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## Spontaneous Membrane Transfer Through Homotypic Synapses Between Lymphoma Cells

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# Spontaneous Membrane Transfer Through Homotypic Synapses Between Lymphoma Cells<sup>1</sup>

Mary Poupot and Jean-Jacques Fournié<sup>2</sup>

Formation of an immunological synapse by T, B, or NK cells is associated with an intercellular transfer of some membrane fragments from their respective target cells. This capture is thought to require effector cell activation by surface recognition of stimulatory ligand(s). However, spontaneous synaptic transfers between homotypic lymphoid cells has never been described. In this study, we show that without adding Ag, resting healthy lymphoid cells and several tumor cell lines are inactive. Conversely, however, some leukemia cell lines including the Burkitt's lymphoma Daudi continuously uptake patches of autologous cell membranes. This intercellular transfer does not involve cytosol molecules or exosomes, but requires cell contact. In homotypic Daudi cell conjugates, this occurs through immunological synapses, involves constitutive protein kinase C and mitogen-activated protein/extracellular signal-regulated kinase activity and strongly increases upon B cell receptor activation. Thus, spontaneous homosynaptic transfer may reflect the hitherto unsuspected autoreactivity of some leukemia cell lines. *The Journal of Immunology*, 2003, 171: 2517–2523.

Upon conjugation to Ag or target cells, B, T, and NK cells establish an immunological synapse. This structure enables the dynamic scan of the target cell surface for ligands to initiate the effector's response. Various supramolecular movements have been characterized on the effector lymphocyte membrane in contact with the target. These comprise peripheral repulsion of CD45, CD43, and LFA-1 molecules and the central concentration of the activating receptors, coreceptors, and associated protein kinases (1, 2). Although initially thought to regulate effector cell activation (3), the synapse also permits to direct the response on its triggering target (4, 5).

This contact area also enables a transient transfer of patches from target membranes to the surface of effector lymphocytes (6–13). However, its physiological significance in the exchange of information between NK or T effectors and their respective target remains unclear, either at the triggering phase or in the response delivery (see reviews, Refs. 14 and 15). Healthy B cells can efficiently extract Ags inserted into cell membranes or even tightly tethered to a synthetic surface (16), which favors their Ag processing and presentation to T lymphocytes. Thus, B cell-mediated synaptic transfer might promote affinity maturation of the immune response (8). Along this line, T cell-mediated synaptic capture could also favor secondary in vivo responses by depriving low-affinity T cell clones of Ag structures from the surface of APC (17).

Innate or innate-like lymphocytes such as NK cells or  $\gamma\delta$  T lymphocytes also strip their target cells through immunological synapses (10, 18), however, for unknown functions. Synaptic transfer mediated by NK cells is determined by both activating ligands and protective HLA alleles on the engaged target, and may take place through inhibitory NK cell synapses (11, 12, 19) and is correlated to the cytolytic response (13). In human HLA-unrestricted V $\gamma$ 9/V $\delta$ 2 CTL cross-reactive to soluble phosphoantigens and to tumoral cells, increasing amounts of either soluble or cell Ag increased their capture of target cell membranes (10). Because these  $\gamma\delta$  T cells share with NK cells the expression of inhibitory receptors for HLA class I molecules on their cell surfaces their trans-synaptic capture activity is also controlled by inhibitory signals such as self-HLA class I on the target cell. Synaptic transfer in various experimental systems is highly sensitive to drugs that block ATP production (12), signal transduction (9, 10), or cytoskeleton remodeling inhibitors (10, 13, 20). De facto, the synaptic transfer constitutes a good read-out for early monitoring of effector cell activation since intensity of the trans-synaptic acquisition correlates to the activation of B, T, or NK cells.

We had previously shown that the HLA class I-negative Daudi Burkitt's lymphoma is a cell target inducing a strong synaptic transfer on either NK or V $\gamma$ 9/V $\delta$ 2  $\gamma\delta$  T cell lines (10, 13). This observation was in concordance with the strong lytic activity exerted on Daudi by these cytolytic subsets (21–23). Although their specific killing was lower than the parental Daudi cytolysis, stable HLA class I<sup>+</sup> transfectants of Daudi still enabled some synaptic transfer on reactive  $\gamma\delta$  T cell lines (10). We hypothesized that the Daudi tumor cell line was particularly prone to synaptic capture by activated effectors, but not by unstimulated lymphocytes. To check this, we first designed a flow cytometry-based assay measuring the background membrane transfer between cells of the same type. We formerly described a method using the stable PKH fluorescent trackers which, once physiologically anchored in plasma membranes by aliphatic chains, do not passively diffuse from labeled cells to nonlabeled cells (24–26). In this study, we unexpectedly found that some lymphoma cell lines including the Daudi Burkitt's lymphoma exert constitutive synaptic transfer within homotypic cell conjugates whereas most healthy lymphoid subsets and several tumoral cell lines are inactive.

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## Materials and Methods

### Reagents and chemicals

The lipophilic fluorochromes PKH26 and PKH67 were purchased from Sigma-Aldrich (St. Louis, MO); the cytoplasmic Cell Tracker Orange CMTMR (5-(and-6)-((4-chloromethyl)benzoyl)amino)tetramethylrhodamine, further referred to as orange-CMTMR, was obtained from Molecular Probes (Eugene, OR). PD98059 and calphostin C were purchased from Calbiochem (Merck Eurolab, Darmstadt, Germany). The mAbs anti-IgM, PE-conjugated anti-CD16, anti-CD19, anti-CD20, anti-CD22, anti-CD40, anti-CD45, PE-conjugated anti-pan TCR  $\alpha\beta$ , and PC5-conjugated anti-pan TCR  $\gamma\delta$  were from Beckman-Coulter-Immunotech (Marseille, France); IgG1- and IgG2a-negative isotype controls were from DAKO (Glostrup, Denmark). The secondary Abs Cya5-conjugated goat anti-mouse (GAM)<sup>3</sup>-IgG2b and Rhod-conjugated GAM-IgG (H + L) were purchased from Caltag Laboratories (Burlingame, CA) and FITC-conjugated GAM-IgG2a was obtained from Beckman-Coulter-Immunotech. Anti-phosphotyrosine (IgG2b, clone PY99) were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse mAb 10G10 against human CD45 (IgG2a) was a kind gift of Prof. S. Valitutti (l'Institut National de la Santé et de la Recherche Médicale, Toulouse, France).

### Cell culture

Daudi and most of the cell lines listed below were cultured between 5 and  $10 \times 10^5$ /ml in RPMI 1640 with Glutamax-I (Life Technologies, Paisley, Scotland), supplemented with 10% FCS (Invitrogen, San Diego, CA), 25 mM HEPES, 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin, and 1 mM sodium pyruvate. The human  $\alpha\beta$  and  $\gamma\delta$  T cell lines were raised in our laboratory and cultured as described elsewhere (10). OCI-Ly8 and VAL cell lines were grown in IMDM with L-glutamine (Life Technologies) containing 100 U/ml penicillin G, 100  $\mu$ g/ml streptomycin, and 1 mM sodium pyruvate, and supplemented with 10% FCS, or with 20% FCS for the KG1 and DAR cell lines. The NKL cell line was maintained in complete RPMI 1640 medium plus 10% of pooled human AB serum and 100 U/ml recombinant human IL-2 (Sanofi-Synthelabo, Paris, France).

### Staining with fluorochromes

Unless otherwise specified, cells were stained with the lipophilic dyes PKH67 (green), PKH26 (red), or with cytoplasmic orange-CMTMR according to the manufacturer's instructions. PKH67-labeled cells were cocultured at various ratios (1:1 when unspecified) either with CMTMR-labeled cells or with PKH26-labeled cells. Cocultures were done in 96-well U-bottom tissue culture plates at a final concentration of  $6 \times 10^5$  cells in 100  $\mu$ l of complete RPMI 1640-10% FCS. Culture plates were then centrifuged 1 min at 700 rpm to promote cell contact and kept 1 h at 37°C in humidified 5% CO<sub>2</sub> in air atmosphere. Cells were then washed twice with PBS containing 0.5 mM EDTA and analyzed by flow cytometry.

### Flow cytometry

After 0 and 60 min of coculture, cells were analyzed by flow cytometry using a FACSCalibur and CellQuest software (BD Biosciences, Mountain View, CA). Live cells were gated on the basis of forward scatter/side scatter parameters and 25,000 events were acquired in each experiment with FL1 channel (log scale) for PKH67 and FL2 channel (log scale) for PKH26. To normalize these experiments, the geometric mean fluorescence intensity (mfi) of PKH67-stained cells was set at ~5,000, while the mfi of PKH26 cells was ~1,000, (~600 V for FL1 and FL2 photomultipliers). With these settings and compensations of 1.7% FL2 (in FL1) vs 28% FL1 (in FL2), dot plots of PKH67/PKH26 unambiguously identified each cell group and permitted to measure the PKH67 mfi of gated red cells (10,000 events per sample). The synaptic transfers were deducted from the PKH67 mfi of gated red cells after 0 and 60 min of coculture.

### Drug treatments

After labeling with PKH and before mixing, cells were treated for 1 h at 37°C either with PD98059 (100  $\mu$ M) or with calphostin C (1  $\mu$ M). When using anti-Ig $\mu$  (4  $\mu$ g/ml), anti-CD19, anti-CD20, anti-CD22, or anti-CD40 (8  $\mu$ g/ml), IgG1 or IgG2a control isotypes (10  $\mu$ g/ml), PKH-stained cells were incubated for 45 min at 4°C and analyzed by flow cytometry.

### Synaptic transfer between fresh lymphoid cells

PBMC and red cells were isolated from healthy donors by density centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were split in two groups which were respectively stained with PKH67 or PKH26 and processed as above. Erythrocytes were processed similarly and analyzed by flow cytometry. Blood-derived NK cells,  $\alpha\beta$  T,  $\gamma\delta$  T, and B lymphocytes were analyzed from PBMC samples which were split in two groups, respectively stained with PKH67 or left unstained. The two groups were mixed together for 1 h at 37°C for synaptic transfer and dissociated by washing with PBS containing 0.5 mM EDTA. Then, the PBMC subsets were revealed by staining for 45 min at 4°C with the following mAbs: anti-CD20-PE (blood B lymphocytes), anti-CD16-PE (NK cells), anti-pan TCR  $\gamma\delta$ -PE ( $\gamma\delta$  T lymphocytes) and anti-pan TCR  $\alpha\beta$ -PE ( $\alpha\beta$  T lymphocytes). Synaptic transfer on these subsets was then analyzed by flow cytometry of the PKH67 mfi of PE<sup>+</sup>-gated cells.

### Confocal microscopy of synaptic transfer

PKH-stained Daudi cells were processed for confocal microscopy as follows. After contact (see above), cells were then gently suspended and plated on poly-L-lysine (Sigma-Aldrich)-coated slides for 5 min at 37°C. After fixation with PBS containing 4% *p*-formaldehyde, cells were washed and directly mounted in PBS containing 90% glycerol and 2% 1,4-diazabicyclo (2.2.2) octane (DABCO; Sigma-Aldrich). Samples were examined using a Carl Zeiss LSM 410 confocal microscope (Jena, Germany).

### Confocal microscopy of the Daudi immunological synapse

Stained Daudi cells were processed for confocal microscopy as above. However, cells were plated on poly-L-lysine (coated slides), fixed with paraformaldehyde and blocked in PBS + 3% BSA. Cells were then permeabilized by incubation for 10 min with 50  $\mu$ l of PBS 3% BSA plus 0.1% saponine. After supernatant aspiration, primary Abs were added in PBS + 1% BSA (45 min, room temperature) and washed prior to incubation with secondary Abs and rinse. Samples were then mounted with PBS-glycerol-DABCO as above and examined using a Carl Zeiss LSM 410 confocal microscope and AIM Imaging Software (Carl Zeiss).

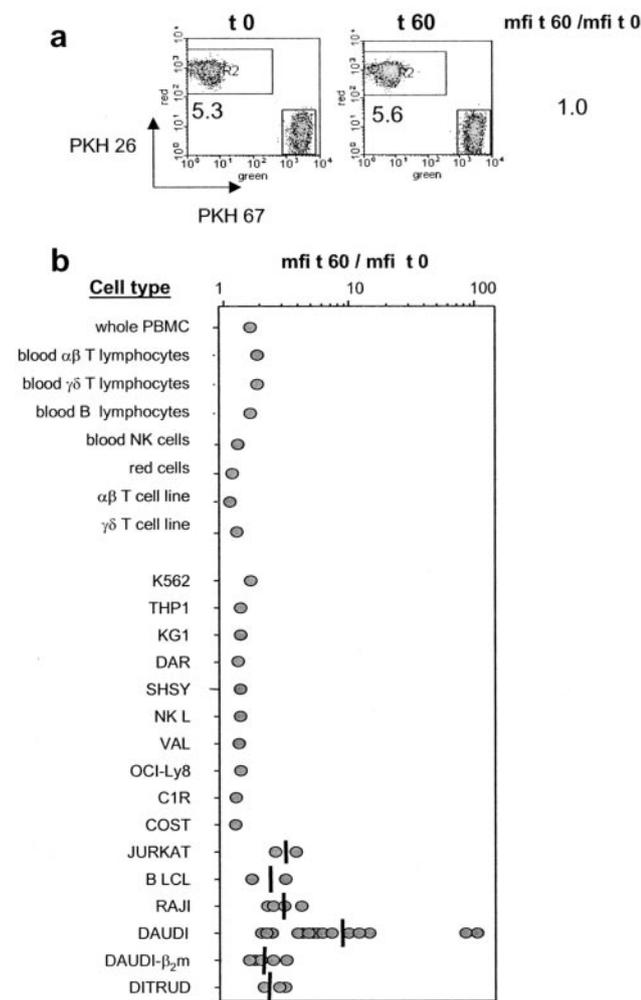
## Results

### Most healthy cells and several tumor cell lines do not exert spontaneous homotypic transfer

To determine whether resting lymphoid cells engaged in homotypic conjugates exchanged their membrane fragments, we modified the assay for synaptic transfer described previously (13) by differently labeling the same cell type as "effector" and as "target." So, each cell line to be tested was cultured in complete culture medium without stimulus and then was split into two equal groups, one of which was stained with PKH26 (red) while the other was stained by PKH67 (green). After coincubation for 1 h, the lack of membrane transfer results in unchanged red and green mfi for each group. Alternatively, membrane exchange was expected to give acquisitions of green PKH67 by red cells and reciprocally, of red PKH26 by green cells. Although applying the same labeling procedure for the two PKH fluorochromes, flow cytometric detection of the green fluorochrome (PKH67) on the FL1 channel was always more sensitive than detection of the red fluorochrome (PKH26) on the FL2 channel (see below). So, we unilaterally focused our comparative analysis to the green acquisition by red cells after 60 min of coculture.

In the absence of CMV-derived pp-65 Ag and APC, a specific  $\alpha\beta$  CTL did not acquire any green fluorescence after the coculture: the green mfi of red CTL remained unchanged after 1 h of coculture with their green homologues (Fig. 1a). The transfer ratio (green mfi of red cells at time *t* 60 of coculture)/(green mfi of red cells at *t* 0 of coculture) was of 1 ( $\pm$  0.8) in several independent experiments. We obtained similar results when testing, without stimulus, the whole PBMC freshly isolated from healthy donors, blood-derived  $\alpha\beta$  T lymphocytes or  $\gamma\delta$  T lymphocytes, blood-derived B lymphocytes, and erythrocytes from various blood groups. This was also found for primary  $\alpha\beta$  and  $\gamma\delta$  T cell lines tested without their APC and respective Ag (Fig. 1b).

<sup>3</sup> Abbreviations used in this paper: GAM, goat anti-mouse; mfi, mean fluorescence intensity;  $\beta_2$ m,  $\beta_2$ -microglobulin; BCR, B cell receptor; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; PKC, protein kinase C.



**FIGURE 1.** Spontaneous homotypic transfer activity in various cell types. *a*, Flow cytometric dot plots of PKH67- vs PKH26-labeled, resting  $\alpha\beta$  CTL after 0 and 60 min of coculture show absence of PKH transfer. Numbers are the green mfi of gated red cells. *b*, Results for different cell types summarized by the (mfi t 60/mfi t 0) ratio (log scale). Vertical bar, Mean of several independent experiments.

We then tested 10 human tumor cell lines, mainly of hemopoietic lineage, and obtained similar results. These included the monomyelocytic THP-1 cell line, the myeloid cell line KG1, the nonhemopoietic glioblastoma DAR and the neuroblastoma SHSY, the IL-2-dependent neoplastic NK-like cell line NKL, the non-Hodgkin's lymphoma VAL and OCI-Ly8 cell lines, the B cell lymphoma C1R, and the ALK<sup>+</sup> anaplastic T cell lymphoma COST cell line. In addition, the BCR-Abl<sup>+</sup> chronic myelogenous leukemia cell line K562 which is highly effective in exosome-mediated intercellular transfer (27), was inactive in homosynaptic transfer assays (Fig. 1*b*). Hence without adding exogenous stimulus, various healthy blood-derived cells, primary lymphoid cell lines, and tumoral cell lines do not spontaneously exchange fragments of their cell membrane, regardless of their ability to secrete exosomes.

#### Spontaneous intercellular exchange of lipophilic fluorochromes between Daudi cells

Unexpectedly however, the homotypic transfer assay also revealed that some tumor cell lines including Daudi spontaneously acquired membrane-bound PKH67 upon 1 h coculture. These were the

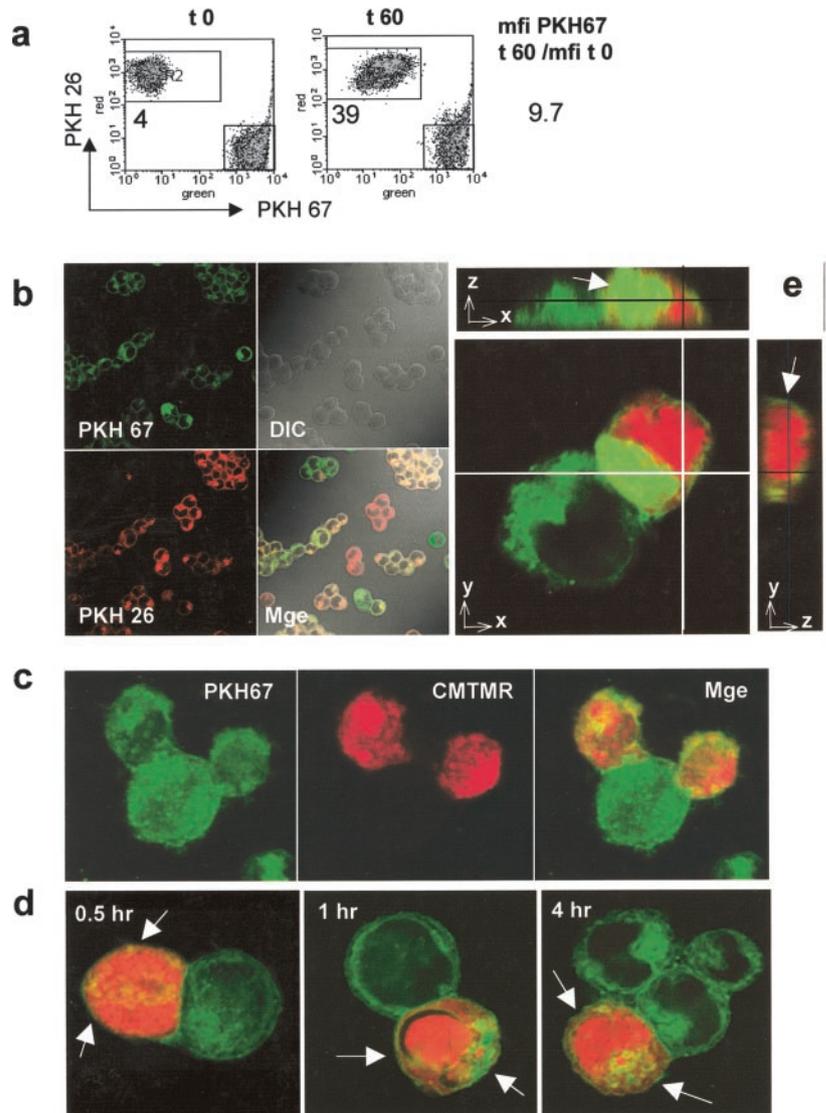
Jurkat thymoma, and the Burkitt's B cell lymphoma Raji, Daudi, the stable  $\beta_2$ -microglobulin ( $\beta_2$ m) transfectant Daudi- $\beta_2$ m (28) and the Daudi-Raji hybrid DITRUD-1 (29). These cells reproducibly acquired green fluorescence when tested in several independent experiments, but most active among these latter was unambiguously the Daudi cell line (Fig. 1*b*). We analyzed further this process using Daudi as a model. It was not an artifact caused by the PKH67 fluorochrome because both PKH were simultaneously exchanged by the cocultured Daudi cells. The clear-cut green fluorescence acquired by the red Daudi cells (a typical 9.7-fold increase is shown in Fig. 2*a*) was parallel to a lower shift of red fluorescence on the green Daudi cells (compare cells in lower right gates from Fig. 2*a*). Along this study, we recorded an interassay variation in green mfi acquisition ratio by red Daudi from 2- to 80-fold, and the average acquisition ratio was of 8-fold. From these data, we used the formula:  $((mfi_{PKH67} \text{ of red Daudi cells at } t \text{ 60 min of coculture}) - (mfi_{PKH67} \text{ of red Daudi cells at } t \text{ 0 min of coculture})) / (mfi_{PKH67} \text{ of green Daudi cells at } t \text{ 0 min of coculture})$  to estimate that within 1 h of coculture at 37°C, all red Daudi cells acquired on average 1–10% of the fluorochrome of their green homologue. This observation was not an artifact due to flow cytometry, because similar conclusions were drawn from fluorescence microscopy analysis. Two sets of Daudi cells respectively stained on their membranes with either PKH26 (red) or with PKH67 (green), coincubated for 1 h in culture medium at 37°C and without dissociation, were prepared on a glass slide and analyzed by fluorescence microscopy. Although few individualized Daudi cells had retained their original green-only or red-only staining pattern, most fields showed large multicellular aggregates stained by both fluorochromes and appeared orange (Fig. 2*b*).

#### Integral membrane patches are transferred between homotypic Daudi cell conjugates

Two sets of Daudi cells were stained on their membranes with PKH67 (green) or at the cytosol with orange-CMTMR, respectively, cocultured as above and analyzed by confocal microscopy. After 1 h, the pictures confirmed that orange-CMTMR-labeled Daudi cells had massively acquired integral patches of green Daudi cell membrane on their own surface (Fig. 2*c*). Increasing the duration of these cocultures progressively led to relocalization of the surface PKH67 labeling of these cells at their cytoplasm (Fig. 2*d*, arrows). Serial confocal sections of such late conjugates were recorded and their Z-stack reconstruction showed that ectopic membrane patches were both located at the recipient cell surface and in its cytosol (Fig. 2*e*, arrows), suggesting that the acquired membrane material was further internalized by the recipient cells. Thus, in culture, Daudi cells spontaneously capture patches of the cell membranes of their homologues.

#### Synaptic transfer involves intercellular membrane exchange, but not cytosol exchange

Because the homotypic conjugation of Daudi cells enabled the spontaneous intercellular transfer of membrane patches, we then questioned whether this process also carried cytosolic material. Daudi cells stained with either membrane PKH67 or cytosolic CMTMR were prepared and analyzed by confocal microscopy as in Fig. 2, *c–e*. Homotypic conjugates with strong transfer of membrane fluorochrome were, however, totally devoid of reciprocal transfer of cytoplasmic CMTMR (Fig. 3*a*). To rule out insufficient CMTMR detection by the confocal-based red channel, the same cocultures were then analyzed by flow cytometry, but the results were identical: no CMTMR was transferred while the mfi PKH67



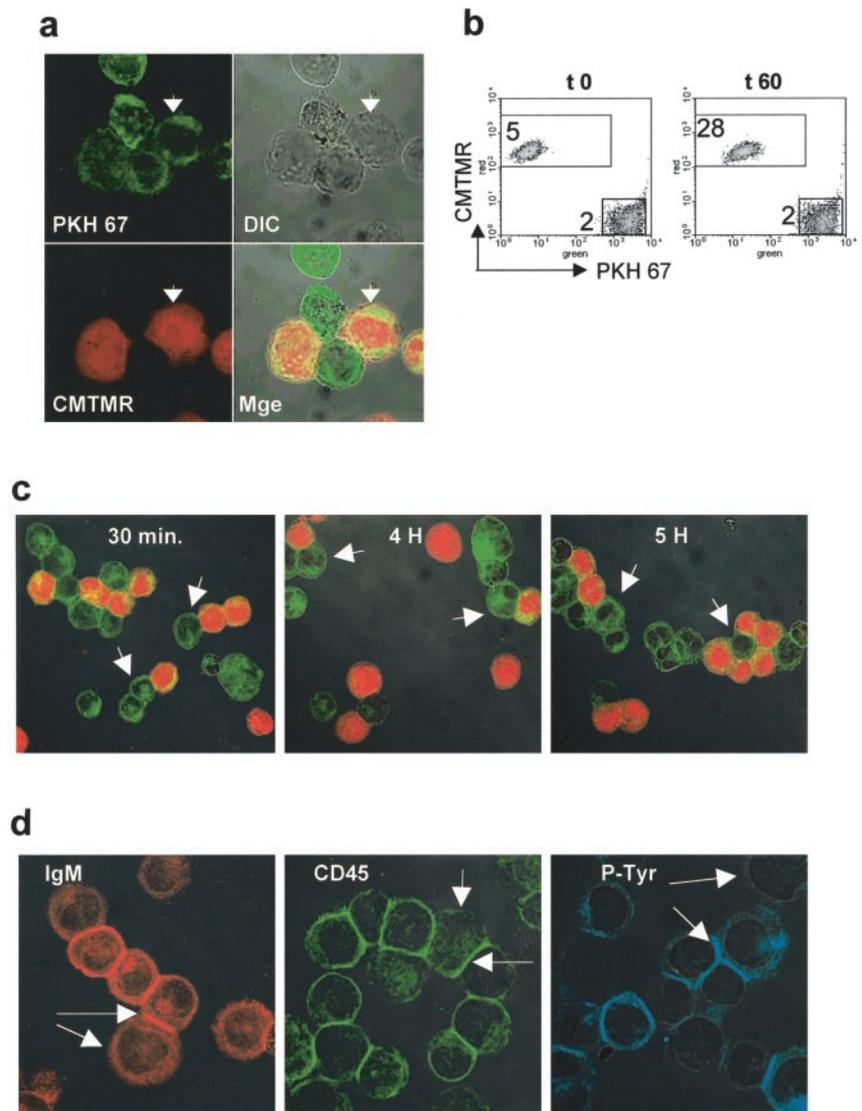
**FIGURE 2.** Spontaneous homotypic transfer activity in Daudi cells. *a*, Flow cytometric dot plots of PKH67- vs PKH26-labeled Daudi cells after 0 and 60 min of coculture show strong PKH67 acquisition by red Daudi cells. Note that the green mfi increase of gated red cells is paralleled by a reciprocal but lower increase of red mfi for gated green cells. *b*, Representative image of a 1 h coculture containing membrane labeled-Daudi, either by PKH67 (green) or PKH26 (red). Although few isolated cells show their original stain, most fields comprised large cell aggregates which merged their respective fluorescence (orange). *c*, Confocal images of an homotypic conjugate between two Daudi cells stained red at the cytosol by CMTMR (*left*), a Daudi cell stained green at the membranes by PKH67 (*middle*), and the transfer evidenced by the merge of both images (*right*). *d*, Progressive internalization of PKH67 acquired by recipient cells from homotypic conjugates in cocultures of increasing durations. Although most pPKH67 is at the cell surface at early time points, it is at the surface and inside at 1 h and mostly internalized at later stages (arrows). *e*, Z-stack reconstitution of serial sections from a homotypic conjugate (1 h cocultures) represented in the (x, y), (x, z), and (y, z) plans, evidencing green fluorochrome at the surface and cytosol (arrows) of the recipient cell reconstituted at the point specified by white lines.

simultaneously shifted (Fig. 3*b*). Because Daudi cells may spontaneously fuse with various other tumor cells and create somatic hybrids (30–33), we asked whether this transfer of membrane-bound fluorochrome merely reflected whole cell fusions. Thus, Daudi cells prepared as above were observed after increasing durations of coculture to detect a delayed cytoplasmic exchange, but no CMTMR transfer was seen until 5 h of coculture whereas PKH67 was acquired along the same period (Fig. 3*c*). Hence, the Daudi cell-mediated homotypic transfer involved intercellular acquisition of cell membrane patches and most probably does not result from somatic cell fusion nor involve a cytosol transfer.

#### Transfer through homotypic synapses between Daudi cells

The fluorochrome transfer was favored by cell contact. Within short cocultures, smears of PKH67 were already present on the few unlabeled cells conjugated to PKH67<sup>+</sup> cells (e.g., on 20% of Daudi cells after 10 min), but not on most isolated unlabeled cells. Accordingly, fluorochrome acquisition by unlabeled Daudi cells mixed with PKH67<sup>+</sup> Daudi cells in cocultures paralleled the progressive formation of new Daudi conjugates. Flow cytometry further confirmed that, comparative to labeled and unlabeled Daudi cells kept separately for 1 h, PKH67 acquisition was stronger within pelleted cocultures than within rotating cocultures (*t* 0 mfi: 4.4, *t* 60 mfi of cells in suspension: 15, *t* 60 mfi of pelleted cells:

37.4, data not shown). Thus, membrane transfer between Daudi cells involved cell conjugates rather than membrane or exosome secretion in the culture medium. Cell contact-dependent membrane transfers have been described in lymphocyte-target conjugates after the formation of their immunological synapse (8). However, because here the conjugates involved the same cell type on each side, whether homotypic conjugates involving two Daudi cells were bound through an immunological synapse was unclear. To address this, we examined the spatial distribution of surface markers in homotypic Daudi conjugates by confocal microscopy. Although immunostainings of the Ig $\mu$  chain or of CD45 in Daudi cultures showed a uniform diffuse pattern on isolated Daudi cells, homotypic cell aggregates frequently showed the enrichment of both B cell receptor (BCR) and CD45 surface markers at the cell interfaces. In addition, single intracellular staining of phosphotyrosine revealed an accumulation of phosphotyrosine-containing proteins below the interfaces of synapsed cells, but not in cells without contact (Fig. 3*d*, arrows). So, both molecular reorganization of surface markers and localized intracellular signaling indicate that the interface of homotypic Daudi cell conjugates correspond to a B cell tumor synapse. Therefore, the membrane uptake between Daudi cells corresponds to a transfer across homotypic immunological synapses.



**FIGURE 3.** Membrane transfer at the immunological synapse between Daudi cells. *a*, CMTMR-labeled cytoplasmic material was not exchanged upon synaptic transfer of PKH67. Confocal images of a 1 h coculture containing Daudi labeled either by the membrane fluorochrome PKH67 (green) or by the cytoplasmic stain CMTMR (red). *b*, Flow cytometry of the same cocultures demonstrate that the green membrane is transferred on red cells but without simultaneous transfer of CMTMR stain on the green cells. *c*, The CMTMR stain does not transfer to conjugated green cells after various time points of the coculture (arrows). *d*, Formation of the synapse between Daudi cells CD45 and BCR do not segregate and accumulate at the contact area, while phosphotyrosines are produced below the synapses.

#### *Spontaneous homotypic transfer of Daudi is active and enhanced by BCR activation*

In nontumoral cells, the trans-synaptic acquisition of cell membrane fragments is tightly controlled by the activation of effector lymphocytes. Although Daudi is a tumoral B cell line, we sought to determine whether the homotypic Daudi's membrane transfer matched such criteria. We tested this activity in Daudi cell cultures containing various stimuli or, conversely, metabolic inhibitors. Microbial stimuli such as LPS (Fig. 4*a*) or lipoteichoic acids (not shown) did not change the intensity of the transfer. However, while several inhibitors including the broad tyrosine kinase inhibitor PP2, the NF- $\kappa$ B-targeting helenalin and gliotoxin, did not affect it (not shown), mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) or protein kinase C (PKC) inhibition by PD98059 or calphostin C, respectively, strongly reduced the homotypic transfer of Daudi (Fig. 4*a*). This phenomenon is thus active and regulated, as for nontumoral immunological synapses. However, by contrast neither thapsigargin nor PMA which strongly increased NK-mediated transfer (13) were effective in increasing the transfer of Daudi. Instead, these reagents slightly reduced it (not shown). Neither of the above treatments gave different results when done prior to or after cell labeling with PKH (data

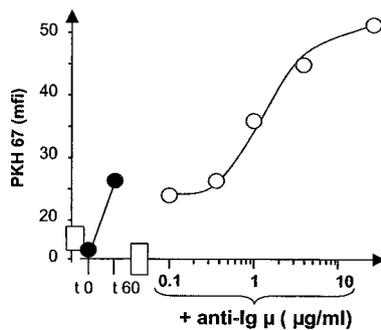
not shown), ruling out artifact of the treatment on the labeling procedure. In addition, none of these drugs modified the fluorescence of unlabeled or PKH-labeled cells. These findings suggested that the spontaneous membrane exchange resulted from self-activation of Daudi cells. To test whether this was receptor-mediated by the Daudi BCR or associated surface coreceptors, we tested the transfer of Daudi in cultures containing mAbs against these markers. As compared to the reference transfer without Ab or with an isotype-matched control Ab, the Daudi BCR cross-linking by anti-Ig $\mu$  strongly enhanced this autologous response (Fig. 4*a*). A similar but lower enhancement was induced by the Ab against the CD19 coreceptor alone, while this did not apply to the other coreceptors CD20, CD22, or CD40, which excluded an Ig-mediated artifact. Furthermore, both anti-BCR and anti-CD19, but no other combination of the above-mentioned Ab, synergized their effects on homotypic transfer (Fig. 4*a*). Titration of the anti-Ig $\mu$  dose effect on PKH67 acquisition demonstrated that BCR activation progressively enhanced the membrane exchange of Daudi ( $EC_{50} = 10^{-8}$  M, Fig. 4*b*). Together, this demonstrated that the spontaneous homosynaptic transfer between Daudi cells involves constitutive PKC and MEK activity and strongly increases upon BCR-mediated activation.

**a**

treatment	target	% transfer**
none	-	100
LPS	0.1 µg / ml	100
	1 µg / ml	103
	10 µg / ml	103
PD98059	100 µM MEK	68
Calphostin C	1 µM pan-PKC	40
isotype control*	-	100
anti-Ig µ*	BCR	560
anti-CD19*	BCR co-receptor	230
anti-CD 20*	Ca channel BCR co-receptor	105
anti-CD22*	inhibitory co-receptor	139
anti-CD40*	BCR co-receptor	133
anti-Ig µ + anti-CD19 *		655
anti-Ig µ + anti-CD20 *		490
anti-Ig µ + anti-CD22 *		514
anti-Ig µ + anti-CD40 *		542

(\*, final conc. 8 µg/ml)

\*\* means from 5 indep. expts

**b**

**FIGURE 4.** BCR activation increases the homotypic transfer between Daudi cells. *a*, By comparison to the reference transfer of PKH67 in culture medium without drug, MEK or PKC inhibition of Daudi cells reduced their transfer activity while it is strongly increased by activation of their BCR-CD19 complex. *b*, Dose-effect titration of the anti-Igµ on PKH67 transfer between Daudi cells; ●, green mfi reference at *t* 0 and *t* 60 without anti-BCR.

## Discussion

So far, synaptic transfers were known to occur in immunological conjugates wherein an Ag cell behaves as “donor” and an activated lymphoid effector acts as “recipient” (14, 15). Without Ag, T cells make synapses with dendritic cells (DC) (34), but whether intercellular transfer ensues remains to be seen (A. Trautmann, unpublished observations). However, without Ag and APC resting lymphocytes do not acquire membrane markers from cells transiently encountered along nonproductive interactions. By using a novel assay for homotypic transfer by autologous healthy blood lymphocytes, this study initially confirmed this assumption and further extended it to several tumoral cell lines of lymphoid and nonlymphoid lineages.

However, it was found in this study that while alone in culture, some leukemia cell lines spontaneously exchanged large amounts of membrane material with their cell homologues. Although clearly not a hallmark of transformed cell lines, this homotypic

transfer is particularly strong between Daudi cells, where it is almost as intense as the synaptic transfer between authentic effector-target cell conjugates. Normal lymphocytes, platelets, DC, tumor cells, and EBV-transformed B lymphocytes secrete endosomal vesicles of small size (80–100 nm) in the culture medium (35–37). However, production of these hydrosoluble exosomes (e.g., ~10% of the MHC of the target cell secreted per day (38)) has a lower yield than the synaptic transfers reported in this study (1–10% of the PKH of the target in 1 h). In addition, the cytosol-labeling fluorochrome orange-CMTMR was not transferred between Daudi cells. Conversely, the K562 erythroleukemia cell line is a potent producer of exosomes (27), but did not make homosynaptic transfer. Most significantly, exosomes are secreted in culture fluids while the transfer described here strictly requires cell contact. Altogether, these differences unambiguously distinguish the homosynaptic transfer described here from the secretion of exosomes.

In the Daudi cell line, this process takes place at tight homotypic contact areas which correspond to tumor cell synapses. Although these homotypic synapses harbored polarization of BCR and related cytoplasmic phosphotyrosine-containing components as already described for healthy B cells (8), they lacked CD45 exclusion at the periphery. Instead, CD45 clearly colocalized with BCR in most of the homotypic Daudi cell conjugates analyzed in this study. This finding is highly reminiscent of the close CD45-TCR association recently detected in central supramolecular activation clusters from early T-B immune synapses (39). In healthy T cells engaging cell targets, CD45 is transiently recruited with the engaged cell-surface TCR (40, 41), after its repulsion at the earliest stages of the activation process (42). CD45 positively regulates the surrounding TCR-triggered tyrosine kinases, thereby sustaining the activation signal (43). Likewise in the Daudi lymphoma, CD45 remained colocalized with BCR and in close vicinity to phosphotyrosine-containing transduction modules (Fig. 3*d*). Thus, we conclude that the synaptic transfer between Daudi cells reflects its spontaneous autoreactivity. Accordingly, the spontaneous Daudi cell autoreactivity involves their PKC/MEK transduction pathway, and increases upon additional activation by anti-Igµ clustering of the BCR. Spontaneous BCR ligands for a defined B lymphoma cell line in culture may have extremely diverse structure and affinity (44). Hence, their elusive elucidation was beyond the scope of this study. The molecular basis underlying the homotypic cell interaction and transfer needs to be clarified, most notably at the issue of its triggering membrane receptor. Although it was found in this study that polyclonal B cell stimuli acting via Toll-like receptors (45), such as the microbial products LPS or lipoteichoic acid (46, 47), were not involved in this response, several other receptors remain to be investigated. Ongoing studies using a variety of BCR-deficient B cells, as well as of non-B cell lymphoma, are currently under way to define the role of the Ag receptor in this process.

Finally, the potential biological relevance of these findings questions the stability of membrane composition in lymphoma cells which perform synaptic transfer. In such a cell, it might comprise the pool of self-made molecules plus that acquired from neighboring cells, which presumably also perform homosynaptic transfer continuously. At this point, we cannot rule out the interference of such homotypic transfers with surrounding cells that enable the establishment of synapses, but without being of tumoral nature. So far, the structure of synapses between healthy lymphocytes and tumoral cells is poorly characterized. Our ongoing studies suggest that these may be built once relevant adhesion factors are involved. Intercellular synaptic transfers had so far been described only between immunological conjugates comprising an effector cell and its target. This novel study now demonstrates that it may also occur spontaneously between autologous cells of some leukemia cell

lines and affect their membrane. In addition, since a recent work shows that ectopic cell surface receptors acquired along synaptic transfer remain functional on the recipient cell surface (48), the present study may raise the issue of the fate for surface receptors transferred between the B lymphoma cells. Ectopic molecules are progressively taken-up intracellularly by the recipient cells. Conceivably, the constitutive exchange of functional surface markers between homotypic cancer cells could contribute to intracellular pathways for sustaining activation of these cells. In addition, the spontaneous intercellular transfer could then constitute a vector to reach intracellular tumoral targets for therapeutic use. Future studies will aim at analyzing the consequences of this tumoral cell activity on the surrounding healthy blood lymphocytes.

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## References

- Bromley, S. K., W. R. Burack, K. G. Johnson, K. Somersalo, T. N. Sims, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 2001. The immunological synapse. *Annu. Rev. Immunol.* 19:375.
- Delon, J. 2000. The immunological synapse. *Curr. Biol.* 10:R214.
- Grakoui, A., S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, and M. L. Dustin. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285:221.
- Davis, S. J., and P. A. van der Merwe. 2001. The immunological synapse: required for T cell receptor signaling or directing T cell effector function? *Curr. Biol.* 11:R289.
- Poo, W. J., L. Conrad, and C. A. Janeway Jr. 1988. Receptor-directed focusing of lymphokine release by helper T cells. *Nature* 332:378.
- Huang, J. F., Y. Yang, H. Sepulveda, W. Shi, I. Hwang, P. A. Peterson, M. R. Jackson, J. Sprent, and Z. Cai. 1999. TCR-mediated internalization of peptide-MHC complexes acquired by T cells. *Science* 286:952.
- Hwang, I., J. F. Huang, H. Kishimoto, A. Brunmark, P. A. Peterson, M. R. Jackson, C. D. Surh, Z. Cai, and J. Sprent. 2000. T cells can use either T cell receptor or CD28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells. *J. Exp. Med.* 191:1137.
- Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature* 411:489.
- Hudrisier, D., J. Riond, H. Mazarguil, J. E. Gairin, and E. Joly. 2001. Cutting edge: CTLs rapidly capture membrane fragments from target cells in a TCR signaling-dependent manner. *J. Immunol.* 166:3645.
- Espinosa, E., J. Tabiasco, D. Hudrisier, and J. J. Fournie. 2002. Synaptic transfer by human  $\gamma\delta$  T cells stimulated with soluble or cellular antigens. *J. Immunol.* 168:6336.
- Sjostrom, A., M. Eriksson, C. Cerboni, M. H. Johansson, C. L. Sentman, K. Karre, and P. Hoglund. 2001. Acquisition of external major histocompatibility complex class I molecules by natural killer cells expressing inhibitory Ly49 receptors. *J. Exp. Med.* 194:1519.
- Carlin, L. M., K. Eleme, F. E. McCann, and D. M. Davis. 2001. Intercellular transfer and supramolecular organization of human leukocyte antigen C at inhibitory natural killer cell immune synapses. *J. Exp. Med.* 194:1507.
- Tabiasco, J., E. Espinosa, D. Hudrisier, E. Joly, A. Vercellone, and J. J. Fournie. 2002. Active trans-synaptic capture of membrane fragments by natural killer cells. *Eur. J. Immunol.* 32:1502.
- Hudrisier, D., and P. Bongrand. 2002. Intercellular transfer of antigen-presenting cell determinants onto T cells: molecular mechanisms and biological significance. *FASEB J.* 16:477.
- Davis, D. M. 2002. Assembly of the immunological synapse for T cells and NK cells. *Trends Immunol.* 23:356.
- Batista, F. D., and M. S. Neuberger. 2000. B cells extract and present immobilized antigen: implications for affinity discrimination. *EMBO J.* 19:513.
- Kedl, R. M., B. C. Schaefer, J. W. Kappler, and P. Marrack. 2002. T cells down-modulate peptide-MHC complexes on APCs in vivo. *Nat. Immunol.* 3:27.
- Davis, D. M., I. Chiu, M. Fasset, G. B. Cohen, O. Mandelboim, and J. L. Strominger. 1999. The human natural killer cell immune synapse. *Proc. Natl. Acad. Sci. USA* 96:15062.
- Zimmer, J., V. Ioannidis, and W. Held. 2001. H-2D Ligand Expression by Ly49A<sup>+</sup> natural killer (NK) cells precludes ligand uptake from environmental cells: implications for NK cell function. *J. Exp. Med.* 194:1531.
- Hwang, I., and J. Sprent. 2001. Role of the actin cytoskeleton in T cell absorption and internalization of ligands from APC. *J. Immunol.* 166:5099.
- Fisch, P., K. Oettel, N. Fudim, J. E. Surfus, M. Malkovsky, and P. M. Sondel. 1992. MHC-unrestricted cytotoxic and proliferative responses of two distinct human  $\gamma\delta$  T cell subsets to Daudi cells. *J. Immunol.* 148:2315.
- Fisch, P., E. Meuer, D. Pende, S. Rothenfusser, O. Viale, S. Kock, S. Ferrone, D. Fradelizi, G. Klein, L. Moretta, et al. 1997. Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human V $\gamma$ 9/V $\delta$ 2 T cells in tumor immunity. *Eur. J. Immunol.* 27:3368.
- Fisch, P., A. Moris, H. G. Rammensee, and R. Handgretinger. 2000. Inhibitory MHC class I receptors on  $\gamma\delta$  T cells in tumour immunity and autoimmunity. *Immunol. Today* 21:187.
- Horan, P. K., and S. E. Slezak. 1989. Stable cell membrane labelling. *Nature* 340:167.
- Wallace, P. K., L. D. Palmer, D. Perry-Lalley, E. S. Bolton, R. B. Alexander, P. K. Horan, J. C. Yang, and K. A. Muirhead. 1993. Mechanisms of adoptive immunotherapy: improved methods for in vivo tracking of tumor-infiltrating lymphocytes and lymphokine-activated killer cells. *Cancer Res.* 53:2358.
- Roussel, C., M. Barbier, V. V. Comte, C. Alcouffe, J. Clement-Lacroix, G. Chancel, and X. Ronot. 2001. Innocuousness and intracellular distribution of PKH67: a fluorescent probe for cell proliferation assessment. *In Vitro Cell. Dev. Biol. Anim.* 37:646.
- Savina, A., M. Vidal, and M. I. Colombo. 2002. The exosome pathway in K562 cells is regulated by Rab11. *J. Cell Sci.* 115:2505.
- Quillet, A., F. Presse, C. Marchiol-Fournigault, A. Harel-Bellan, M. Benbunan, H. Ploegh, and D. Fradelizi. 1988. Increased resistance to non-MHC-restricted cytotoxicity related to HLA A, B expression: direct demonstration using  $\beta_2$ -microglobulin-transfected Daudi cells. *J. Immunol.* 141:17.
- Klein, G., J. Zeuthen, R. Ber, and I. Ernberg. 1982. Human lymphoma-lymphoma hybrids and lymphoma-leukemia hybrids. II. Epstein-Barr virus induction patterns. *J. Natl. Cancer Inst.* 68:197.
- Viale, O., P. van der Bruggen, E. Meuer, R. Kunzmann, H. Kohler, R. Mertelsmann, T. Boon, and P. Fisch. 1996. Recognition by human V $\gamma$ 9/V $\delta$ 2 T cells of melanoma cells upon fusion with Daudi cells. *Immunogenetics* 45:27.
- Lubbert, M., C. W. Miller, L. Crawford, and H. P. Koeffler. 1988. p53 in chronic myelogenous leukemia: study of mechanisms of differential expression. *J. Exp. Med.* 167:873.
- Klein, G., G. Clements, J. Zeuthen, and A. Westman. 1976. Somatic cell hybrids between human lymphoma lines. II. Spontaneous and induced patterns of the Epstein-Barr virus (EBV) cycle. *Int. J. Cancer* 17:715.
- Klein, G., P. Terasaki, R. Billing, R. Honig, M. Jondal, A. Rosen, J. Zeuthen, and G. Clements. 1977. Somatic cell hybrids between human lymphoma lines. III. Surface markers. *Int. J. Cancer* 19:66.
- Revy, P., M. Sospedra, B. Barbour, and A. Trautmann. 2001. Functional antigen-independent synapses formed between T cells and dendritic cells. *Nat. Immunol.* 2:925.
- Amigorena, S. 2001. Exosomes derived from dendritic cells. *J. Soc. Biol.* 195:25.
- Amigorena, S. 1998. Anti-tumour immunotherapy using dendritic-cell-derived exosomes. *Res. Immunol.* 149:661.
- 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.
- 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.
- Freiberg, B. A., H. Kupfer, W. Maslanik, J. Delli, J. Kappler, D. M. Zaller, and A. Kupfer. 2002. Staging and resetting T cell activation in SMACs. *Nat. Immunol.* 3:911.
- Montixi, C., C. Langlet, A. M. Bernard, J. Thimonier, C. Dubois, M. A. Wurbel, J. P. Chauvin, M. Pierres, and H. T. He. 1998. Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J.* 17:5334.
- Leitenberg, D., Y. Boutin, D. D. Lu, and K. Bottomly. 1999. Biochemical association of CD45 with the T cell receptor complex: regulation by CD45 isoform and during T cell activation. *Immunity* 10:701.
- Leupin, O., R. Zaru, T. Laroche, S. Muller, and S. Valitutti. 2000. Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes. *Curr. Biol.* 10:277.
- Johnson, K. G., S. K. Bromley, M. L. Dustin, and M. L. Thomas. 2000. A supramolecular basis for CD45 tyrosine phosphatase regulation in sustained T cell activation. *Proc. Natl. Acad. Sci. USA* 97:10138.
- Cumbers, S. J., G. T. Williams, S. L. Davies, R. L. Grenfell, S. Takeda, F. D. Batista, J. E. Sale, and M. S. Neuberger. 2002. Generation and iterative affinity maturation of antibodies in vitro using hypermutating B-cell lines. *Nat. Biotechnol.* 20:1129.
- Bernasconi, N. L., E. Traggiai, and A. Lanzavecchia. 2002. Maintenance of serological memory by polyclonal activation of human memory B cells. *Science* 298:2199.
- Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in TLR4 gene. *Science* 282:2085.
- Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem.* 274:17406.
- Tabiasco, J., A. Vercellone, F. Megetto, D. Hudrisier, P. Brousset, and J. J. Fournie. 2003. Acquisition of viral receptor by NK cells through immunological synapse. *J. Immunol.* In press.