino, and L. Philipson. 1991. Cloning of a growth arrest-specific and transforming growth factor β-regulated gene, TI 1, from an epithelial cell line. *Mol. Cell. Biol. 11:5338.*

3. Miyake, M., M. Koyama, M. Seno, and S. Ikeyama. 1991. Identification of the motility-related protein (MRP-1), recognized by monoclonal antibody M31-15, which inhibits cell motility. *J. Exp. Med. 174:1347.*

4. Imai, T., and O. Yoshie. 1993. C33 antigen and M38 antigen recognized by monoclonal antibodies inhibitory to syncytium formation by human T cell leukemia virus type 1 are both members of the transmembrane 4 superfamily and associate with each other and with CD4 or CD8 in T cells. *J. Immunol. 151:6470.*

5. Bell, G. M., W. E. Seaman, E. C. Niemi, and J. B. Imboden. 1992. The OX-44 molecule couples to signaling pathways and is associated with CD2 on rat T lymphocytes and a natural killer cell line. *J. Exp. Med. 175:527.*

6. Levy, S., S. C. Todd, and H. T. Maecker. 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu. Rev. Immunol. 16:89.*

7. Stipp, C. S., T. V. Kolesnikova, and M. E. Hemler. 2001. EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily. *J. Biol. Chem. 276:40545.*

8. Charrin, S., F. Le Naour, M. Oualid, M. Billard, G. Faure, S. M. Hanash, C. Boucheix, and E. Rubinstein. 2001. The major CD9 and CD81 molecular partner: identification and characterization of the complexes. *J. Biol. Chem. 276:14329.*

9. Clark, K. L., Z. Zeng, A. L. Langford, S. M. Bowen, and S. C. Todd. 2001. PGRL is a major CD81-associated protein on lymphocytes and distinguishes a new family of cell surface proteins. *J. Immunol. 167:5115.*

10. Stipp, C. S., D. Orlicky, and M. E. Hemler. 2001. FPRP, a major, highly stoichiometric, highly specific CD81- and CD9-associated protein. *J. Biol. Chem. 276:4853.*

11. Maecker, H. T., and S. Levy. 1997. Normal lymphocyte development but delayed humoral immune response in CD81-null mice. *J. Exp. Med. 185:1505.*

12. Miyazaki, T., U. Muller, and K. S. Campbell. 1997. Normal development but differentially altered proliferative responses of lymphocytes in mice lacking CD81. *EMBO J. 16:4217.*

13. Todd, S. C., S. G. Lipps, L. Crisa, D. R. Salomon, and C. D. Tsoukas. 1996. CD81 expressed on human thymocytes mediates integrin activation and interleukin 2-dependent proliferation. *J. Exp. Med. 184:2055.*

14. Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton. 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science 244:359.*

15. Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, et al. 1998. Binding of hepatitis C virus to CD81. *Science 282:938.*

16. Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating. 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. *J. Virol. 73:6235.*

17. 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.*

18. 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.*

19. Pan, B. T., and R. M. Johnstone. 1983. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell 33:967.*

20. Denzer, K., M. J. Kleijmeer, H. F. Heijnen, W. Stoorvogel, and H. J. Geuze. 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci. 113:3365.*

21. Monleon, I., M. J. Martinez-Lorenzo, L. Monteagudo, P. Lasierra, M. Taules, M. Iturralde, A. Pineiro, L. Larrad, M. A. Alava, J. Naval, et al. 2001. Differential secretion of Fas ligand- or APO$_2$ ligand/TNF-related apoptosis-inducing ligand-carrying microvesicles during activation-induced death of human T cells. *J. Immunol. 167:6736.*

22. Blanchard, N., D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo, and C. Hivroz. 2002. TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/ζ complex. *J. Immunol. 168:3235.*

23. Cao, Y., A. Merling, U. Karsten, and R. Schwartz-Albiez. 2001. The fucosylated histo-blood group antigens H type 2 (blood group O, CD173) and Lewis Y (CD174) are expressed on CD34$^+$ hematopoietic progenitors but absent on mature lymphocytes. *Glycobiology 11:677.*

24. Ott, M., S. Emiliani, C. Van Lint, G. Herbein, J. Lovett, N. Chirmule, T. McCloskey, S. Pahwa, and E. Verdin. 1997. Immune hyperactivation of HIV-1-infected T cells mediated by Tat and the CD28 pathway. *Science 275:1481.*

25. Van Lint, C., S. Emiliani, M. Ott, and E. Verdin. 1996. Transcriptional activation and chromatin remodeling of the HIV-1 promoter in response to histone acetylation. *EMBO J. 15:1112.*

26. Iyer, S. B., L. E. Hultin, J. A. Zawadzki, K. A. Davis, and J. V. Giorgi. 1998. Quantitation of CD38 expression using QuantiBRITE beads. *Cytometry 33:206.*

27. Kartenbeck, J., H. Stukenbrok, and A. Helenius. 1989. Endocytosis of simian virus 40 into the endoplasmic reticulum. *J. Cell Biol. 109:2721.*

28. Raposo, G., H. W. Nijman, W. Stoorvogel, R. Liejendekker, C. V. Harding, C. J. Melief, and H. J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med. 183:1161.*

29. 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.*

30. 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 of multivesicular bodies and α-granules. *Blood 94:3791.*

31. Thery, C., M. Boussac, P. Veron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, and S. Amigorena. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol. 166:7309.*

32. Rabesandratana, H., J. P. Toutant, H. Reggio, and M. Vidal. 1998. Decay-accelerating factor (CD55) and membrane inhibitor of reactive lysis (CD59) are released within exosomes during In vitro maturation of reticulocytes. *Blood 91:2573.*

33. Mack, M., A. Kleinschmidt, H. Bruhl, C. Klier, P. J. Nelson, J. Cihak, J. Plachy, M. Stangassinger, V. Erfle, and D. Schlondorff. 2000. Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat. Med. 6:769.*

34. Denzer, K., M. van Eijk, M. J. Kleijmeer, E. Jakobson, C. de Groot, and H. J. Geuze. 2000. Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface. *J. Immunol. 165:1259.*

35. Maecker, H. T., S. C. Todd, E. C. Kim, and S. Levy. 2000. Differential expression of murine CD81 highlighted by new anti-mouse CD81 monoclonal antibodies. *Hybridoma 19:15.*

36. Andria, M. L., C. L. Hsieh, R. Oren, U. Francke, and S. Levy. 1991. Genomic organization and chromosomal localization of the TAPA-1 gene. *J. Immunol. 147:1030.*

37. Vidal, M., P. Mangeat, and D. Hoekstra. 1997. Aggregation reroutes molecules from a recycling to a vesicle-mediated secretion pathway during reticulocyte maturation. *J. Cell Sci. 110:1867.*

38. Fernandez-Borja, M., R. Wubbolts, H. Calafat, H. Janssen, N. Divecha, S. Dusseljee, and J. Neefjes. 1999. Multivesicular body morphogenesis requires phosphatidyl-inositol 3-kinase activity. *Curr. Biol. 9:55.*

39. Pages, F., M. Ragueneau, R. Rottapel, A. Truneh, J. Nunes, J. Imbert, and D. Olive. 1994. Binding of phosphatidylinositol-3-OH kinase to CD28 is required for T-cell signalling. *Nature 369:327.*

40. Wolfers, J., A. Lozier, G. Raposo, A. Regnault, C. Thery, C. Masurier, C. Flament, S. Pouzieux, F. Faure, T. Tursz, et al. 2001. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat. Med. 7:297.*

41. Andreola, G., L. Rivoltini, C. Castelli, V. Huber, P. Perego, P. Deho, P. Squarcina, P. Accornero, F. Lozupone, L. Lugini, et al. 2002. Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J. Exp. Med. 195:1303.*