Direct stimulation of naïve T cells by membrane vesicles from antigen-presenting cells: Distinct roles for CD54 and B7 molecules

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T cell stimulation usually requires direct contact with viable antigen-presenting cells (APCs). However, we show here that small exosome-like membrane vesicles shed from APCs can be recognized by naïve CD8$^+$ T cells in the absence of viable APCs. T cell antigen receptor-dependent binding of vesicles by CD8$^+$ cells is MHC class I/peptide-specific and requires that the vesicles coexpress intercellular adhesion molecule 1 (ICAM-1, CD54), although not B7 (B7-1). In the absence of B7, T cell binding of vesicles is nonimmunogenic. By contrast, vesicles expressing both ICAM-1 and B7 are strongly immunogenic and cause purified APC-depleted CD8$^+$ cells to mount peptide-specific proliferative responses and differentiate into effector cells.

Under physiological conditions T cell activation occurs in the T-dependent areas of secondary lymphoid tissues and is presumed to require direct contact with viable antigen-presenting cells (APCs) (1–3). Presentation of antigen by APCs involves degradation (''processing'') of native proteins, followed by loading of immunogenic peptides onto class I and II MHC molecules. Peptide loading of MHC class I molecules can also involve a process of ''cross-presentation'' (cross priming) through APC uptake of antigenic material, notably minor H and tumor-associated antigens, from other cells (4). Until recently it has been assumed that cross-presentation involves phagocytosis of dying cells. For tumor rejection, however, it is now apparent that strong immune responses can be elicited by small membrane vesicles (exosomes) secreted by viable tumor cells (5). Exosomes are also produced by several types of normal cells, including DCs, B cells, T cells, and immature erythrocytes, and appear to be secreted by viable cells after fusion of multivesicular endosomes with the plasma membrane (6–9).

The observation that exosomes are highly immunogenic for tumor-specific CD8$^+$ cells has focused attention on how these structures are handled by the immune system. Currently, there is little if any evidence that T cells can recognize exosomes in the absence of APCs (6–9). Instead, the prevailing view is that exosomes are first ingested by APCs and then processed, thus degrading proteins into peptides for loading onto APC MHC class I molecules. Exosomes from APCs can also transfer preformed MHC/peptide complexes to other APCs (9).

Under defined conditions, it is clear that T cells, including naïve T cells, can be stimulated in the absence of APCs. This is apparent from the finding that T cells can proliferate *in vitro* after exposure to cross-linked-specific MHC/peptide complexes (10) or anti-T cell antigen receptor (TCR) plus anti-CD28 mAbs (11, 12). For this reason, one could envisage that if exosomes and/or other subcellular material from APCs were engineered to display a high concentration of MHC/peptide plus appropriate costimulatory ligands, this material would be directly immunogenic for T cells in the absence of intact APCs. In favor of this idea, we show here that purified naïve CD8$^+$ cells can recognize small (100 nm) vesicles shed by APCs, including mature DCs and artificial APCs constructed from transfected *Drosophila* (Dros) cells. Vesicle binding by CD8$^+$ cells is highly specific and requires that the vesicles coexpress two different ligands, namely specific MHC class I/peptide complexes and intercellular adhesion molecule 1 (ICAM-1). To be directly immunogenic, however, the vesicles also have to express a third ligand, B7.

Materials and Methods

Animals. C57BL/6J (B6) and BALB/cByJ mice were purchased from the Jackson Laboratory. 2C TCR transgenic mice on a normal B6 and B6.CD28$^{-/-}$ background (13) were bred and maintained at The Scripps Research Institute.

Cell Lines and Culture Media. Dros cells transfected with mouse molecules were prepared and maintained as described (14).

Chemicals, Peptides, and mAbs. PMA and Ionomycin were purchased from Calbiochem and 5,6-carboxyfluorescein diacetate succinimyl ester (CFSE) was purchased from Sigma. Transwells (3-μm pore size) were purchased from Costar (Corning). QL9 (QLSPFPFDL), p2Ca (LSPFPFDL), and P1A (LPYLGWLVF) peptides were purchased from Sigma-Genosys (The Woodlands, TX). Anti-lymphocyte function-associated antigen 1 (LFA-1) (M17/4), anti-CD40 (HM40-3), anti-B7-1 (16-10A1), phycoerythrin (PE)-conjugated anti-B7-1 (16-10A1), and streptavidin were purchased from PharMingen. Anti-ICAM-1 (YN1/1.7.4) was purchased from e-Bioscience (San Diego). PE-conjugated goat anti-mIgG Ab, Rhodamine Red-X-conjugated goat anti-hamster Ab, and horseradish peroxidase-conjugated streptavidin were purchased from Jackson ImmunoResearch. Goat anti-Hamster Ab was purchased from Caltag Laboratories (Burlingame, CA). Anti-clonotypic 2C TCR (1B2) (15), anti-L^d (30-5-7) (16), anti-mCD8 (3.168), and anti-CD4 (RL172) mAbs were prepared in our laboratory as ascites fluid. The biotinylated 1B2 mAb was prepared in our laboratory. Anti-IL-2 (JES6-1A12), biotinylated anti-IL-2 (JES6-5H4), anti-IFN-γ (R4-6A2), and biotinylated anti-IFN-γ (XMG1.2) were purchased from PharMingen for ELISA. Dynabeads M-450 coated with sheep anti-rat IgG was purchased from Dynal. Recombinant IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF) were purchased from e-Bioscience.

Preparation of T Cells and Dendritic Cells. Purified CD8$^+$ T cells were prepared from lymph nodes by using a mixture of mAbs plus complement followed by removal of dead cells as described (17). T cells were cultured in RPMI medium supplemented with 10% heat-inactivated FCS, 10 mM Hepes, glutamin, antibiotics, and 2-Me (5×10^{-5} M). BALB/c DCs were generated from bone marrow cells as described (6), with some modifications. Briefly, bone marrow cells were treated with anti-CD8 (3.168)

Abbreviations: APC, antigen-presenting cell; CFSE, 5,6-carboxyfluorescein diacetate succinimyl ester; CTL, cytotoxic T lymphocyte; DC, dendritic cell; Dros, *Drosophila*; ICAM-1, intercellular adhesion molecule 1; LFA-1, lymphocyte function-associated antigen 1; TCR, T cell antigen receptor.

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and anti-CD4 (RL172) mAbs plus complement to remove T cells and then cultured in RPMI medium overnight. The nonadherent cells were cultured in fresh medium containing IL-4 (10 ng/ml) and GM-CSF (1,000 units/ml) for 7 days to prepare immature DCs; supernatants from these cultures were used to prepare membrane vesicles. For activation of DCs, immature DCs were resuspended in PBS and were treated with anti-mCD40 mAb plus goat anti-hamster Ab for 2 days (18), followed by removal of culture supernatant to prepare membrane vesicles.

Preparation of Membrane Vesicles and Peptide Loading. Dros cells were first cultured with $CuSO_4$ (1 mM) at room temperature to induce expression of transfected mouse molecules (14, 19). After 2 days of induction, culture supernatant was centrifuged at $2,000 \times g$ for 30 min to remove cell debris, followed by passage through a Nalgene filter unit (0.45-μm pore size), and then ultracentrifuged at $100,000 \times g$ to pellet membrane vesicles; the pellet was resuspended in buffer (10 mM Hepes, pH 7.5/100 mM NaCl). In some experiments, suspensions of membrane vesicles were filtered by using Whatman syringe filters with different pore sizes (0.8, 0.4, or 0.2 μm) to remove aggregates that may have formed during the ultracentrifugation step. The concentration of membrane vesicles was determined by measuring the protein concentration of the suspension with a Bio-Rad DC Protein Assay kit. The same procedure was used to prepare membrane vesicles from culture supernatant of DCs. For peptide loading, membrane vesicles were cultured with graded concentrations of peptide (usually 1–10 μM) for 2 h at room temperature and then added to T cells.

***In Vitro* Binding Assays.** For experiments involving direct T/APC interaction, 1×10^6 purified $CD8^+$ T cells were incubated with 1×10^6 peptide-loaded Dros APCs in a volume of 0.5 ml for 1 h at 37°C in a 24-well plate, then stained with mAbs on ice for fluorescence-activated cell sorting (FACS) analysis. For experiments where T cells and APCs were separated in transwells, 1×10^6 purified $CD8^+$ T cells in a 24-well plate were incubated with 4×10^6 Dros APCs placed separately in the transwell for 1 h at 37°C, followed by mAb staining of T cells for FACS analysis.

For experiments using membrane vesicles prepared from culture supernatant, 1×10^5 purified $CD8^+$ T cells were incubated with 20 μg of peptide-loaded membrane vesicles in a volume of 0.1 ml for 40 min at 37°C in a 96-well plate. After incubation, T cells were washed by centrifugation and stained with mAbs on ice for fluorescence-activated cell sorting analysis. In the experiments using mAbs and drugs for inhibition, $CD8^+$ T cells were pretreated with the mAb or drugs for 1 h before use.

***In Vitro* Stimulation of 2C $CD8^+$ T Cells.** In experiments using intact Dros cells as APCs, 5×10^4 purified $CD8^+$ T cells were incubated with 2.5×10^4 Dros APCs in a 96-well plate (14). When membrane vesicles were used for stimulation, 5×10^4 purified $CD8^+$ T cells were incubated with varying concentrations of membrane vesicles in a final volume of 0.1 ml. After 24 h, 100 μl of fresh warm medium was added to the cultures for further incubation. [^{3}H]thymidine (^{3}HTdR) at 1 μCi/ml (1 Ci = 37 GBq) was added to the cultures 8 h before harvest. CFSE labeling of the cells was performed as described (20).

ELISA and Cytotoxic T Cell Killing Assay. Cells were cultured as for proliferation assessment. For measuring cytokine production, culture supernatants were initially stored at −20°C, then thawed and used for ELISA as described (21); recombinant mIL-2 and mIFN-γ were used as standards. For the cytotoxic T lymphocyte (CTL) killing assay, activated T cells were collected after 60 h of culture and used in the JAM assay (22). As a target, ^{3}H-labeled P815 tumor cells (1×10^4 per well) were used.

Results

Recent studies showed that, during T/APC interaction, T cells were able to rapidly absorb molecules from APCs and then internalize the absorbed ligands (17, 23–25). This process was ligand specific and could be mediated either by TCR/MHC/peptide or CD28/B7 interaction, although not by LFA-1/ICAM-1 interaction. Because both specific and "bystander" molecules on APCs were absorbed, T cells appeared to absorb APC-derived molecules as small membrane blebs, these structures being pinched off the surface of APCs during T/APC conjugate formation. Surprisingly, however, preliminary experiments showed that T cells displayed weak but significant absorption of APC molecules under conditions where cell/cell interaction was prevented, i.e., after separation of T cells from APCs in transwells (24).

In the experiments described below, we determined which particular cell-surface molecules are involved in TCR-mediated absorption of molecules from APCs during T/APC culture in transwells. As T cells, purified $CD8^+$ 2C TCR transgenic cells on an H-2^b background were used (14, 26). These cells are reactive to several MHC class I L^d-associated peptides, including the strong QL9 peptide and the weaker p2Ca peptide (27, 28). These peptides are not recognized by naïve 2C cells in association with autologous K^b or D^b molecules, thereby eliminating the possibility of cross-presentation of these peptides by T/T interaction between 2C cells. To avoid the possibility of absorption via CD28/B7 interaction (see above), in some experiments we used 2C cells on a $CD28^{-/-}$ background. For naïve 2C cells, however, absorption via CD28/B7 interaction was usually so low that normal 2C cells and $CD28^{-/-}$ 2C cells could be used interchangeably for TCR-mediated absorption. As APCs, we used Dros cells transfected with L^d plus B7-1 (L^d.B7-1 APC), L^d plus ICAM-1 (L^d.B7.ICAM-1 APC), or L^d plus B7-1 plus ICAM-1 (L^d.B7-1.ICAM-1 APC) loaded with 10 μM QL9 peptide. We also used DCs as APCs.

T Cell Absorption of APC Molecules Through Transwells Requires ICAM-1. Confirming previous findings (17), strong 2C $CD8^+$ cell uptake of both B7-1 and L^d occurred when 2C cells were in direct contact (intact APCs) with either L^d.B7-1 or L^d.B7-1.ICAM-1 Dros APCs (Fig. 1*a*); no uptake occurred in the absence of QL9 peptide or with APCs lacking L^d (data not shown). When 2C cells were separated from L^d.B7-1.ICAM-1 Dros APCs in transwells, weak but significant uptake of both B7-1 and L^d by 2C cells occurred, although only with addition of QL9 peptide (Fig. 1*a*; data not shown). Uptake of B7-1 and L^d through transwells was most noticeable with activated 2C cells (cells pretreated with PMA plus ionomycin), although uptake was also clearly apparent with naïve cells (Fig. 1*a Upper*). Significantly, uptake via transwells was abolished by adding anti-LFA-1 mAb to the cultures (Fig. 1*a Lower*), implying a crucial role for LFA-1/ICAM-1 interaction in absorption. In support of this notion, absorption via transwells occurred only with L^d.B7-1.ICAM-1 APCs and not with L^d.B7-1 APCs (Fig. 1*a*). For T cell absorption after direct T/APC interaction (intact APCs), by contrast, LFA-1/ICAM-1 interaction was irrelevant (Fig. 1*a*).

Based on the above findings, TCR-mediated uptake of APC-derived molecules by 2C cells was critically dependent on LFA-1/ICAM-1 interaction, but only when absorption occurred via transwells and not after direct T/APC interaction. Because passage of APC-derived molecules through transwell membranes was probably quite limited, for further studies we prepared purified material from Dros cell supernatants.

Purification of Soluble Membrane Vesicles from Dros APCs. Using a protocol described for preparing exosomes from mammalian cells (5, 6), 48-h supernatants from viable Dros APCs (cells

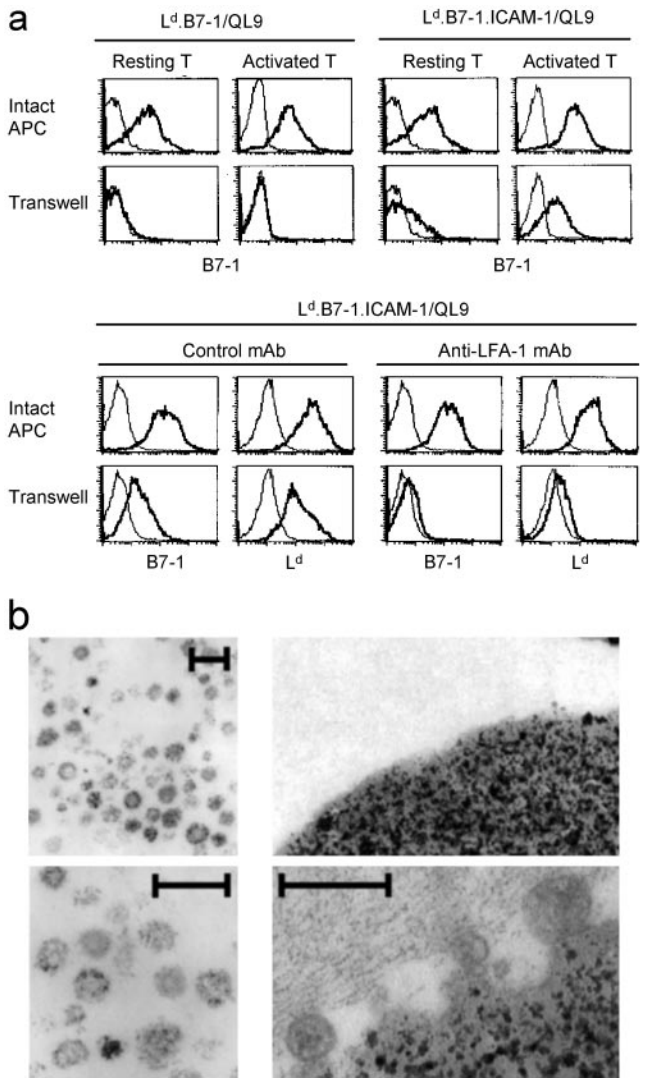

Fig. 1. TCR-mediated absorption of membrane vesicles. (*a*) Absorption of B7-1 by purified naïve or activated $CD8^+$ 2C cells after incubation for 1 h with L^d.B7-1 or L^d.B7-1.ICAM-1 Dros APCs loaded with QL9 peptide at 10 μM; activated T cells were prepared by preculturing cells for 12 h with PMA plus ionomycin. T cells and APCs were either cultured together (intact APCs) or separated from each other by placing APCs in a transwell (pore size 3 μm); Dros cells are large (>20 μm) and were unable to pass through the Transwell membrane. After culture, T cells were stained for B7-1, L^d, and CD8 and then examined by flow cytometry. The data show staining of gated $CD8^+$ cells. (*a Upper*) B7-1 staining of resting vs. activated 2C cells after culture with L^d.B7-1 vs. L^d.B7-1.ICAM-1 Dros APCs. (*a Lower*) B7-1 and L^d staining of activated 2C cells cultured with L^d.B7-1.ICAM-1 Dros APCs in the absence or presence of anti-LFA-1 mAb (5 ng/ml). (*b*) Morphology of membrane vesicles. Culture supernatants from L^d.B7-1.ICAM-1 Dros APCs were depleted of cell debris, then ultracentrifuged. (*Left*) Electron microscopic view of pelleted material is shown at low (*Upper*) and high (*Lower*) magnification. (Bar, 200 nm.) Much of the material in the pellet shows the morphology of membrane vesicles; cell debris, more prominent in other fields, is also present. (*Right*) Electron microscopic view of magnetic beads that were coated with anti-ICAM-1 mAb (*Lower*) or an Ig isotype-matched control mAb (*Upper*) before incubation with the above membrane vesicles.

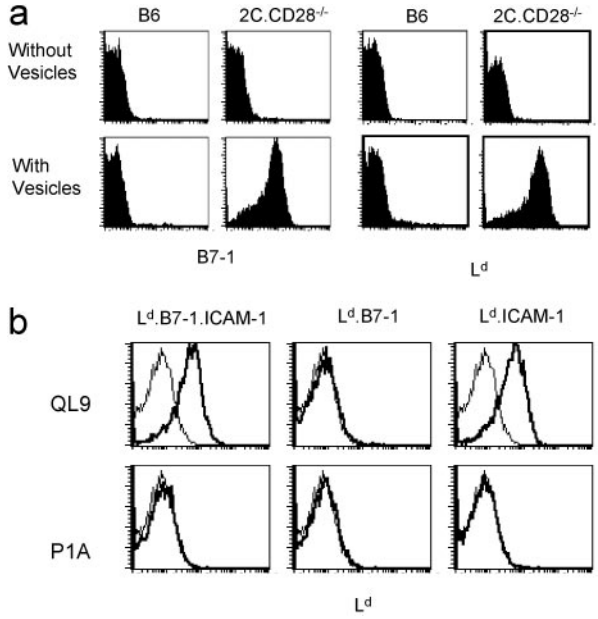

Fig. 2. TCR-mediated absorption of membrane vesicles requires LFA-1/ICAM-1 interaction. (*a*) Absorption of QL9-loaded L^d.B7-1.ICAM-1 Dros APCs membrane vesicles by resting B6 and 2C.$CD28^{-/-}$ $CD8^+$ cells. T cells were incubated for 45 min with 200 μg/ml L^d.B7-1.ICAM-1 membrane vesicles that had been loaded with 10 μM QL9 peptide; control cells were incubated in the absence of vesicles. The T cells were then washed, stained for B7-1, L^d, and CD8, and examined by flow cytometry. The data show representative staining for B7-1 and L^d on gated $CD8^+$ cells. (*b*) 2C cell absorption of vesicles derived from L^d.B7-1.ICAM-1, L^d.B7-1, and L^d.ICAM-1 Dros APCs. 2C cells on a normal (not a $CD28^{-/-}$) background were incubated with membrane vesicles and then stained as for *a*; vesicles were loaded either with QL9 or control PIA peptide at 10 μM. The data show representative staining for L^d on gated $CD8^+$ cells after incubation with vesicles derived from the indicated Dros cells.

cultured at room temperature) were lightly centrifuged to remove cells and cell debris and then ultracentrifuged at 100,000 × *g*; protein yields in the pellets were 1–3 μg per 10^6 cells. By electron microscopy, the pelleted material consisted of a relatively homogeneous preparation of small (≈100 nm) particles resembling membrane vesicles (Fig. 1*b Left*). When derived from L^d.B7-1.ICAM-1 Dros APCs, the vesicles prepared from these cells adhered to beads coated with anti-ICAM-1 mAb but not to uncoated beads (Fig. 1*b Right*). Hence, at least for ICAM-1, the vesicles expressed the transfected molecules. Based on morphology, the vesicles released from Dros APCs closely matched the description of typical exosomes derived from mammalian cells (8). To be conservative, however, we will refer to the material released from Dros APCs as membrane vesicles rather than exosomes.

T Cell Binding of Purified Membrane Vesicles. To measure T cell binding, membrane vesicles were first loaded with peptides by culturing purified vesicles with QL9 or control P1A peptides for 2 h and then added to purified 2C T cells. Because these peptides do not bind to the K^b and D^b molecules on 2C cells (see above), peptide-loaded vesicles were added to 2C cells without prior removal of unbound peptide.

In contrast to the weak absorption seen in transwell cultures, incubating either normal 2C $CD8^+$ or $CD28^{-/-}$ 2C $CD8^+$ cells with QL9-loaded purified soluble membrane vesicles released from L^d.B7-1.ICAM-1 Dros APCs led to strong T cell uptake of B7-1 and L^d, both for naïve 2C (Fig. 2*a*) and activated 2C cells (data not shown); no uptake occurred with control normal B6 $CD8^+$ cells (Fig. 2*a*). Binding to 2C cells was dose dependent and reached a plateau with high concentrations of vesicles. Uptake of L^d (Fig. 2*b*) and B7-1 (data not shown) by 2C cells also occurred with vesicles prepared from L^d.ICAM-1 Dros APCs but not with vesicles from L^d.B7-1 Dros APCs, thus confirming that binding required LFA-1/ICAM-1 interaction but not CD28/B7-1 interaction; note that normal ($CD28^+$) 2C cells, rather than 2C.$CD28^{-/-}$ cells, were used

in Fig. 2*b*. For both L^d.ICAM-1 and L^d.B7-1.ICAM-1 Dros APCs, absorption by 2C cells was peptide specific, being strong with QL9 peptide but undetectable with control P1A peptide (Fig. 2*b*); this latter peptide binds strongly to L^d but is not recognized by the 2C TCR (17, 29).

T Cell Activation by Vesicles Requires Both B7 and ICAM-1. As shown in Fig. 3*a*, normal ($CD28^+$) 2C $CD8^+$ cells gave undetectable proliferative responses to QL9-loaded vesicles prepared from either L^d.B7-1 or L^d.ICAM-1 Dros APCs. In marked contrast, strong proliferation occurred with L^d.B7-1.ICAM-1 vesicles; the response was QL9 peptide specific and undetectable with P1A peptide (Fig. 3*a*; data not shown). Thus, in contrast to binding, the capacity of vesicles to elicit T cell proliferation required coexpression of ICAM-1 and B7. Further evidence that both of these ligands were essential for proliferation is shown in Fig. 3*b*. Here it can be seen that, for responses elicited by L^d.B7-1.ICAM-1 vesicles, proliferative responses of normal 2C $CD8^+$ cells were abolished by addition of anti-LFA-1 mAb and that, unlike normal 2C cells, $CD28^{-/-}$ 2C cells were totally unresponsive to the vesicles. Note that, in these and other experiments, passing the purified vesicles through filters of pore size as small as 0.2 μm failed to reduce the extent of proliferation. It should be emphasized that 2C cells were totally unresponsive to free QL9 peptide and also to QL9-loaded vesicles from K^b.B7-1.ICAM-1 APCs (data not shown). These findings indicate that stimulation by QL9-loaded L^d.B7-1.ICAM-1 vesicles is mediated by the vesicles *per se* rather than by nonbound peptide in the vesicle preparations.

With regard to kinetics, proliferative responses elicited by L^d.B7-1.ICAM-1 vesicles were of short duration, reaching a peak on day 2 and then declining abruptly (Fig. 3*c*). These kinetics contrasted with the protracted response elicited by intact APCs (Fig. 3*c*). As measured by CFSE labeling, however, proliferation elicited by vesicles was extensive, i.e., up to eight divisions by 63 h (Fig. 3*d*); proliferation at 15 h was undetectable. The brevity of the response to membrane vesicles may simply reflect that the vesicles are rapidly destroyed by proteases in the culture medium. In support of this idea, prolonged proliferative responses occurred when vesicles were added to the cultures at daily intervals (rather than as a single dose on day 0; data not shown).

In the above experiments, relatively high concentrations of vesicles, i.e., 100 μg of total protein per ml (10 μg per well), were added to naïve 2C cells. Titration experiments showed that proliferation of naïve T cells fell to low levels when the concentration of vesicles was reduced to <10 μg/ml (Fig. 3*e*; data not shown). With short-term (30 min) pretreatment of naïve T cells with PMA, 5- to 10-fold lower concentrations of vesicles elicited strong proliferation (Fig. 3*e*), presumably because PMA treatment enhanced LFA-1/ICAM-1 interaction.

In the preceding experiments, vesicles were loaded with a high concentration of QL9 peptide (10 μM). Strong proliferative responses also occurred when the concentration of QL9 peptide was reduced to 0.1 μM; with QL9 at 0.001 μM, responses fell to undetectable levels (Fig. 3*f*). It should be noted that quite strong proliferative responses occurred when vesicles were pulsed with p2Ca peptide (Fig. 3*f*), which is a much "weaker" peptide for 2C cells than QL9 (28).

Vesicles Derived from Dendritic Cells. To assess whether the above data were relevant to normal APCs, we prepared membrane vesicles from DCs. Using standard procedures, immature DCs were prepared by culturing BALB/c (L^d) bone marrow cells with granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 for 1 week *in vitro*; subsequently, some of the immature DCs were cultured for 2 days with anti-CD40 mAb, thereby generating mature DCs. Supernatants from immature and mature DCs were then ultracentrifuged to prepare membrane

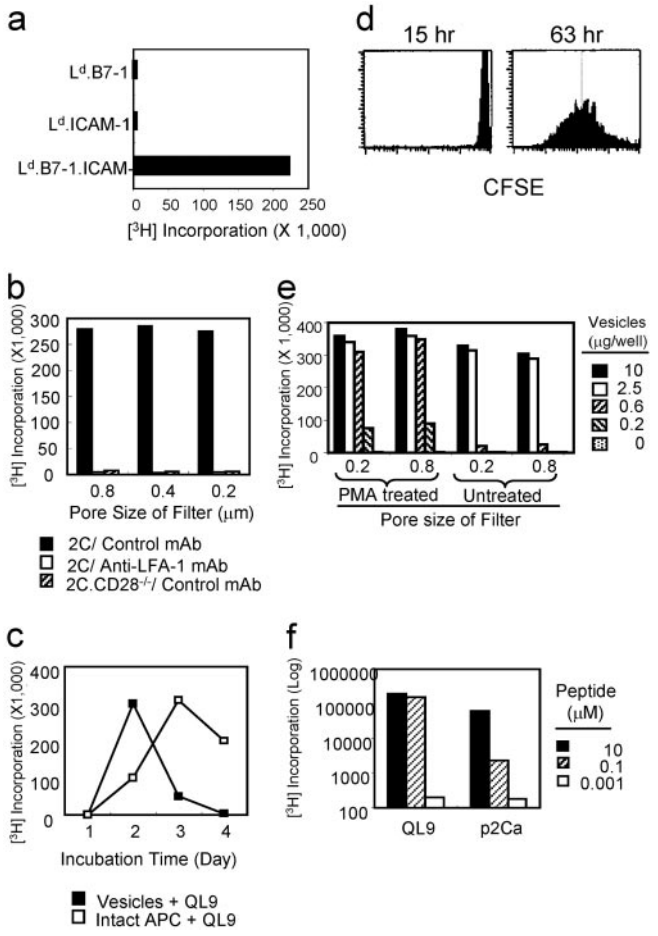

Fig. 3. Accessory molecules required for proliferative responses of naïve 2C cells to peptide-loaded membrane vesicles. (*a*) Purified resting normal ($CD28^+$) 2C $CD8^+$ T cells (5×10^4 per well) were cultured with membrane vesicles (100 μg/ml, 10 μg per well) purified from L^d.B7-1, L^d.ICAM-1, and L^d.B7-1.ICAM-1 Dros APCs loaded with 10 μM QL9 peptide in a 96-well plate. The cultures were pulsed with ^{3}HTdR after 40 h, and ^{3}HTdR incorporation was measured after 48 h. The data show mean cpm values for triplicate cultures; SDs were very small and are not shown. (*b*) Purified normal 2C or 2C.$CD28^{-/-}$ $CD8^+$ T cells were cultured with QL9-loaded L^d.B7-1.ICAM-1 membrane vesicles as described in *a*. For mAb blocking, 2C T cells were preincubated with 5 μg/ml anti-LFA-1 mAb or anti-CD45 mAb (as an isotype control mAb) for 30 min on ice before culture, and the mAbs were left in the cultures during incubation at 37°C. For size fractionation of resuspended vesicles after ultracentrifugation, solutions of membrane vesicles were filtered by using syringe filters with the pore sizes indicated. After filtration, protein concentrations in the filtrates were measured and, after loading with QL9 peptide (10 μM), the vesicles were added to the cultures at 10 μg per well. As in *a*, the data show mean levels of ^{3}HTdR incorporation (cpm) for triplicate cultures. (*c*) Purified resting 2C T cells (5×10^4 per well) were cultured with intact L^d.B7-1.ICAM-1 Dros APCs (loaded with 0.01 μM QL9; see *Materials and Methods*) or with purified L^d.B7-1.ICAM-1 membrane vesicles (loaded with 10 μM QL9) at 100 μg/ml. The cultures were pulsed with ^{3}HTdR for 8 h before harvest. The data show ^{3}HTdR incorporation (mean cpm of triplicate cultures) on days 1–4 of culture. (*d*) CFSE-labeled purified resting normal 2C $CD8^+$ T cells were cultured with L^d.B7-1.ICAM-1 membrane vesicles as described in *a* for 15 or 63 h and then stained with CD8 mAb. Histograms show CFSE staining on gated $CD8^+$ T cells. The data are representative of several experiments. (*e*) PMA-treated or untreated purified 2C T cells (5×10^4 per well) were cultured with various concentrations of membrane vesicles (L^d.B7-1.ICAM-1) loaded with 10 μM QL9 peptide for 48 h. For PMA treatment, resting 2C T cells were incubated with PMA (50 ng/ml) for 30 min at 37°C and then washed thoroughly; as in *b*, suspensions of membrane vesicles were filtered by using syringe filters with the indicated pore sizes before addition to 2C cells. As in *a*, the data show mean cpm values for ^{3}HTdR incorporation on day 2 of culture. (*f*) Resting purified normal 2C T cells were cultured as in *a* with 100 μg/ml L^d.B7-1.ICAM-1 membrane vesicles; vesicles were loaded with QL9 or p2Ca peptide at the concentrations shown. ^{3}HTdR incorporation was measured on day 2.

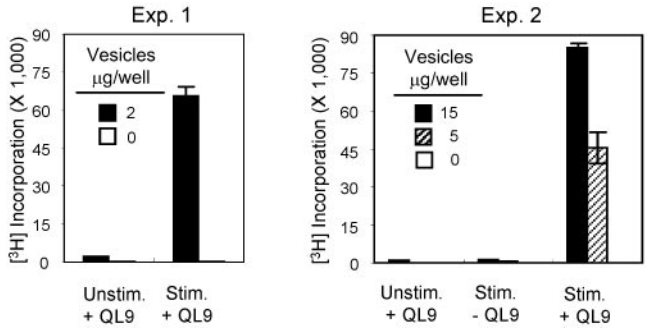

Fig. 4. Proliferative responses of naïve 2C CD8$^+$ cells to membrane vesicles secreted by immature vs. mature BALB/c DCs. Culture supernatants prepared from immature (Unstim.) and mature (Stim.) BALB/c DCs (*Materials and Methods*) were ultracentrifuged to prepare membrane vesicles and then loaded (+QL9) or not loaded (−QL9) with QL9 peptide (10 μM). In the two experiments shown, purified 2C CD8$^+$ cells (5×10^4 per well) were cultured with membrane vesicles at the concentration indicated. The data show mean levels of ^{3}HTdR incorporation (cpm) for triplicate cultures.

vesicles. The immunogenicity of DC vesicles was assessed by examining their capacity to induce proliferation of purified naïve 2C CD8$^+$ cells.

With vesicles prepared from immature (Fig. 4, Unstim.) DCs, proliferative responses of 2C CD8$^+$ cells were very low, even with addition of QL9 peptide. Quite different results were found with vesicles from mature (Fig. 4, Stim.) DCs. Thus, these vesicles elicited strong proliferative responses, although only with addition of exogenous QL9 peptide. The finding that the immunogenicity of DC vesicles was far higher for mature than immature DCs is consistent with the evidence that mature DCs have much higher expression of costimulatory/adhesion molecules than immature DCs.

Generation of Effector Function. In addition to stimulating proliferation, membrane vesicles from APCs were able to induce differentiation of naïve 2C cells into effector cells, both for Dros APCs (Fig. 5) and DCs (data not shown). Thus, when loaded with QL9 peptide, vesicles from L^d.B7-1.ICAM-1 Dros APCs caused naïve 2C CD8$^+$ cells to synthesize IFN-γ (Fig. 5*a*) and IL-2 (Fig. 5*b*) and to differentiate into CTL (Fig. 5*c*). Effector cell generation was also apparent with vesicles loaded with p2Ca peptide, although responses were clearly weaker with p2Ca than QL9 peptide (Fig. 5*c*). For CTL generation, responses were abolished by addition of 1B2 anti-clonotypic mAb to the cultures (Fig. 5*c*).

Discussion

The key conclusion from the above experiments is that exosome-like membrane vesicles shed by APCs can be directly immunogenic for purified naïve CD8$^+$ cells. Both for normal APCs (mature DCs) and transfected Dros cells, membrane vesicles from APCs lead to strong proliferative responses and differentiation into effector cells. Such stimulation is highly ligand specific and requires three different receptor/ligand interactions, namely a combination of TCR/MHC/peptide, LFA-1/ICAM-1, and CD28/B7 interactions. For stimulation, the data support a two-step model where vesicles are first bound to T cells by the combined effects of TCR/MHC/peptide and LFA-1/ICAM-1 interactions; B7-1 on the vesicles then interacts with CD28 and delivers a unique "second signal," thus inducing T cell activation and proliferation. The implication therefore is that, for responses to membrane vesicles, the functions of LFA-1 and CD28 are quite distinct: LFA-1 acts solely as an adhesion molecule and CD28 solely as a costimulatory molecule. Paradoxically, this functional distinction between LFA-1 and CD28 is far less clear for responses directed to intact APCs (11, 14, 30).

For DCs, it is notable that membrane vesicles from mature L^{d+} DCs were only directly immunogenic for 2C cells when pulsed with exogenous peptide. With intact L^{d+} APCs, by contrast, presentation of endogenous peptides by these cells leads to strong proliferation of 2C cells in the absence of exogenous peptide (27, 28). This discrepancy may reflect that preparation of membrane vesicles/exosomes leads to rapid dissociation of preexisting peptide/MHC complexes. Alternatively, membrane vesicles may be intrinsically less immunogenic than whole APCs, e.g., because certain costimulatory/adhesion molecules are poorly represented on membrane vesicles.

Our finding that membrane vesicles can be directly immunogenic for naïve CD8$^+$ cells contrasts with the report that stimulation of naïve CD4$^+$ cells by peptide-pulsed vesicles from a DC line, D1, required the presence of viable APCs (9). This discrepancy could reflect an essential difference between CD4$^+$ and CD8$^+$ cells. Alternatively, the density of ICAM-1 and/or B7 on exosomes from D1 cells could be too low to induce direct T cell activation.

As *in vitro*, we have found that peptide-pulsed L^{d+} vesicles are strongly immunogenic for naïve 2C CD8$^+$ cells transferred to B6

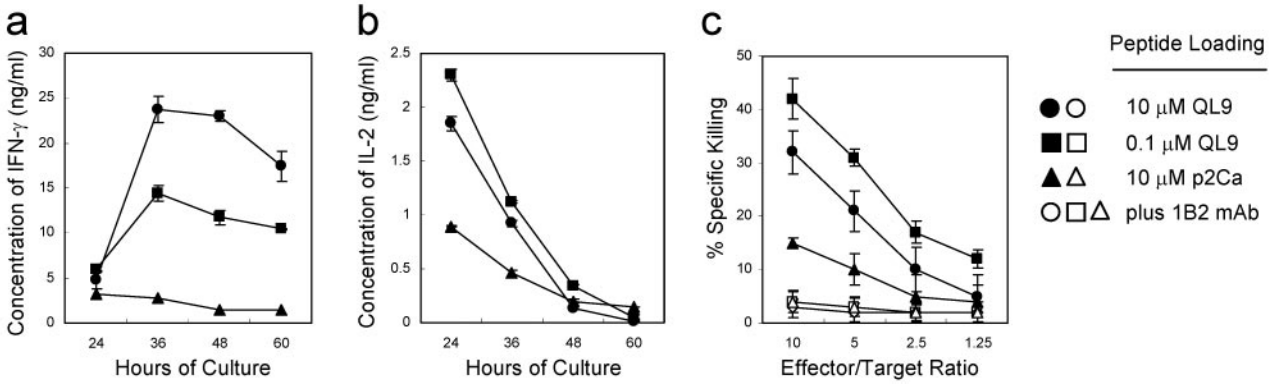

Fig. 5. Effector functions of 2C CD8$^+$ cells stimulated with Dros APC membrane vesicles. As for proliferation, 2C CD8$^+$ cells at 5×10^4 cells per well were stimulated with 100 μg/ml L^d.B7-1.ICAM-1 membrane vesicles loaded with QL9 or p2Ca peptides at the concentrations shown. Concentrations of IFN-γ (*a*) and IL-2 (*b*) in culture supernatants collected at 24–60 h are shown; cytokines were measured by ELISA. The data represents means (±SD) of triplicate cultures. (*c*) CTL activity of 2C cells cultured with vesicles as above for 60 h. The data show mean percent of lysis (±SD) of P815 (L^d) target cells for triplicate cultures; lysis was measured over 4 h. In some cultures, the activated 2C cells were preincubated with 1B2 anti-clonotypic mAb (10 μg/ml). Note that P815 cells were not supplemented with exogenous peptide.

hosts (data not shown). This finding also applies in MHC class $I^{-/-}$ hosts, indicating that processing by host APCs is not required. To be immunogenic *in vivo*, however, the vesicles do not need to express B7 or ICAM-1; only expression of MHC (L^d) and specific peptide (QL9) are essential. These findings are in line with a model where, under *in vivo* conditions, vesicles are rapidly absorbed to the surface of host APCs (9); T cells then react to preformed peptide/MHC complexes on the APC-bound vesicles and receive costimulation from endogenous B7 and ICAM-1 molecules on the APCs. In support of this model we have found that the failure of 2C $CD8^+$ cells to respond to vesicles lacking B7 and ICAM-1 *in vitro* can be overcome by adding MHC class $I^{-/-}$ APCs (data not shown).

With regard to physiological relevance, we have found that the direct immunogenicity of peptide-pulsed membrane vesicles is not unique to secreted exosomes but also applies to vesicles prepared from sonicated APCs (unpublished data). This finding could explain the paradox that proliferative responses to pathogens *in vivo* can continue for a week or more even though differentiation of T cells into CTL is presumed to cause rapid destruction of APCs (30–32). Here, one can envisage that late primary responses are driven by subcellular material released from killed APCs. On a practical point, we have found that, as *in vitro*, peptide-pulsed vesicles injected *in vivo* lead to strong production of effector cells that cause rapid destruction of tumor cells (unpublished data). Hence appropriately engineered vesicles could be used therapeutically as a vaccine for tumor rejection and/or memory cell generation.

In conclusion, we show here that peptide/MHC complexes expressed on membrane vesicles can be directly immunogenic for naïve $CD8^+$ cells *in vitro*; stimulation occurs in the absence of APCs and is independent of exogenous cytokines or mAb ligation. For direct immunogenicity, the key requirement is that the vesicles coexpress both B7 and ICAM-1 in addition to peptide/MHC.

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