Direct stimulation of T cells by membrane vesicles from antigen-presenting cells

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Activation of naïve T cells generally requires T cell receptor-mediated contact with MHC-bound peptides on viable antigen-presenting cells such as dendritic cells (DC). Here evidence is presented that dissociated cell membrane fragments from a DC line can be used as an effective substitute for viable DC. Ultracentrifuged material derived from sonicates of IFN-γ-matured DC is enriched in small membrane vesicles that closely resemble exosomes. When complexed with MHC class I-restricted specific peptide, vesicles from DC sonicates generate strong responses by purified naïve CD8+ cells in vitro in the absence of normal antigen-presenting cells and can also efficiently prime T cells for tumor rejection in vivo. Both in terms of total yields from DC and relative immunogenicity, membrane vesicles from DC sonicates are much more effective than classic exosomes and may be a valuable tool for tumor immunotherapy.

Results

Preparation of Membrane Vesicles. To prepare membrane vesicles from DC2.4 cells, cells were disrupted with a Dounce homogenizer. After removal of nuclei by light centrifugation, supernatants were sonicated and then centrifuged at 10,000 × g. Thereafter, the supernatants were subjected to ultracentrifugation (100,000 × g) for 1 h. Electronmicroscopic examination of the pellet material showed a heterogeneous mixture of membrane fragments and small organelles. Based on examining multiple sections throughout the pellet, approximately one-third of the material had the morphology of small (50- to 100-nm) round membrane vesicles (Fig. LA Right). Of the remaining material, the lighter (upper) portion of the pellet contained ribosomes and small irregular membrane fragments whereas the heavier (lower) portion consisted mostly of larger membrane fragments. Interestingly, the round membrane vesicles closely resembled classic exosomes released from intact DC2.4 cells (Fig. 1A Left). For the functional studies discussed below, pellets of ultracentrifuged membrane vesicles from DC2.4 sonicates and DC2.4 exosomes were resuspended in saline.

Expression of Costimulatory Molecules. Surface expression of MHC class I and costimulatory molecules on intact DC2.4 cells was only modest but became conspicuous after overnight incubation with various Toll-like receptor agonists or IFN-γ (data not shown). IFN-γ treatment was particularly effective, and, unless stated otherwise, all DC2.4 cells used to prepare membrane vesicles from cell sonicates were preincubated overnight with IFN-γ. As shown by labeling of DC2.4 cells with carboxyfluorescein succinimidyl ester (CFSE) before sonication, the membrane vesicles from the cells expressed MHC class I (data not shown) as well as several costimulatory/adhesion molecules, including CD54, CD80, and CD86 (Fig. 1B).

Binding to T Cells. As found previously for exosomes (23, 24), naïve CD8+ T cells were able to bind membrane vesicles from DC2.4 cells in vitro but only in the presence of specific peptide. Thus, naïve 2C TCR transgenic CD8+ T cells, which have specificity for MHC class I Kb peptide SIYRYYGL (SIYR) peptide, showed strong binding of CFSE-labeled DC2.4 (Kb) vesicles in the presence of

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Abbreviations: TCR, T cell receptor; APC, antigen-presenting cell; DC, dendritic cell; LN, lymph node; CFSE, carboxyfluorescein succinimidyl ester.

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SIYR peptide (vesicles/SIYR) (Fig. 1C). By contrast, uptake of vesicles/SIYR by normal polyclonal B6 CD8+ cells was negligible.

**Immunogenicity of Membrane Vesicles Versus Exosomes.** In previous studies, purified naïve 2C CD8+ cells responded well in the absence of APC to peptide-pulsed exosomes released from transfected *Drosophila* cells and also from normal DC (23). These data applied to 2C responses to MHC class I L4 and the strong QL9 peptide. DC2.4 exosomes and SIYR peptide also elicited proliferation from purified 2C CD8+ cells, albeit at a low level (Fig. 1D). This response correlates with Kb/SIYR being a weaker ligand for the 2C TCR than L4/QL9 (25). Significantly, membrane vesicles prepared from DC2.4 sonicates were strongly stimulatory for 2C CD8+ cells (Fig. 1D).

The key finding in the above experiment is that, in the presence of specific peptide, sonicates from DC2.4 cells were strongly stimulatory for naïve 2C CD8+ cells in *vitro* in the absence of APC. Sonicates were clearly superior to exosomes in two respects. First, in terms of protein concentration, sonicates were more potent than exosomes by a factor of 10- to 30-fold. Second, total yields of immunogenic material per 10^6 cells were ~50-fold higher for sonicates than for exosomes (Fig. 1D, below graph). All of the experiments discussed below refer to vesicles prepared from ultracentrifuged DC2.4 sonicates. For simplicity these preparations are referred to as “vesicles.”

**Features of in Vitro Responses to Vesicles.** Vesicles prepared from unstimulated DC2.4 were poorly immunogenic for 2C cells, as seen by low proliferative responses to SIYR peptide (Fig. 2A). In contrast, vesicles prepared from IFN-γ-induced DC2.4 cells were highly immunogenic for 2C cells, correlating with the higher level of costimulatory molecules on these cells.

The above findings applied to peptide addition after vesicle preparation. Substantial, although lower, responses were elicited by...
IFN-γ-induced DC2.4 cells that were peptide-pulsed before sonication (Fig. 2B). The addition of extra peptide during culture considerably enhanced proliferation of 2C cells in response to the vesicles (Fig. 2B), implying significant elution of Kb-associated peptide during vesicle preparation. In general, adding peptide both before and after vesicle preparation was only slightly more effective than adding peptide just during T cell culture. Therefore, peptide was routinely added to vesicles only during the culture period with T cells.

In most experiments SIYR peptide was added to culture at 0.32 μM. This concentration of peptide was nonstimulatory in the absence of vesicles yet elicited nearly optimal responses in the presence of vesicles (Fig. 2C Upper). Higher concentrations of peptide (≥1 μM) induced proliferation of 2C CD8+ cells in the absence of vesicles, presumably reflecting peptide presentation by the responding cells themselves. With respect to vesicle concentration, optimal proliferative responses of 2C CD8+ cells occurred with vesicles at 10 μg/ml for both [3H]thymidine incorporation and total yields of live cells (Fig. 2C Lower). However, proliferation was observed with concentrations of vesicles as low as 0.3 μg/ml.

Stimulation of CD8+ cells by vesicles was strongly peptide-specific. Thus, 2C cells responded well to vesicles/SIYR but not to vesicles/SIINFEKL peptide (Fig. 2D Upper). Conversely, OT-1 CD8+ cells responded to vesicles/SIINFEKL but not to vesicles/SIYR (Fig. 2D Lower).

Because DC2.4 cells express a variety of costimulatory/adhesion molecules, it was of interest to determine which of these molecules were important during vesicle stimulation of 2C cells. Proliferative responses to vesicles/SIYR were blocked or greatly reduced by anti-CD11a mAb (Fig. 2E), indicating the importance of both CD28/B7 and lymphocyte function-associated antigen 1 (LFA-1)/CD54 interactions. Inhibition by anti-CD2 mAb was minimal, suggesting little or no contribution from CD2. As expected, proliferation was abolished by 1B2 anticoniotypic mAb and also by anti-CD8 mAb.

In the above experiments the responding T cells were highly purified and depleted of APC; implying that when loaded with specific peptide the vesicles were directly immunogenic for 2C cells. Hence, it was of interest to compare the response of 2C CD8+ cells to vesicles versus intact APC. This comparison is shown in Fig. 3. Here 2C CD8+ cells were cultured with titrated doses of vesicles/SIYR versus titrated numbers of intact IFN-γ-induced DC2.4 cells/SIYR. In both situations, 2C responses generally reached a peak on day 3 of culture. With intact DC2.4 cells/SIYR as APC, optimal proliferative responses required 6 x 10^5 cells per milliliter (1 x 10^5 per well). With vesicles/SIYR, comparable responses occurred with 3 μg/ml vesicles. These findings applied to [3H]thymidine incorporation, CFSE dilution, cell viability, and fold expansion of the responding T cells (Fig. 3 and data not shown). With SIYR peptide alone there was significant proliferation of a proportion of 2C cells, but most of the responding cells were nonviable by day 3 of culture (Fig. 3).

The above findings refer to T cell proliferation. Similar results were observed for differentiation of 2C cells into effector cells (Fig. 4). Thus, for vesicles/SIYR at 10 μg/ml and intact DC2.4 cells at 6.25 x 10^4 per milliliter, comparable 2C responses occurred with regard to IL-2 synthesis (Fig. 4A), IFN-γ synthesis (Fig. 4B), granzyme B synthesis (Fig. 4C), and lysis of peptide-loaded target cells (P815 cells expressing Ld plus QL9 peptide) (Fig. 4D).

Based on the above findings we conclude that vesicles plus peptide are strongly stimulatory for naive CD8+ cells by all parameters measured. Qualitatively, responses to vesicles versus intact APC were indistinguishable.

**Fig. 3.** Comparison of the stimulatory activity of membrane vesicles versus intact DC2.4 cells. The data show proliferation of 2C CD8+ cells cultured with graded doses of vesicles plus 0.32 μM SIYR peptide (vesicles/SIYR), graded doses of intact irradiated IFN-γ-induced DC2.4 cells plus 0.32 μM SIYR (cells/SIYR), or graded amounts of SIYR without vesicles (SIYR alone). Proliferation was measured by [3H]-incorporation at 24–96 h (A), CFSE dilution of 2C cells at 72 h (B), 2C cell viability at 72 h (C), and fold expansion of 2C cells at 72 h (D) (relative to the number of cells initially cultured) in response to 3-fold dilutions of vesicles/SIYR (32 to 0.32 μg/ml) and 2-fold dilutions of cells/SIYR (60 to 3.9 x 10^5 per milliliter), or 3-fold dilutions of SIYR alone (10 to 0.1 μM). 2C cells were cultured at 5 x 10^4 per well in 0.2-ml wells (A and D) or at 2 x 10^5 per well in 1-ml wells (B and C).

**Fig. 4.** Development of effector function of 2C CD8+ cells stimulated by membrane vesicles versus intact DC2.4 cells. Purified 2C CD8+ cells were stimulated with vesicles (10 μg/ml) plus 0.32 μM SIYR, intact irradiated IFN-γ–induced DC2.4 cells (6.25 x 10^4 per milliliter) plus 0.32 μM SIYR or with 0.32 μM SIYR alone (A and B). 2C CD8+ cells were cultured at 5 x 10^4 per well, and culture supernatants were collected at 8–72 h to measure IL-2 (A) and IFN-γ (B) by ELISA. (C) Granzyme B synthesis was measured by culturing CFSE-labeled 2C CD8+ cells at 2 x 10^5 per well for 3 days with the above stimuli followed by fixing and permeabilizing the cells before staining for granzyme B. (D) Cells were cultured as for C, washed, and used as effector cells in a 51Cr-release assay with P815 (H-2d) cells pulsed for 1 h with 10 μM QL9 peptide.
vesicles, i.e., proliferative responses were apparent even with low doses of were injected i.v. and vesicles
spleen and LN, which contrasted with almost undetectable prolif-

One day later mice were injected i.v. with 0.2 nmol SIYR or with 240
vesicles plus a fixed amount of 0.2 nmol SIYR peptide or with SIYR

In Vivo Responses. To examine responses in vivo, naïve CFSE-
labeled 2C CD8\(^+\) cells were transferred i.v. into syngeneic B6 mice. One day later the recipients were injected i.v. with graded doses of vesicles plus a fixed amount of 0.2 nmol SIYR peptide or with SIYR alone. As shown in Fig. 5, a modest dose of vesicles (40 \(\mu\)g per mouse) plus peptide led to significant proliferation of TCR clono-
type\(^+\) (1B2\(^+\)) 2C cells as indicated by CFSE dilution and cell

Efficient tumor rejection also applied to T cells that were activated
with membrane vesicles relative to injection of peptide alone as in A. (C) CFSE-labeled 2C CD8\(^+\) cells (Ly5.2) were injected i.v. into B6.Ly5.1 recipients (4 \(\times\) 10\(^6\) per mouse). One day later mice were injected i.v. with 0.2 nmol SIYR or with 240 \(\mu\)g of vesicles plus 0.2 nmol SIYR. Mice were killed 3 days later. Numbers of donor Ly5.2\(^+\) CD8\(^+\) cells in spleen and LN relative to host Ly5.1\(^+\) CD8\(^+\) cells are shown. (D) CFSE versus CD44 expression for donor Ly5.2\(^+\) CD8\(^+\) 2C cells is shown for the same mice as in C. For C and D there were two mice per group. (E) CFSE-labeled Thy1.2\(^-\)-marked 2C CD8\(^+\) cells were injected into Thy1.1\(^-\)-marked B6 recipients at 4 \(\times\) 10\(^6\) cells per mouse. One day later mice were injected with PBS (Control), 0.1 nmol SIYR alone, 2.5 nmol SIYR plus 100 \(\mu\)g of poly I:C, or vesicles plus 0.1 nmol SIYR. SIYR plus poly I:C was injected i.p., whereas all other samples were injected into both footpads. Mice were killed 3 days later. CFSE profiles versus CD44 or CD43 expression of Thy1.2\(^-\) CD8\(^+\) cells are shown for inguinal LN or spleen, respectively. Data are representative of at least two independent experiments.

To examine effector function in vivo, we examined rejection of DP1 tumor cells, which are EL4 (H-2\(^d\)) cells transfected with an SIYR peptide minigene (26). Preliminary experiments established that DP1 cells were rejected by normal syngeneic B6 mice but grew

well in TAP\(^{-/-}\) mice (data not shown); because TAP\(^{-/-}\) mice are selectively depleted of CD8\(^+\) T cells (27), DP1 rejection is presum-
ably controlled largely by CD8\(^+\) cells.

To measure tumor rejection, doses of 4 \(\times\) 10\(^6\) naïve 2C cells were transferred i.v. to B6.TAP\(^{-/-}\) mice. After 1 day the host mice were injected i.v. with vesicles/SIYR or with SIYR alone; controls received PBS. At 3 days after 2C injection the hosts were injected s.c. with 2 \(\times\) 10\(^6\) DP1 tumor cells. In three separate experiments, two of which are shown in Fig. 6A, there was no reduction in tumor growth when 2C cells were injected alone or were coinjected with SIYR without vesicles, even with a high dose of 10 nmol peptide. With a modest dose of 80 \(\mu\)g of vesicles plus 0.2 nmol peptide per mouse, tumor growth was undetectable in 50% (four of eight) of the hosts (two of four in each of two experiments), and in the remaining hosts tumor growth was clearly reduced (Fig. 6A Upper). With a higher dose of vesicles (220 \(\mu\)g) tumor rejection was 100% (no growth in four of four mice) (Fig. 6A Lower).

The above data refer to tumor rejection by T cells primed in vivo. Efficient tumor rejection also applied to T cells that were activated by vesicles/SIYR in vitro before transfer in vivo. Here 2C cells were cultured for 3 days in vitro with vesicles plus a low concentration of SIYR (0.32 \(\mu\)M) or with a high concentration of SIYR (10 \(\mu\)M) without peptide. After transfer to B6 hosts (without peptide), the donor (Thy1.1\(^-\)-marked) 2C cells activated by vesicles/peptide were clearly apparent in blood by FACS analysis at day 14 after

Fig. 5. Stimulation of 2C CD8\(^+\) cells by vesicles plus peptide in vivo. (A) CFSE-labeled 2C CD8\(^+\) cells were injected i.v. into syngeneic B6 recipients at 8 \(\times\) 10\(^6\) cells per mouse. One day later mice were injected i.v. with PBS (Control), 0.2 nmol SIYR, or titrated doses of vesicles plus 0.2 nmol SIYR. Mice were killed 3 days later and analyzed for CFSE dilution of 1B2\(^+\) CD8\(^+\) cells in spleen and LN. Data for LN are shown. (B) Expansion of 1B2\(^+\) CD8\(^+\) cells in spleen and LN of mice injected with membrane vesicles relative to injection of peptide alone as in A. (C) CFSE-labeled 2C CD8\(^+\) cells (Ly5.2) were injected i.v. into B6.Ly5.1 recipients (4 \(\times\) 10\(^6\) per mouse). One day later mice were injected i.v. with 0.2 nmol SIYR or with 240 \(\mu\)g of vesicles plus 0.2 nmol SIYR. Mice were killed 3 days later. Numbers of donor Ly5.2\(^+\) CD8\(^+\) cells in LN and spleen relative to host Ly5.1\(^+\) CD8\(^+\) cells are shown. (D) CFSE versus CD44 expression for donor Ly5.2\(^+\) CD8\(^+\) 2C cells is shown for the same mice as in C. For C and D there were two mice per group. (E) CFSE-labeled Thy1.2\(^-\)-marked 2C CD8\(^+\) cells were injected into Thy1.1\(^-\)-marked B6 recipients at 4 \(\times\) 10\(^6\) cells per mouse. One day later mice were injected with PBS (Control), 0.1 nmol SIYR alone, 2.5 nmol SIYR plus 100 \(\mu\)g of poly I:C, or vesicles plus 0.1 nmol SIYR. SIYR plus poly I:C was injected i.p., whereas all other samples were injected into both footpads. Mice were killed 3 days later. CFSE profiles versus CD44 or CD43 expression of Thy1.2\(^-\) CD8\(^+\) cells are shown for inguinal LN or spleen, respectively. Data are representative of at least two independent experiments.
ple and specific, with peptide added to vesicles or to isolated membranes. The immunogenic vesicles were obtained from a transfected cell line (unpublished observations). Here separation of the sonicates on sucrose gradients, plus the ability to retard vesicle immunogenicity with a pre-sonication trypsin treatment, implied that the vesicles were derived largely from the plasma membrane.

As for exosomes, vesicle immunogenicity from sonicates was strictly peptide-dependent and peptide-specific, with peptide added to cells before sonication or, more effectively, to the vesicles at the time of T cell stimulation. Notably, the immunogenicity of the vesicles after peptide loading applied to purified naïve CD8+ cells in the absence of APC. Under these conditions, T cell stimulation required that, in addition to MHC/peptide, the vesicles coex-
nonessential amino acids, and antibiotics. DP1 cells were cultured in the same medium plus 0.5 μg/ml G-418.

Peptides. SIYR (SIYRYYGL), QL9 (QLSPFPFDL), and SIINFEKL peptides were purchased from Sigma–Genosys, and purity was ≥95%.

Preparation of Membrane Vesicles. Where indicated, DC2.4 cells were cultured with 10 ng/ml recombinant murine IFN-γ with or without 2.5 μM SIYR peptide for 24 h. Cells were then washed with PBS and resuspended in Dounce buffer with protease inhibitors. The cell suspension was incubated for 10 min at 4°C and transferred to Dounce homogenizer, and 30 strokes were delivered. Immediately thereafter, tonicity was restored to 0.15 M NaCl (final). The supernatant was recovered, diluted with PBS, and sonicated. This suspension was centrifuged at 10,000 × g to remove the nuclear fraction (pellet). The supernatant was recovered, diluted with PBS, and sonicated. This suspension was centrifuged at 10,000 × g to remove mitochondria, any remaining nuclei fragments, and larger cell debris. The supernatant was recovered and centrifuged at 100,000 × g to pellet the vesicles. The pellet was resuspended in 1–2 ml of PBS. To remove possible aggregates thereafter, samples were spun at 7,500 × g for 5 min.

To prepare CFSE-labeled membrane vesicles, DC2.4 cells were cultured as described above, washed, and resuspended in warm PBS/0.1% BSA. Cells were then labeled with 10 μl of 5 mM Vybrant CFDA SE Cell Tracer kit per milliliter of cell suspension for 20 min at 37°C. Cells were washed as described for T cells and used for membrane vesicle preparation.

Exosomes were isolated from culture supernatant of DC2.4 cells used for preparation of membrane vesicles and were purified as described (23).

Cell Purification. 2C and OT-1 CD8+ cells were purified from LN by using a negative selection kit (Miltenyi Biotec).

CFSE Labeling of T cells. 2C CD8+ cells were resuspended in 37°C PBS containing 0.1% BSA at 1–2 × 10^6 cells per milliliter and incubated with 1 μl of 5 mM CFSE per milliliter for 10 min at 37°C. Labeling was terminated by adding excess ice-cold PBS containing 10% FCS, and cells were then washed three times before use.

Antibodies and Flow Cytometry Analysis. The following antibodies were used: phycoerythrin-conjugated anti-CD3 (145-2C11, Becton Dickinson), anti-CD8α (53-6.7, Becton Dickinson), anti-CD43 (1B11, Becton Dickinson), and anti-granzyme B (GB12, Caltag); allophycocyanin-conjugated anti-CD44 (IM7), anti-CD45.2 (104), and anti-CD90.2 (HSI51); Alexa Fluor 405-conjugated anti-CD4 (RM4-5, Caltag); biotin-conjugated anti-CD54 (YN1/1.7.4), anti-CD80 (16-10A1), anti-CD86 (Michel-17), anti-IL-2 (JES6-51H4), and anti-IFN-γ (XMGl2); unconjugated anti-IL-2 (JES61A12) and anti-IFN-γ (RA-6A2). Antibodies were purchased from eBioscience unless otherwise stated. Cy5-conjugated 1B2 mAb was prepared by using a Cy5 labeling kit.

For intracellular staining of granzyme B, GolgiStop was added to cells for the last 5 h of incubation. Cells were then washed, stained for surface markers, fixed, permeabilized, washed, and analyzed. For staining of in vivo-activated cells, RBCs were lysed before surface marker staining. Streptavidin-coated beads were used to detect surface markers on membrane vesicles. Beads were coated with biotinylated anti-CD54, anti-CD80, anti-CD86, or isotype control mAbs. Coated beads were incubated with CFSE-labeled membrane vesicles, washed twice, and analyzed for bound material.

In Vitro Stimulation of 2C CD8+ T Cells. In most experiments, 5 × 10^4 purified 2C CD8+ T cells were incubated with varying concentrations of membrane vesicles for 72 h. [3H]Thymidine at 1 μCi/ml (1 Ci = 37 GBq) was added to the cultures 8 h before harvest. When intact DC2.4 cells were used as stimulators, DC2.4 cells were incubated for 24 h with 10 ng/ml recombinant murine IFN-γ and 2.5 μM SIYR peptide, irradiated, washed, and added to wells with 2C CD8+ cells plus 0.32 μM free SIYR peptide.

Measurement of Proliferation of 2C CD8+ Cells in Vivo. CFSE-labeled 2C CD8+ cells were injected i.v. The next day mice were injected i.p. with membrane vesicles given either i.v. or s.c. (both footpads). As a positive control, in some experiments SIYR and poly I:C were injected i.p. Mice were killed 3 days later. Donor cells were identified by antigenotypic 1B2 mAb staining or by using Thy1.1/1.2 or Ly5.1/2 differences between donor and host.

ELISA and Cytotoxic T Cell Assays. A total of 5 × 10^4 of 2C CD8+ T cells per well were incubated with membrane vesicles (10 μg/ml) plus 0.32 μM SIYR, intact DC2.4 cells (1.25 × 10^5 per well), plus 0.32 μM SIYR or with 0.32 μM SIYR alone. DC2.4 cells were treated as described for proliferation assays. Culture supernatants were collected and used for ELISA as described (32).

To assay cytotoxic T lymphocyte activity, T cells were collected after 68 h of culture and used in a standard 51Cr-release assay. As a target, 51Cr-labeled P815 cells (1 × 10^4 per well) pulsed with 1 h with 10 μM QL9 peptide were used.

Tumor Rejection. TAP-/- mice were injected i.v. with naïve 2C CD8+ cells (4 × 10^6 per mouse). The next day membrane vesicles were injected i.v. Three days after vesicle injection mice were injected s.c. with 2 × 10^5 DP1 cells. DP1 cells express a minigene encoding SIYR peptide (26).

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