The Journal of Immunology

Synaptic Transfer by Human γδ T Cells Stimulated with Soluble or Cellular Antigens

Eric Espinosa, Julie Tabiasco, Denis Hudrisier and Jean-Jacques Fournié

J Immunol 2002; 168:6336-6343; ;
doi: 10.4049/jimmunol.168.12.6336
http://www.jimmunol.org/content/168/12/6336

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Synaptic Transfer by Human γδ T Cells Stimulated with Soluble or Cellular Antigens[1]

Eric Espinosa, Julie Tabiasco, Denis Hudrisier, and Jean-Jacques Fournié[2]

B, αβ T, and NK lymphocytes establish immunological synapses (IS) with their targets to enable recognition. Transfer of target cell-derived Ags together with proximal molecules onto the effector cell appears also to occur through synapses. Little is known about the molecular basis of this transfer, but it is assumed to result from Ag receptor internalization. Because human γδ T cells recognize soluble nonpeptidic phosphoantigens as well as tumor cells such as Daudi, it is unknown whether they establish IS with, and extract molecules from, target cells. Using flow cytometry and confocal microscopy, we show in this work that Ag-stimulated human Vγ9/Vδ2 T cells conjugate to, and perform molecular transfer from, various tumor cell targets. The molecular transfer appears to be linked to IS establishment, evolves in a dose-dependent manner in the presence of either soluble or cellular Ag, and requires γδ TCR ligation, Src family kinase signaling, and participation of the actin cytoskeleton. Although CD45 exclusion characterized the IS performed by γδ T cells, no obvious capping of the γδ TCR was detected. The synaptic transfer mediated by γδ T cells involved target molecules unrelated to the cognate Ag and occurred independently of MHC class I expression by target cells. From these observations, we conclude thatm despite the particular features of γδ T cell activation, both synapse formation and molecular transfer of determinants belonging to target cell characterize γδ T cell recognition of Ags. ***The Journal of Immunology,*** **2002, 168: 6336–6343.**

Upon Ag recognition, B and T cells establish with target cells a tight and dynamic area of contact termed immunological synapse (IS).[3] Within IS, proteins rearrange into supramolecular complexes organized in a central core of TCR signaling modules surrounded by a ring of adhesion molecules that maintain cell contact (1–4). Consequent to their recognition, peptide/MHC complexes (5, 6) or membrane Ags (7) appear to be internalized by T and B lymphocytes, respectively. This phenomenon is presumably the result of engaged receptor down-modulation (8). Along with this acquisition, not only Ags but also unrelated molecules from target cell surface are transferred onto the effector lymphocyte surface (6, 7, 9–12). Capture of membrane fragments occurs within minutes after conjugate formation, passes through the IS, depends on TCR signaling, and takes place before functional responses (9). Like B and T cells, NK cells establish IS with target cells lacking appropriate MHC class I expression (13, 14). In this case as well, NK cell activation by target cell induces the capture of target cell membrane fragments (67) and MHC proteins (15, 16). Both activatory and inhibitory surface signals and remodeling of the cytoskeleton control this transfer (10, 17, 18).[4] Thus, activation-induced synaptic transfer constitutes a novel aspect of the molecular dynamics at the IS of B, αβ T, and NK cells.

Whether or not γδ T cells share these properties is unknown. Around 1–5% of lymphocytes in peripheral blood of healthy human adults consist in γδ T cells, most of which express TRGV2/TRDV2-encoded γδ TCR (referred to here as γ9δ2 lymphocytes) (see Ref. 19 for recent review). The reactivity of this subset is mostly MHC unrestricted (20–23) and is directed toward a broad spectrum of Ags comprising, on the one hand, soluble nonpeptidic ligands and, on the other hand, activated, virally infected, or cancer cells such as the β_2-microglobulin (β_2m)$^-$ Burkitt lymphoma Daudi (24–28). Surface receptors for MHC class I (NK cell MHC receptor (NMR)) expressed by most γδ T cells regulate the recognition process (29–32).

Although structurally diverse, the nonpeptide Ags activating γ9δ2 cells have low m.w. and hydrophilic structures. These comprise either natural or synthetic pyrophosphoesters referred to as phosphoantigens (33, 34), therapeutic aminobisphosphonates (35), and natural or synthetic alkylamines (36). Natural phosphoantigens such as 3-formyl-1-butyl pyrophosphate (3fbPP) were isolated from various bacteria including *Mycobacterium tuberculosis* (37) and *Escherichia coli* (38). Among other powerful phosphoantigens, isopentenyl pyrophosphate (IPP) is a ubiquitous metabolite (39), and the synthetic agonist bromohydrin pyrophosphate (BrHPP) was recently produced in our laboratory (40). The phosphoantigen receptor on γδ T cells was unambiguously demonstrated as the Vγ9/Vδ2 TCR (41, 42), but the molecular basis of this recognition remains unclear. Presumably, the Vγ9/Vδ2 TCR might accommodate distinct phosphoantigens thanks to the direct binding of phosphate groups to the positive charges of adequately exposed germline residues at the surface of the Ag-binding region (42–44). Activation of γ9δ2 T cells also requires cell-to-cell contact but not MHC class I expression, and thus might involve presentation by an as-yet-unidentified molecule (22, 23). An attractive candidate in this respect is the uncharacterized phosphatase activity involved in activation by phosphoantigens (45). In marked contrast with αβ T cells, γδ T cell activation by phosphoantigens does not result in TCR down-modulation (46, 47), which questions the ability of the γδ T cell to mediate synaptic capture of

Institut National de la Santé et de la Recherche Médicale, Centre Hospitalier Universitaire Purpan, Toulouse, France

Received for publication February 11, 2002. Accepted for publication April 8, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by institutional grants from Institut National de la Santé et de la Recherche Médicale, l'Association pour la Recherche sur le Cancer (contract no. 5665), and the European Union Cluster for Tuberculosis Vaccine.

[2] Address correspondence and reprint requests to Dr. Jean-Jacques Fournié, Institut National de la Santé et de la Recherche Médicale, BP3028 Centre Hospitalier Universitaire Purpan, 31024 Toulouse, France. E-mail address: fournie@toulouse.inserm.fr

[3] Abbreviations used in this paper: IS, immunological synapse; PP, pyrophosphate; BrHPP, bromohydrin PP; CMTMR, chloromethylbenzoylaminotetramethyl-rhodamine; 3fbPP, 3-formyl-1-butyl-PP; IPP, isopentenyl PP; MFI, mean fluorescence intensity; NMR, NK cell MHC receptor; β_2m, β_2-microglobulin; CytD, cytochalasin D; PPi, inorganic PP.

0022-1767/02/$02.00

target cell Ags. We sought to investigate this point using γδ T cells stimulated with either soluble or cellular Ags. This paper reports flow cytometry and confocal microscopy data evidencing synaptic transfer by Ag-stimulated γ9δ2 T cells.

Materials and Methods

Ags and Abs

BrHPP and 3fbPP were synthesized in our laboratory as previously described (37, 40). Synthetic IPP was obtained from Sigma-Aldrich (St. Louis, MO). Mouse mAb against human Vγ9 TCR, human IgM (clone AF6), and secondary goat anti-mouse Ab ($F(ab')_2$) conjugated to rhodamine were from Immunotech (Marseille, France). Mouse mAb 10G10 against human CD45 was a kind gift of P. Valitutti (Institut National de la Santé et de la Recherche Médicale, Toulouse, France).

Cell culture

PBL from healthy donors were separated by density centrifugation on Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Uppsala, Sweden), washed three times, and then resuspended at 10^6/ml in RPMI complete culture medium (RPMI 1640 with Glutamax-I (Life Technologies, Paisley, U.K.), supplemented with 10% FCS, 25 mM HEPES, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 1 mM sodium pyruvate).

Polyclonal γ9δ2 T cell lines were specifically raised by incubating PBL (10^6/ml) in culture medium with 3fbPP (10 nM) plus 100 U/ml IL-2 (Sanofi-Synthélabo, Labège, France) during 20 days. The expansion of γ9δ2 T cells was followed by cytometric analysis, and only cultures showing >95% TCR Vδ2-positive cells were used for subsequent experiments.

The THP-1 cell line was cultivated in RPMI complete culture medium plus 50 μM 2-ME. Raji, Daudi, OCI-Ly8, VAL, and C1R cell lines were grown in complete RPMI culture medium.

TNF-α release assay

TNF-α release by γδ T cell lines was measured using a bioassay described elsewhere (40).

Analysis of membrane capture by flow cytometry

Cells were stained with green lipophilic dye PKH67 (Sigma-Aldrich) or orange chloromethylbenzoylaminotetramethyl-rhodamine (CMTMR; Molecular Probes, Eugene, OR) according to the manufacturer's instructions. PKH-labeled target cells were cocultured at various ratio (1:1 when unspecified) with CMTMR-labeled T cells in 96-well U-bottom tissue culture plates at a final concentration of 4×10^5 cells in 100 μl with the indicated concentration of phosphoantigen. Cells were centrifuged for 1 min at 700 rpm to promote cell contact and incubated for 1 h at 37°C. Cells were then washed twice with PBS containing 0.5 mM EDTA and analyzed by flow cytometry. When specified, γδ T cells were pretreated with cytochalasin D (CytD), 10^{-6} M (Calbiochem, Merck-Eurolab, Fontenay-sous-Bois, France) for 1 h at 37°C or with 20 μM pyrophosphate (PP)2 (Sigma-Aldrich) for 15 min at 37°C before addition of target cells, and then cocultured with these drugs.

Analysis of synaptic transfer or IS by confocal microscopy

Cells were stained as described above. Equal numbers of γδ T cells and target cells (usually 2×10^5) were cocultured after centrifugation (1 min at 700 rpm) in 96-well U-bottom tissue culture plates for 1 h at 37°C (synaptic transfer experiments) or for 15 min (IS analysis) in specified conditions. Cells were then gently resuspended and plated on poly-L-lysine (Sigma-Aldrich)-coated slides for 5 min at 37°C. After fixation with PBS containing 4% paraformaldehyde, cells were washed, blocked in PBS supplemented with 1% BSA (Sigma-Aldrich), and processed for immunofluorescence. Primary Abs were added in PBS/1% BSA for 1 h at room temperature and then washed extensively. Secondary Ab were added in PBS/1% BSA for 30 min, washed extensively in PBS, and then mounted in PBS containing 90% glycerol and 2% 1–4-diazabicyclo (2.2.2) octane (Sigma-Aldrich). Samples were examined using a Zeiss LSM 410 confocal microscope (Zeiss, Jena, Germany). The presented pictures were representative of results from at least three different experiments.

Results

Phosphoantigen-stimulated γδ T cells actively capture membrane fragments from accessory THP-1 cells

It is well known that, while γδ T cells can be stimulated with soluble Ags, the presence of accessory cells, including the γδ T cells themselves, is required. The myelomonocytic THP-1 cell line exerts such accessory function for the response of polyclonal γ9δ2 T cells to nonpeptide Ags (48). Because recognition of target cell by αβ T, B, and NK cells involved the capture of target cell molecules, we determined whether such a molecular transfer occurred in conditions where THP-1 cells were cocultured with γδ T lymphocytes activated by the soluble Ag BrHPP. To unambiguously identify each cell type, THP-1 were stained green with the lipophilic dye PKH67, which inserts in cell membrane, while γδ T cells were stained orange with the cytoplasmic dye CMTMR. The PKH67 fluorescence of gated live cells allowed us to discriminate between the $PKH67^{bright}$ population corresponding to THP-1 cells and the $PKH67^{low}$ population corresponding to γδ T lymphocytes (Fig. 1*a*). After 1 h of coculture, the MFI_{PKH67} of the γδ T cells cultured with THP-1 cells alone (mean fluorescence intensity (MFI) = 11 measured on gated CMTMR-positive T cells) reproducibly increased when BrHPP was added to the coculture (MFI = 18), indicating the capture of THP-1-derived membrane lipids by activated γδ T cells. The MFI_{PKH67} of γδ T cells dropped to 6 in cocultures where both BrHPP and the Src family kinase inhibitor PP2 were added (Fig. 1*a*). The capture of THP-1 membrane fragment by unstimulated γδ T cells was weak but reproducible and was lowered by the addition of PP2 to the coculture (see Fig. 4*b*). Increasing the ratio of THP-1:γδ T cells also increased the amount of target cell lipids acquired by BrHPP-stimulated γδ T cells; the MFI_{PKH67} reached 44 at the highest E:T ratio tested (Fig. 1*b*). As expected from the weak reactivity of unstimulated γδ T cells to THP-1 cells alone, this capture measured in these conditions was marginally affected by the above cell ratio. When a large excess of

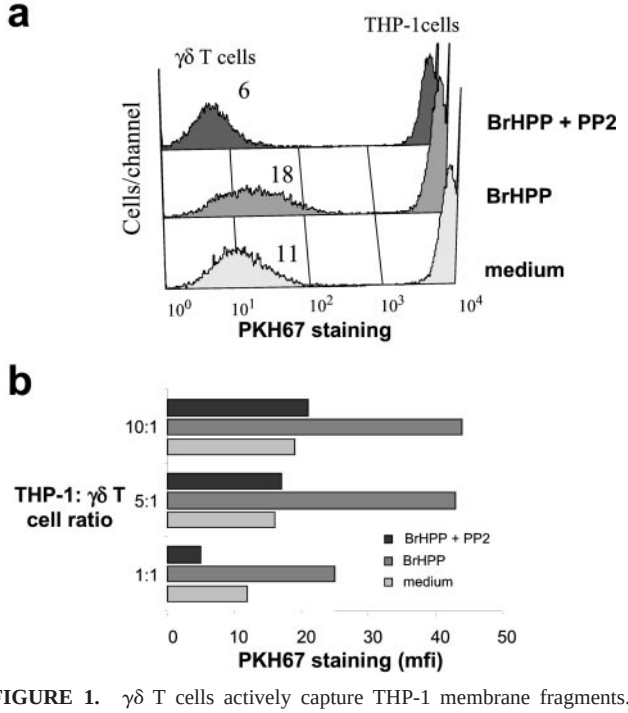

FIGURE 1. γδ T cells actively capture THP-1 membrane fragments. THP-1 cells and γδ T cells were labeled with the membrane PKH67 dye and cytoplasmic CMTMR dye, respectively. Equal numbers of labeled THP-1 and γδ T cells were cocultured in the specified conditions (200 nM BrHPP and 10 μM PP2) for 1 h at 37°C. *a*, transfer of PHK67 staining followed by flow cytometry of the cocultured THP-1 and γδ T cells. Numbers indicate MFI_{PKH67} for γδ T cells (gated as $CMTMR^+$ cells). *b*, Effect of the THP-1:γδ T cell ratio on PKH67 capture in the same conditions as above. These data are representative of four experiments.

THP-1 cells was used the effect of PP2 on membrane capture by activated γδ T cells was slightly less marked, possibly reflecting consumption by THP-1 cells (Fig. 1*b*). Nevertheless, PP2 reduced membrane capture to levels observed with unstimulated γδ T cells. Altogether, these data indicated that, like other lymphocyte populations, γδ T cells activated by soluble phosphoantigens capture membrane fragments of accessory THP-1 cells.

Ag density controls the extent of membrane capture by activated γδ T cells

γδ T cells respond to different concentrations of the soluble phosphoantigens IPP, BrHPP, and 3fbPP as well as to the β_2m-deficient Burkitt's lymphoma cell line Daudi (24, 49). To probe the qualitative and quantitative relationship between antigenic stimulation of γδ T cells and membrane capture further, we tested various doses of these Ags in cocultures of γδ T cells and THP-1 cells as above. The transfer of THP-1 membrane fragments onto γδ T cells augmented with increasing concentrations of BrHPP, IPP, or 3fbPP in a dose-dependent manner. Half-maximal capture correlated well to the known biological activity of these compounds, as assessed in functional assays (33, 37, 39). Thus, BrHPP or 3fbPP induced half-maximal THP-1 membrane capture at roughly 10^3-fold lower concentrations than IPP (i.e., 200 or 50 nM vs 200 μM, respectively; Fig. 2*a*).

Then PKH67-labeled Daudi cells alone were used as a stimulus instead of soluble Ags. As a control, γδ T cells and Daudi tumor cells were incubated separately and mixed immediately before flow cytometric analysis (duration of cell contact: 0 min; Fig. 2*b*, ○). By comparison, after 1 h of coculture, the MFI_{PKH67} of γδ T cells was markedly increased. As observed above with soluble Ags an increase in Ag density, as obtained in increasing the Daudi:γδ T cell ratio, resulted in a more efficient transfer of Daudi determinants on γδ T cells (Fig. 2*b*). Interestingly, at the same target cell:γδ T cell ratio, the capture of THP-1 membrane fragment in the presence of high phosphoantigen concentrations was equivalent to the capture of Daudi cell membrane alone. Thus, regardless of the stimulus (soluble or cellular), γδ T cell activation resulted in a marked capture of membrane fragments from cells to which they were conjugated.

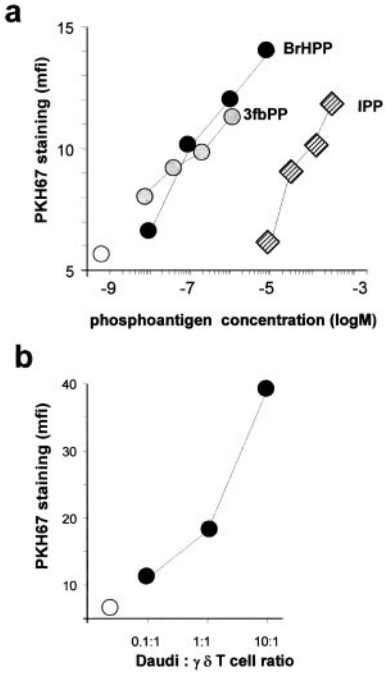

FIGURE 2. Ag density controls membrane transfer by γδ T cells. *a*, Dose effect of phosphoantigen concentration. The indicated concentrations of phosphoantigens were added to cocultures of equal proportions of PKH67-labeled THP-1 and CMTMR-labeled γδ T cells. The MFI_{PKH67} on gated γδ T cells is reported. Phosphoantigens were BrHPP (●), IPP (◇) and 3fbPP (shaded circles). Negative control (○) was obtained by measuring the MFI_{PKH67} of gated γδ T cells without phosphoantigen. *b*, Effect of Daudi:γδ T cell ratio on PKH67 transfer to γδ T cells. Experiments were conducted as above by replacing THP-1 and phosphoantigens with Daudi cells alone. ○, MFI_{PKH67} of γδ T cells before exposure with Daudi cells.

γδ T cells acquire molecules from target cells after synapse formation

To visualize the process of molecular transfer between γδ T cells and either THP-1 or Daudi cells, we repeated the experiments presented above and analyzed them by confocal microscopy. A hallmark of IS performed by αβ T and B lymphocytes in the presence of Ag is the exclusion of CD45 from the area of contact with target cells. Thus, we used an anti-CD45 mAb to analyze conjugates between γδ T and THP-1 cells in the presence or absence of BrHPP. As shown in Fig. 3, the uniform CD45 staining observed on γδ T cells conjugated to THP-1 in the absence of BrHPP was replaced by a discontinuous staining, CD45 being largely excluded from the contact area when BrHPP was added. A comparable CD45 exclusion was observed in conjugates formed between γδ T cells and Daudi (Fig. 3, *a* and *b*). Thus, CD45 exclusion applies to γδ T cell synapse as well.

However, this highly typical characteristic of IS (50) was not associated with capping of γδ TCR at the contact area (Fig. 3, *a* and *b*). When PKH67-labeled THP-1 cells were cocultured with CMTMR-labeled γδ T cells, a clear PKH67 staining was observed on T cells when BrHPP was present but not in its absence. Interestingly, the presence of PKH67 on T cells was the most noticeable at the level of the IS (Fig. 3*a*). Captured membrane patches spread out of the contact area at the γδ T cell surface, localizing at the membrane and submembrane levels of the IS (Fig. 3*c*, arrows). In the presence of PP2 or CytD, BrHPP-induced membrane capture was markedly inhibited (Fig. 3*c*). Conjugates of γδ T cells and THP-1 cells were observed in each experiment, although they were more frequent with BrHPP. In contrast, PP2 and CytD significantly prevented conjugate formation (Table I). In agreement with the former cytometry experiments, membrane capture by unstimulated T cells was very limited, whereas large green patches derived from THP-1 membranes were observed at the surface of BrHPP-stimulated γδ T cells. Similarly conjugated γδ T cells were less abundant in cocultures containing BrHPP and PP2 or BrHPP and CytD (Table I). In neither condition did γδ T cells without synaptic engagement harbor any green patch of THP-1 membrane (data not shown). The γδ T cells conjugated to Daudi cells also led to a synaptic transfer of PKH67 dye, which increased in the presence of BrHPP and decreased in the presence of PP2 or CytD as found above with THP-1 cells (Fig. 3*d*). To document the molecular transfer of membrane markers from Daudi to γδ T cells further, the conjugates were analyzed for surface expression of IgM. For comparison, these cocultures were also done in medium containing either PP2 or CytD. As shown in Fig. 3*e*, a clear transfer of IgM from Daudi to γδ T cell surface was observed except when PP2 or CytD was added (Fig. 3*e*).

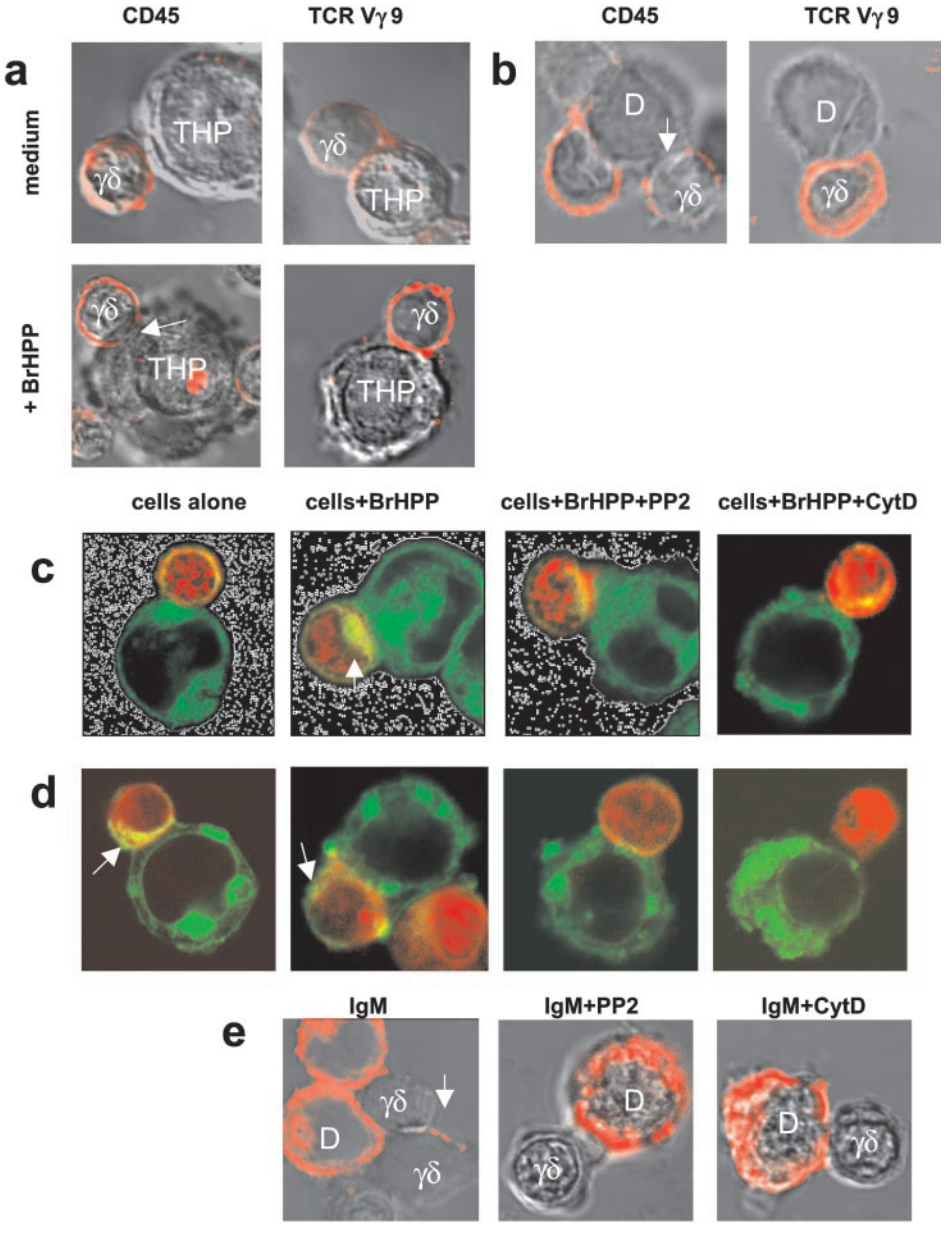

FIGURE 3. Confocal microscopy analysis of synaptic transfer by γδ T cells. *a* and *b*, Fluorescence and differential interference contrast overlays of unstained THP-1 (*a*) or Daudi (*b*) and γδ T cells left for 15 min at 37°C in culture medium, fixed on poly-L-lysine-coated slides, stained with primary anti-CD45 or anti-TCRVγ9 plus rhodamine-conjugated secondary Ab, and then analyzed by confocal microscopy. Arrows indicate CD45 exclusion from the γδ T cell contact surface. *c* and *d*, PKH67-labeled THP-1 cells (*c*) or PKH67-labeled Daudi cells (*d*) were cocultured with γδ T cells stained red with CMTMR for 1 h at 37°C in the presence of the indicated drugs, then fixed onto poly-L-lysine-coated slides and analyzed by confocal microscopy. Arrows indicate PKH67 patches transferred onto γδ T cells. *e*, Fluorescence and differential interference contrast overlays of unstained Daudi and γδ T cells prepared as for *b* in the presence of the indicated drugs, then labeled with anti-IgM mAb and rhodamine-conjugated secondary Ab. Arrows indicate IgM patches. For clarity, γδ T cells, Daudi, and THP-1 are respectively indicated by γδ, D, and THP labels.

Altogether these results indicated that activated γδ T cells establish an IS with target or accessory cells through which theycapture target cell surface molecules including lipids and proteins such as IgM. This process was referred to as synaptic transfer.

Table I. *Percentage of conjugated γδ T cells in cocultures and of conjugates exhibiting synaptic transfer*[a]

Coculture	γδ in Conjugates (%)	Conjugates Exhibiting Synaptic Transfer (%)
γδ T cells with THP-1		
Medium alone	54 ± 7	68 ± 6
BrHPP (100 nM)	77 ± 8	94 ± 5
BrHPP (100 nM) + PP2	22 ± 7	63 ± 11
BrHPP (100 nM) + CytD	20 ± 7	35 ± 11
γδ T cells with Daudi		
Medium alone	51 ± 6	67 ± 7
Medium + PP2	19 ± 4	31 ± 9
Medium + CytD	27 ± 4	31 ± 13

[a] At least 100 γδ T cells were analyzed visually for each condition. The numbers give the mean and SD from three independent experiments.

Phosphoantigen inhibitors reduced synaptic transfer by γδ T cells

We had previously shown that phosphoantigen recognition involves dephosphorylation by an as-yet-unidentified phosphatase (45). Because the efficiency of synaptic transfer by γδ T lymphocytes correlated with Ag potency, we tested the effect of phosphatase inhibitors on the capture process. At millimolar (nontoxic; data not shown) concentrations, levamisole and inorganic PP (PPi) severely inhibited the release of TNF-α by BrHPP-stimulated γδ T cells but did not affect mitogenic (PHA) activation of γδ T cells (Fig. 4*a*). When tested in membrane capture assays, levamisole and PPi markedly inhibited synaptic transfer. In addition, a strong inhibition of PKH67 transfer was also obtained when the Src family kinase inhibitor PP2 or the cytoskeleton remodeling inhibitor CytD were used (Fig. 4*b*). Altogether, the pharmacological control of membrane capture indicates that synaptic transfer requires the selective recognition

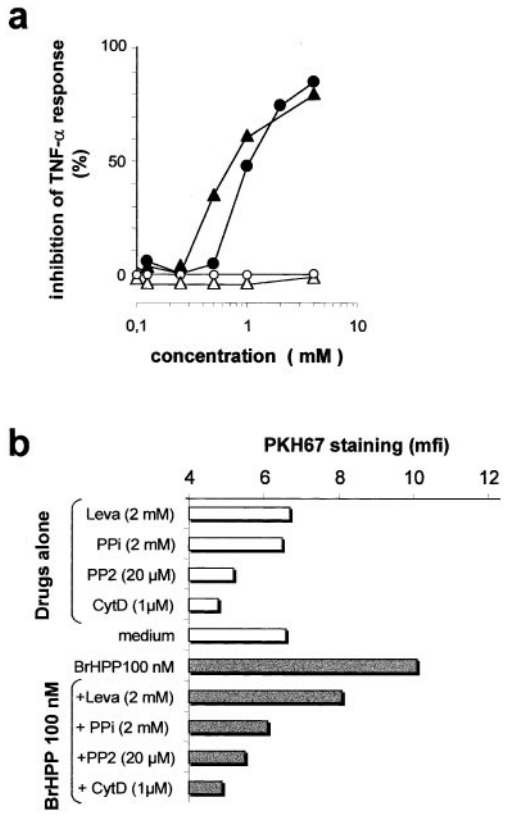

FIGURE 4. Phosphoantigen inhibitors reduce synaptic transfer by BrHPP-activated γδ T cells. *a*, Levamisole (●) and PPi (▲) inhibit BrHPP-induced but not PHA-induced (○ and △, respectively) γδ T cell activation as monitored in a standard TNF-α release assay. Percentage of inhibition was calculated by normalizing the values obtained for each experimental measurement to the maximal (300 pg/ml) and basal (4 pg/ml) levels. *b*, MFI_{PKH67} of gated γδ T cells in the indicated culture conditions. $PKH67^+$ THP-1 cells and $CMTMR^+$ γδ T cells (at a 1:1 ratio) were cocultured for 1 h at 37°C alone or in the presence of the indicated drugs. These data are representative of three experiments.

by the TCR of phosphoantigens and subsequent γδ T cell activation involving Src family kinase activity and remodeling of the actin cytoskeleton.

Activation-induced synaptic transfer from Daudi cells

As indicated by the above results, γδ T cells readily capture membrane fragments from the Daudi lymphoma in the absence of phosphoantigen added. Addition of PP2 or BrHPP, respectively, inhibited and enhanced the synaptic transfer by γδ T cells in 1 h of coculture with Daudi (Fig. 5*a*). As the β_2m-deficient Burkitt's lymphoma Daudi does not express MHC class I molecules, its recognition by T cells may be controlled not only by the γ9δ2 TCR but also by NK receptors for MHC class I expressed at the γδ T cell surface (29–32). Therefore, we compared the extent of synaptic transfer exerted by γδ T cells coincubated either with Daudi or with β_2m transfectants of Daudi cells that express surface MHC class I molecules (51). The MHC class I molecule expression was verified before each transfer assay by immunofluorescence using W6/32 mAb (data not shown). When cocultured for 1 h with β_2m^+ Daudi, PKH67 was transferred onto γδ T cells in quantitatively similar extent as with untransfected Daudi. This active capture process was again reduced by PP2 and increased by BrHPP (Fig. 5*b*). Therefore, surface expression of MHC class I molecules by engaged targets did not affect synaptic transfer by γδ T cells, which appears to be mediated mainly by the γ9δ2 TCR.

Synaptic transfer from other tumor cells

The above data suggested that activated γδ T cells capture membrane fragments from tumor cells regardless of MHC class I expression. To strengthen this observation, we tested other tumoral cell lines that elicit different biological responses from γδ T cells. We used the MHC class I-negative K562 cell line, which is lysed by γδ T cells in an NK-like TCR-independent fashion (52). The non-Hodgkin lymphomas VAL and OCI-Ly8 were chosen as efficiently killed targets by γδ T cells (28), and Raji (29) and C1R (data not shown) were chosen as nonlysed cell lines. The γδ T cell responses to those tumor cell lines are summarized in Table II. Only a marginal synaptic transfer from C1R occurred, which was PP2 resistant and increased by BrHPP. All other tumor cells induced a strong transfer onto γδ T cells, which was moderately increased in the presence of BrHPP. In addition, the membrane capture observed for these tumor cell lines was only slightly inhibited by PP2 (Fig. 5*c*). Thus, in agreement with the former results, γδ T cells captured membrane fragments from most of their conjugated tumor targets, regardless of whether these latter expressed MHC class I molecules.

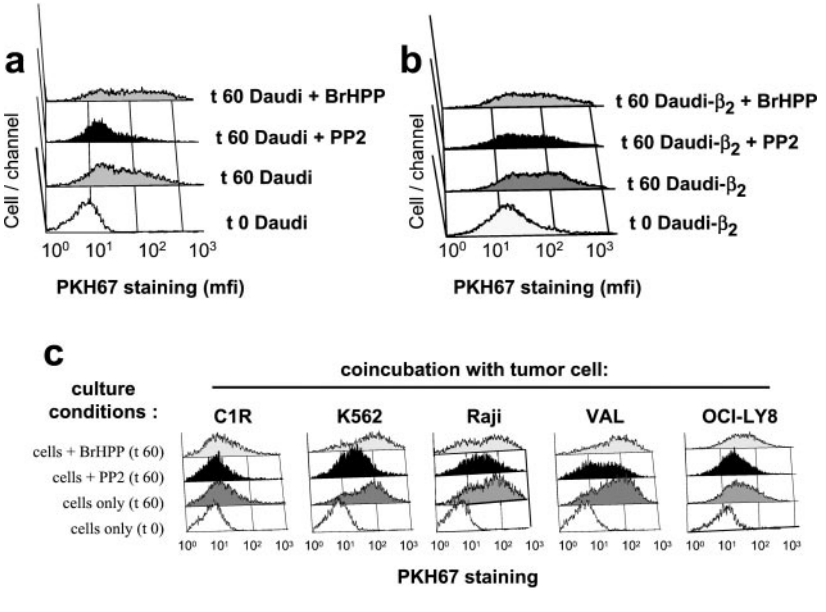

FIGURE 5. Daudi Burkitt lymphoma and several other tumor cells stimulate synaptic transfer by γδ T cells. *a*, Daudi lymphoma cells were labeled with PKH67 and γδ T cells were labeled with CMTMR. Equal numbers of γδ T cells and Daudi cells were cultured separately for 1 h at 37°C, then mixed and immediately analyzed by cytometry (t = 0 min) or cocultured for 1 h at 37°C (t = 60 min) in medium containing the specified drugs before flow cytometry. *b*, The same assays as above, using Daudi-β_2m^+ cells instead of Daudi cells. *c*, The same series of experiments as above using the specified tumor cell lines.

Table II. *Biological responses of γδ T cells to tumor cells used in this study*

Target Cell Lines	Selective Amplification from PBL Cultures	Specific Lysis	Ref.	Synaptic Transfer from Target Cell
Daudi (MHC class I^-)	+	+	30	+
Raji (MHC class I^+)	−	−	30	+
OCI-Ly8 (MHC class I^-)	−	+	29	+
VAL (MHC class I^-)	−	+	29	+
K562 (MHC class I^-)	−	+	30	+
C1R (MHC class I^-)	ND	−		−

Discussion

The conjugation of activated αβ T cells (5, 6, 9), B cells (7, 53), and NK cells (15, 16, 18)[4] by their respective Ags involves the stripping of target cell membrane fragments that convey cell surface proteins including Ags. The present study demonstrates for the first time that, once activated by either soluble or cellular Ags, human γδ T cells also capture target cell molecules. Because cell-to-cell contact is required for γδ T cell activation by soluble nonpeptide Ags, we first used the THP-1 myelomonocytic cell line (providing a convenient accessory cell) in the presence of phosphoantigens (48, 54) as a γδ T cell stimulus. Activation by phosphoantigens triggered an active transfer of THP-1 membrane patches by the γδ T cell effectors. We also analyzed this process in a second model involving the innate γδ T cell reactivity for tumor cells such as Daudi, in the absence of exogenously supplied phosphoantigens. In this case as well, γδ T cells conjugated to Daudi acquired molecules from the target cell surface.

ISs are tight contact zones that reactive lymphocytes establish with their cell targets. In this contact area, some molecules are enriched and others are excluded. For instance, exclusion of CD45 from the cell-cell contact region is a hallmark of IS (4, 50). Based on this criterion, γδ T cells seem to establish authentic IS with their conjugated targets. The building of IS also relies upon the highly dynamic remodeling of the actin cytoskeleton, mediated by inter alia ATP, Ca^{2+}, and protein kinase C (2, 17). Consequently, inhibitors targeting the cytoskeleton such as CytD profoundly affect the ability of lymphoid cells to set ISs, to transduce activation signals, and to internalize ligands from their surface receptors (10, 55). In this respect, the synaptic transfer by γδ T cells was crucially sensitive to inhibitors of actin cytoskeleton. This is very reminiscent of TCR- and CD28-mediated ligand uptake as performed by αβ T cells (10) or NMR-mediated capture by NK cells.[4] Because an IS may appear in the absence of Ag (56), we wanted to ascertain whether cognate phosphoantigen recognition was necessary in our model. γδ T cell activation by BrHPP involves hydrolysis of the PP group by an unidentified phosphatase and release of the inactive monophosphate byproduct (45). We thus used levamisole as phosphohydrolase inhibitor (57) and PPi. Presumably by competing with PP moiety of phosphoantigens, PPi was found to specifically inhibit phosphoantigen-induced activation. Both molecules inhibited phosphoantigen activation and impaired BrHPP-induced membrane capture. Hence, the synaptic transfers was subordinated to the Ag recognition mechanism, including phosphoantigen hydrolysis.

In line with previous studies (9),[4] the potent inhibition caused by PP2 demonstrated that synaptic transfer required Src family kinase activity. For T cells, the level of TCR occupancy by Ag is usually translated into an Ag dose-dependent activation of protein tyrosine kinases (58); therefore, we assume that Src family kinase activation was triggered by γδ TCR engagement. The occupancy of γδ TCR by phosphoantigens cannot be monitored as for αβ T cells (46, 47). Nevertheless, synaptic transfer strictly followed the dose-dependent γδ T cell activation by either soluble or cell Ags, and was markedly inhibited by inhibitors of phosphoantigen recognition. Furthermore, the experimental conditions allowing synaptic transfer to occur had previously been shown to induce a sustained signaling in polyclonal γδ T cell (46). Altogether, these results allow us to conclude that γδ T cells do not apparently differ from other lymphocyte populations in their manner to interact with targets and to capture surface molecules from them (4). Thus, in γδ T cell-target cell conjugates, TCR triggering led γδ T cells to establish an IS, to reorganize their actin cytoskeleton, and to initiate sustained intracellular signaling, enabling synaptic transfer of target cell molecules onto the γδ T cell.

However, the following lines of evidence seem to diverge from this standard concept. Synapse formation by αβ T cells is thought to concentrate locally both TCR and signaling molecules at central supramolecular activation clusters (1, 3, 59). Although γδ T cells apparently established typical IS with target cells, no macroscopic γδ TCR polarization at the IS was noticed. However, this situation was previously reported, and either high dissociation rates of cognate complexes or different kinetics of synapse establishment and TCR polarization were proposed to explain the absence of TCR enrichment in IS (50, 60). In the present model, it is also possible that the phosphoantigen concentration or Ag density at the tumor cell surface was high enough to trigger both sustained signaling and synaptic transfer without the need for enrichment of γδ TCR. In the case of $CD4^+$ or $CD8^+$ T cells, ligand uptake is thought to be the consequence of αβ TCR internalization (5, 7, 9). However, recognition of potent phosphoantigen agonists by cell surface γδ TCR does not involve γδ TCR down-modulation (46, 47). Therefore, γδ T cell-mediated synaptic transfer induced by antigenic stimulation is not the mere consequence of TCR internalization.

The recognition of Daudi cells is mediated by the γδ TCR (24–26, 61). Accordingly, coculture of Daudi cells with γ9δ2 T cells led to a synaptic transfer that was slightly increased by adding BrHPP to the coculture or was abrogated by γδ T cell treatment with PP2. As already reported for αβ CTL (9) and NK cells,[4] the capture of target cell membrane fragments by γδ T cells occurred via the IS. Thus, synaptic transfer is unrelated to the nature of the γδ TCR ligand, as soluble or cellular Ags activated a comparable membrane capture by γδ T cells.

Exposure of γδ T cells to increasing numbers of Daudi targets increased synaptic transfer correspondingly. This observation is consistent with the so-called "kiss and run" phenomenon underlying the serial target cell killing by CTL. During this process we observed the capture of target cell markers such as B cell-derived Ig on the γδ T cell surface. This capture can be visualized as small IgM aggregates distributed on the surface of γδ T cells. The role that IgM or other surface molecules may play once they are acquired by T cells is elusive and requires further investigation.

Recognition of Daudi by γδ T cells involves the γ9δ2 TCR (26) but consequent activation is controlled by NMR, as testified by the higher sensitivity of Daudi as compared with Daudi-β_2m^+ transfectants to γδ T cell-mediated lysis (29–32). In contrast to cytotoxicity assays, γδ T cells captured membrane material from Daudi and Daudi-β_2m^+ tumor cells to similar extents. Therefore, the expression of MHC class I molecules at the Daudi cell surface does not hamper the synaptic transfer. A panel of tumor cell lines, selected according to their MHC class I molecule expression and effector responses (Table II), reinforced theses observations. As for Daudi cells, synaptic transfer occurred with these tumor cells regardless of their MHC class I molecule expression. Furthermore, no correlation appeared between the ability of those cell lines to be subject to synaptic transfer and their ability to trigger cytotoxicity or amplification. This dissociation from effector responses was already noticed using perforin-deficient T cells (9) or B cell models (7). So human γδ T cells exerted synaptic transfer from tumor cells, but without strong inhibitory effect delivered by their NMR. This suggests that synaptic transfer is an early process occurring while the T cell is scanning its conjugated cell surface. Nevertheless, as shown with C1R cells, not all tumoral cell lines allow this phenomenon. The difference with the highly controlled synaptic transfer performed by NK cells (18)[4] may be due to the dominant activatory signal delivered by γδ TCR. In addition, γδ T cell response to lymphoma often involves CD28 coengagement (62–64), which also potentiates the synaptic transfer (6). γδ TCR-mediated or NK receptor-mediated target cell recognition may lead to synaptic transfer. Phosphoantigen addition frequently enhances the γδ T cell responses to non-Hodgkin lymphoma (28). Also, γδ T cells exert quantitatively similar synaptic transfer from TCR-dependent and -independent cell targets Daudi and K562, respectively. Current studies in our laboratory aim at delineating the respective contributions and outcomes of TCR and NK receptor signals in this IS and the associated transfer.

By visualizing membrane bridges between CTL and target cell surface, Stinchcombe et al. (11) proposed that the continuity of fused membrane bilayers enabled the direct molecular diffusion from target to effector cell at the level of the IS. Taken together, our observations fully agreed with Stinchcombe's model of membrane ripping by nonselective lateral diffusion (11). Furthermore, this process permits the transfer of target cell surface molecules such as IgM in proper orientation. Synaptic transfer induced by activation of γδ T cells depended upon the highly active establishment of a functional IS. By allowing the local formation of membrane continuities, the synapse most likely enables lateral diffusion of surface molecules originating from the target, regardless of TCR internalization. However, the physiological role of this process still remains unclear. By acquiring target cell-derived Ags, γδ T cells like αβ T cells may become themselves subjected to CTL lysis or fratricide killing (5). Hence, synaptic transfer could favor the termination of immune responses via lymphocyte exhaustion (6). Alternatively, a competition between reactive T cells for Ag stripping from APC surfaces might drive affinity maturation of the in vivo immune response (65). However, in the present context this hypothesis seems unlikely because no biased selection of lymphocytes bearing higher-affinity receptors was observed in response to phosphoantigen stimulation evaluated by the size profiles of their TCRVδ2 CDR3 (66). Future work will now focus on the elucidation of the physiological role of synaptic transfer by γδ T cells.

Acknowledgments

We thank P. S. Valitutti (Institut National de la Santé et de la Recherche Médicale) and Sanofi-Synthélabo for providing mAbs and IL-2, and we acknowledge the expert technical assistance of Sabina Mueller for confocal microscopy imaging.

References

1. Monks, C. R., B. A. Freiberg, H. Kupfer, N. Sciaky, and A. Kupfer. 1998. Three-dimensional segregation of supramolecular activation clusters in T cells. *Nature 395:82.*
2. Dustin, M. L., and A. S. Shaw. 1999. Costimulation: building an immunological synapse. *Science 283:649.*
3. 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.*
4. 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.*
5. 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.*
6. 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.*
7. Batista, F. D., D. Iber, and M. S. Neuberger. 2001. B cells acquire antigen from target cells after synapse formation. *Nature 411:489.*
8. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature 375:148.*
9. 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.*
10. 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.*
11. Stinchcombe, J. C., G. Bossi, S. Booth, and G. M. Griffiths. 2001. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity 15:751.*
12. Davis, S. J., and P. A. van der Merwe. 2001. The immunological synapse: required for T cell receptor signalling or directing T cell effector function? *Curr. Biol. 11:R289.*
13. Davis, D. M., I. Chiu, M. Fassett, G. B. Cohen, O. Mandelboim, and J. L. Strominger. 1999. The human natural killer cell immune synapse. *Proc. Natl. Acad. Sci. USA 96:15062.*
14. Vyas, Y. M., K. M. Mehta, M. Morgan, H. Maniar, L. Butros, S. Jung, J. K. Burkhardt, and B. Dupont. 2001. Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated noncytolytic and cytolytic interactions. *J. Immunol. 167:4358.*
15. 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.*
16. 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.*
17. Dustin, M. L., and J. A. Cooper. 2000. The immunological synapse and the actin cytoskeleton: molecular hardware for T cell signaling. *Nat. Immunol. 1:23.*
18. Zimmer, J., V. Ioannidis, and W. Held. 2001. H-2D ligand expression by $Ly49A^+$ natural killer (NK) cells precludes ligand uptake from environmental cells: implications for NK cell function. *J. Exp. Med. 194:1531.*
19. Hayday, A. C. 2000. γδ cells: a right time and a right place for a conserved third way of protection. *Annu. Rev. Immunol. 18:975.*
20. Schild, H., N. Mavaddat, C. Litzenberger, E. W. Ehrich, M. M. Davis, J. A. Bluestone, L. Matis, R. K. Draper, and Y. H. Chien. 1994. The nature of major histocompatibility complex recognition by γδ T cells. *Cell 76:29.*
21. Holoshitz, J., N. C. Romzek, Y. Jia, L. Wagner, L. M. Vila, S. J. Chen, J. M. Wilson, and D. R. Karp. 1993. MHC-independent presentation of mycobacteria to human γδ T cells. *Int. Immunol. 5:1437.*
22. Lang, F., M. A. Peyrat, P. Constant, F. Davodeau, J. David-Ameline, Y. Poquet, H. Vie, J. J. Fournie, and M. Bonneville. 1995. Early activation of human Vγ9Vδ2 T cell broad cytotoxicity and TNF production by nonpeptidic mycobacterial ligands. *J. Immunol. 154:5986.*
23. Morita, C. T., E. M. Beckman, J. F. Bukowski, Y. Tanaka, H. Band, B. R. Bloom, D. E. Golan, and M. B. Brenner. 1995. Direct presentation of nonpeptide prenyl pyrophosphate antigens to human γδ T cells. *Immunity 3:495.*
24. Fisch, P., M. Malkovsky, S. Kovats, E. Sturm, E. Braakman, B. S. Klein, S. D. Voss, L. W. Morrissey, R. De Mars, W. J. Welch, et al. 1990. Recognition by human Vγ9/Vδ2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science 250:1269.*
25. Davodeau, F., M. A. Peyrat, M. M. Hallet, J. Gaschet, I. Houde, R. Vivien, H. Vie, and M. Bonneville. 1993. Close correlation between Daudi and mycobacterial antigen recognition by human γδ T cells and expression of V9JPC1γ/V2DJCδ-encoded T cell receptors. *J. Immunol. 151:1214.*
26. Bukowski, J. F., C. T. Morita, Y. Tanaka, B. R. Bloom, M. B. Brenner, and H. Band. 1995. Vγ2Vδ2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. *J. Immunol. 154:998.*
27. Kobayashi, H., Y. Tanaka, J. Yagi, H. Toma, and T. Uchiyama. 2001. γδ T cells provide innate immunity against renal cell carcinoma. *Cancer Immunol. Immunother. 50:115.*

28. Sicard, H., T. Al Saati, G. Delsol, and J. J. Fournie. 2001. Synthetic phosphoantigens enhance human Vγ9Vδ2 T lymphocytes killing of non-Hodgkin's B lymphoma. *Mol. Med. 7:711.*
29. 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 Vγ9/Vδ2 T cells in tumor immunity. *Eur. J. Immunol. 27:3368.*
30. Poccia, F., B. Cipriani, S. Vendetti, V. Colizzi, Y. Poquet, L. Battistini, M. Lopez-Botet, J. J. Fournie, and M. L. Gougeon. 1997. CD94/NKG2 inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive Vγ9Vδ2 T lymphocytes. *J. Immunol. 159:6009.*
31. Halary, F., M. A. Peyrat, E. Champagne, M. Lopez-Botet, A. Moretta, L. Moretta, H. Vie, J. J. Fournie, and M. Bonneville. 1997. Control of self-reactive cytotoxic T lymphocytes expressing γδ T cell receptors by natural killer inhibitory receptors. *Eur. J. Immunol. 27:2812.*
32. Carena, I., A. Shamshiev, A. Donda, M. Colonna, and G. D. Libero. 1997. Major histocompatibility complex class I molecules modulate activation threshold and early signaling of T cell antigen receptor-γ/δ stimulated by nonpeptidic ligands. *J. Exp. Med. 186:1769.*
33. Espinosa, E., C. Belmant, H. Sicard, R. Poupot, M. Bonneville, and J. J. Fournié. 2001. $Y2K^{+}1$ state-of-the-art on non-peptide phosphoantigens, a novel category of immunostimulatory molecules. *Microbes Infect. 3:645.*
34. Tanaka, Y., M. B. Brenner, B. R. Bloom, and C. T. Morita. 1996. Recognition of nonpeptide antigens by T cells. *J. Mol. Med. 74:223.*
35. Kunzmann, V., E. Bauer, J. Feurle, F. Weissinger, H. P. Tony, and M. Wilhelm. 2000. Stimulation of γδ T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. *Blood 96:384.*
36. Bukowski, J. F., C. T. Morita, and M. B. Brenner. 1999. Human γδ T cells recognize alkylamines derived from microbes, edible plants and tea: implication for innate immunity. *Immunity 11:57.*
37. Belmant, C., E. Espinosa, R. Poupot, M. A. Peyrat, M. Guiraud, Y. Poquet, B. M., and J. J. Fournié. 1999. 3-formyl-1-butyl diphosphate, a novel mycobacterial metabolite stimulating human γδ T cells. *J. Biol. Chem. 274:32079.*
38. Feurle, J., E. Espinosa, S. Eckstein, F. Pont, V. Kunzmann, J. J. Fournie, M. Herderich, and M. Wilhelm. 2002. *Escherichia coli* produces phosphoantigens activating human γδ T cells. *J. Biol. Chem. 277:148.*
39. Tanaka, Y., C. T. Morita, E. Nieves, M. B. Brenner, and B. R. Bloom. 1995. Natural and synthetic non-peptide antigens recognized by human γδ T cells. *Nature 375:155.*
40. Espinosa, E., C. Belmant, F. Pont, B. Luciani, R. Poupot, F. Romagné, H. Brailly, M. Bonneville, and J. J. Fournié. 2001. Chemical synthesis and biological activity of bromohydrin pyrophosphate, a potent stimulator of human γδ T cells. *J. Biol. Chem. 276:18337.*
41. Davodeau, F., M. A. Peyrat, M. M. Hallet, I. Houde, H. Vie, and M. Bonneville. 1993. Peripheral selection of antigen receptor junctional features in a major human γδ subset. *Eur. J. Immunol. 23:804.*
42. Bukowski, J. F., C. T. Morita, H. Band, and M. B. Brenner. 1998. Crucial role of TCRγ chain junctional region in prenyl pyrophosphate antigen recognition by γδ T cells. *J. Immunol. 161:286.*
43. Allison, T. J., C. C. Winter, J. J. Fournié, M. Bonneville, and D. N. Garboczi. 2001. Structure of a human γδ T cell receptor. *Nature 411:820.*
44. Miyagawa, F., Y. Tanaka, S. Yamashita, B. Mikami, K. Danno, M. Uehara, and N. Minato. 2001. Essential contribution of germline-encoded lysine residues in Jγ1.2 segment to the recognition of nonpeptide antigens by human γδ T cells. *J. Immunol. 167:6773.*
45. Belmant, C., E. Espinosa, F. Halary, Y. Tang, M. A. Peyrat, H. Sicard, A. Kozikowski, R. Buelow, R. Poupot, M. Bonneville, and J. J. Fournié. 2000. A chemical basis for recognition of nonpeptide antigens by human δ T cells. *FASEB J. 14:1669.*
46. Sireci, G., E. Espinosa, C. Di Sano, A. Salerno, J. J. Fournié, and F. Dieli. 2001. Differential activation of human γδ cells by nonpeptide phosphoantigens. *Eur. J. Immunol. 31:1628.*
47. Lafont, V., J. Liautard, M. Sable-Teychene, Y. Sainte-Marie, and J. Favero. 2001. Isopentenyl pyrophosphate, a mycobacterial non-peptidic antigen, triggers delayed and highly sustained signaling in human γδ T lymphocytes without inducing down-modulation of T cell antigen receptor. *J. Biol. Chem. 276:15961.*
48. Miyagawa, F., Y. Tanaka, S. Yamashita, and N. Minato. 2001. Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human γδ T cells by aminobisphosphonate antigen. *J. Immunol. 166:5508.*
49. Sturm, E., E. Braakman, P. Fisch, R. J. Vreugdenhil, P. Sondel, and R. L. Bolhuis. 1990. Human Vγ9-Vδ2 T cell receptor-γδ lymphocytes show specificity to Daudi Burkitt's lymphoma cells. *J. Immunol. 145:3202.*
50. 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.*
51. 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 β_2-microglobulin-transfected Daudi cells. *J. Immunol. 141:17.*
52. Ferrarini, M., S. Heltai, E. Toninelli, M. G. Sabbadini, C. Pellicciari, and A. A. Manfredi. 1995. Daudi lymphoma killing triggers the programmed death of cytotoxic Vγ9/Vδ2 T lymphocytes. *J. Immunol. 154:3704.*
53. Batista, F. D., and M. S. Neuberger. 2000. B cells extract and present immobilized antigen: implications for affinity discrimination. *EMBO J. 19:513.*
54. Ottones, F., J. Dornand, A. Naroeni, J. P. Liautard, and J. Favero. 2000. Vγ9Vδ2 T cells impair intracellular multiplication of *Brucella suis* in autologous monocytes through soluble factor release and contact-dependent cytotoxic effect. *J. Immunol. 165:7133.*
55. Valitutti, S., M. Dessing, K. Aktories, H. Gallati, and A. Lanzavecchia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy: role of T cell actin cytoskeleton. *J. Exp. Med. 181:577.*
56. 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.*
57. McComb, R. B., G. N. Bowers, Jr., and S. Posen. 1979. *Alkaline Phosphatase*. Plenum, New York.
58. Muller, S., S. Demotz, C. Bulliard, and S. Valitutti. 1999. Kinetics and extent of protein tyrosine kinase activation in individual T cells upon antigenic stimulation. *Immunology 97:287.*
59. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science 283:680.*
60. Krummel, M., C. Wulfing, C. Sumen, and M. M. Davis. 2000. Thirty-six views of T-cell recognition. *Philos. Trans. R. Soc. London B 355:1071.*
61. 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 γ/δ T cell subsets to Daudi cells. *J. Immunol. 148:2315.*
62. Testi, R., and L. L. Lanier. 1989. Functional expression of CD28 on T cell antigen receptor γ/δ-bearing T lymphocytes. *Eur. J. Immunol. 19:185.*
63. Lafont, V., J. Liautard, A. Gross, J. P. Liautard, and J. Favero. 2000. Tumor necrosis factor-α production is differently regulated in γδ and αβ human T lymphocytes. *J. Biol. Chem. 275:19282.*
64. Li, Y., S. A. Newby, J. V. Johnston, K. E. Hellstrom, and L. Chen. 1995. Protective immunity induced by B7/CD28-costimulated γδ T cells to the EL-4 lymphoma in allogenic athymic mice. *J. Immunol. 155:5705.*
65. Kedl, R. M., B. C. Schaefer, J. W. Kappler, and P. Marrack. 2002. T cells downmodulate peptide-MHC complexes on APCs in vivo. *Nat. Immunol. 3:27.*
66. Boullier, S., Y. Poquet, F. Halary, M. Bonneville, J. J. Fournie, and M. L. Gougeon. 1998. Phosphoantigen activation induces surface translocation of intracellular CD94/NKG2A class I receptor on $CD94^{-}$ peripheral Vγ9Vδ2 T cells but not on $CD94^{-}$ thymic or mature γδ T cell clones. *Eur. J. Immunol. 28:3399.*
67. Tabiasco, J., E. Espinosa, D. Hudrisier, E. Joly, A. Vercellone, and J.-J. Fournié. 2002. Active trans-synaptic capture of membrane fragments by natural killer. *Eur. J. Immunol. In press.*