T Cells Can Use Either T Cell Receptor or CD28 Receptors to Absorb and Internalize Cell Surface Molecules Derived from Antigen-presenting Cells

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Abstract

At the site of contact between T cells and antigen-presenting cells (APCs), T cell receptor (TCR)–peptide–major histocompatibility complex (MHC) interaction is intensified by interactions between other molecules, notably by CD28 and lymphocyte function-associated antigen 1 (LFA-1) on T cells interacting with B7 (B7-1 and B7-2), and intracellular adhesion molecule 1 (ICAM-1), respectively, on APCs. Here, we show that during T cell–APC interaction, T cells rapidly absorb various molecules from APCs onto the cell membrane and then internalize these molecules. This process is dictated by at least two receptors on T cells, namely CD28 and TCR molecules. The biological significance of T cell uptake of molecules from APCs is unclear. One possibility is that this process may allow activated T cells to move freely from one APC to another and eventually gain entry into the circulation.

Key words: T cell receptor • CD28 • absorption • internalization • antigen presenting cells

Introduction

T cell interaction with specific peptides bound to MHC molecules on APCs causes naive T cells to proliferate and differentiate into effector cells (1, 2). Stimulation of T cells by APCs is thought to involve at least two signaling events: signal 1 induced via TCR recognition of peptide–MHC complexes, and signal 2 elicited by CD28 molecules on T cells interacting with B7 (B7-1 or B7-2) on APCs. In addition to CD28–B7, interactions between several other molecules on T cells and complementary molecules on APCs appear to augment T cell stimulation (3–6). Whether these additional interactions, typified by LFA-1 on T cells binding to intracellular adhesion molecule 1 (ICAM-1)[1] on APCs, activate further signaling pathways or serve as adhesion molecules or both is still controversial.

Interaction between T cells and APCs leads to tight conjugate formation and rapid segregation of supramolecular activation clusters at the T cell contact site (7–11). Lateral movement of cell surface molecules on T cells into supramolecular activation clusters is known to include TCR, CD2, CD4/8, LFA-1, and CD28 molecules, and may apply to a variety of other molecules. Although the individual binding affinities involved in T–APC molecular interactions are thought to be low, the overall avidity of these interactions at the site of cell–cell contact is presumably very high and may serve to potentiate both the strength and duration of T cell signaling.

The formation of tight conjugates between T cells and APCs raises the question of how these cells subsequently disengage themselves, and whether the cells retain or exchange their respective cell surface molecules. On this latter point, there is extensive literature documenting that T cells can absorb various molecules, especially MHC and Ig molecules, from APCs. Studies in the early 1970s demonstrated that alloreactive T blast cells generated during a graft-versus-host reaction in vivo expressed Ig molecules (12, 13). Further studies showed that the Ig molecules were of donor origin, and it was hypothesized that these molecules represented specific alloantibodies attached to fragments of host MHC molecules bound to antigen-specific receptors on T cells. Other in vitro studies confirmed this scenario and demonstrated that alloreactive blast cells had binding

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[1]*Abbreviations used in this paper:* B6, C57BL/6J; DC, dendritic cell; ICAM, intracellular adhesion molecule; MFI, mean fluorescence intensity; PI, propidium iodide.

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specificity for MHC molecules released from APCs by nitrogen cavitation (14–16); such binding was antigen specific. For T cells specific for MHC class II–restricted conventional antigens, absorption of class II molecules from APCs was shown to require contact with specific antigen (17, 18). Later studies with bone marrow chimeras indicated that donor-derived thymocytes were able to acquire host MHC molecules, apparently from thymic epithelial cells (19, 20). Information on whether T cells can absorb additional molecules from APCs and other cells is sparse, although in one study MHC class II–restricted T cell clones acquired both MHC class I and II molecules from APCs (21).

In this paper, we have reexamined the capacity of T cells to absorb cell surface molecules from APCs and defined the receptor–ligand interactions involved. The data show that exposure to APCs causes T cells, including naive cells, to rapidly absorb a variety of cell surface molecules from APCs. Absorption of these molecules can be mediated by either TCR–peptide–MHC or by CD28–B7 interaction, and applies both in vivo and in vitro. Significantly, molecules absorbed to the cell surface are rapidly internalized.

Materials and Methods

Animals. C57BL/6J (B6), B10.D2, DBA/2J, and BALB/cByJ mice were purchased from The Jackson Laboratory. CD28$^{-/-}$ (22) and ICAM-1$^{-/-}$ (23) mice were purchased from The Jackson Laboratory and maintained at The Scripps Research Institute. 2C transgenic mice (24) were bred at The Scripps Research Institute. The 2C CD28$^{-/-}$ mice were generated in our laboratory. CB-17 SCID mice were purchased from Taconic Farms. Lewis rats were purchased from Harlan.

Cell Lines and Media. *Drosophila* APCs were generated and maintained as described previously (25). RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics was used for T cell culture.

Peptides. QL9 (QLSPFPFDL) and SIYR (SIYRYYGL) peptides were synthesized, purified, and quantitated as described previously (25, 26). *Drosophila* APCs and dendritic cells (DCs) were pulsed with the peptides for 2 h at room temperature before use.

Cell Purification. T cells were purified by mAb plus C treatment as described previously (27). For purification of total T cells, LN cells were treated with a mixture of anti-HSA (J11D) and anti-IA^b (28-16-8s) mAbs plus a mixture of guinea pig and rabbit C for 45 min at 37°C. For purification of CD8$^+$ T cells, anti-CD4 mAb (RL174) was added along with the above mAbs and C. For purification of 2C CD8$^+$ T cells, positive panning on anti-CD8 mAb (3.168)-coated plates was performed after mAb plus C treatment. Rat T cells were enriched by panning LN cells on mouse anti–rat IgG–coated plates. After 1-h incubation at 4°C, unbound cells were recovered and used. For preparation of LPS blasts, spleen cells were treated with a mixture of anti-Thy1.2 (J1j), anti-CD4, and anti-CD8 mAbs plus C and then cultured for 20 h with LPS (10 μg/ml) followed by Ficoll gradient separation to remove dead cells. Preactivated T cells were prepared by culturing purified T cells with PMA (10 nM) and ionomycin (370 ng/ml) for 14 h. DCs were purified as described by Inaba et al. (28).

Culture Conditions. For overnight stimulation of 2C T cells (see Fig. 1), purified resting 2C T cells (2×10^5) were incubated with transfected *Drosophila* APCs or LPS blasts (8×10^4) plus 1 μM QL9 peptide in a 24-well plate.

For short-term culture, purified resting T cells (2.5×10^6) or preactivated T cells (6×10^5) were mixed with transfected 6×10^5 *Drosophila* cells, with or without 1 μM QL9 peptide in a 48-well plate, centrifuged for 1 min at 120 *g*, and incubated at 37°C for 1 h. When DCs were used as APCs, T cells in the above numbers were incubated with 5×10^5 DCs with or without 1 μM SIYR peptide in a 48-well plate for the indicated period of time.

For overnight stimulation of rat T cells with mouse DCs, 2×10^5 B-depleted rat T cells were incubated with 2×10^4 DCs purified from DBA/2J spleens.

Antibodies and Flow Cytometry Analysis. Anti-L^d (30-5-7 [29]), anti-clonotypic 2C TCR (1B2 [30]), anti-B220 (RA 3-3A1), anti-Vβ8.2 (F23.2 [31]), and anti–TCR-β (H57 [32]) mAbs were prepared in ascites form. The following mAbs were purchased from PharMingen: anti–LFA-1 (M17/4); FITC-conjugated anti-CD8 (53-6.7); FITC-conjugated anti–B7-1 (16-10A1); FITC-conjugated anti–rat CD8 (OX-8); PE-conjugated anti–B7-1 (16-10A1); PE-conjugated anti–B7-2 (GL1); PE-conjugated anti–ICAM-1 (3E2); PE-conjugated anti-K^d (SF1-1.1); PE-conjugated anti-IA^b (AF6-120.1); PE-conjugated anti-IA^d (AMS-32.1); PE-conjugated anti–rat TCR-α/β (R73); PE-conjugated anti–human B7-1 (BB1); PE-conjugated anti-HLA (G46-2.6); PE-conjugated anti–human ICAM-1 (HA58); PE-conjugated streptavidin; and Cychrome™-conjugated anti–rat CD4 (OX-35). Cy5-conjugated anti-CD8 (3.168) mAb was prepared using a Cy5 labeling kit (Amersham). Biotinylated anti-L^d mAb was prepared in our laboratory.

Unless stated otherwise, for FACS® analysis cell surface staining was performed with FITC-conjugated anti-CD4 or anti-CD8 mAbs, along with PE-conjugated mAbs specific for other cell surface markers. After 20-min incubation on ice, cells were washed and resuspended in FACS® buffer (1× PBS, 25 mM Hepes, 2.5% horse serum) containing 4 μg/ml propidium iodide (PI). FACSort™ (Becton Dickinson) was used for flow cytometry and FACS Vantage™ (Becton Dickinson, CA) was used for cell sorting. The data shown refer to staining of viable (PI$^-$) cells.

In Vivo Stimulation of Rat T Cells. Doses of 2×10^7 B cell–depleted LN T cells from Lewis rats were intravenously injected into irradiated (350 cGy) CB-17 SCID mice (Taconic Farms). The activated T cells were recovered from LNs, spleen, and thoracic duct lymph (33) 2 or 3 d after injection, and used for cell surface staining.

Reverse Transcription PCR. Total RNA preparation and cDNA synthesis were performed as described previously (34). The conditions used for PCR were as follows: denaturation at 91°C for 30 s, annealing at 55°C for 30 s, and polymerization at 72°C for 1 min for 30 cycles. The primers used for PCR were as follows: (1) B7-1 sense, ACCTCAATAGACTCTTACTAGT; B7-1 antisense, CCGCTCTAACTTAGAGGCTG (2); B7-2 sense, ATGCTGTTTCCGTGGAGACG; B7-2 antisense, CCGCTCTAACTTAGAGGCTG (3); glyceraldehyde 3-phosphate dehydrogenase sense, TGATGGGTGTGAACCACGAG; and glyceraldehyde 3-phosphate dehydrogenase antisense, TCAGTGTAGCCCAAGATGCC.

Laser Confocal Microscopy. PMA and ionomycin–stimulated or resting T cells were incubated with transfected *Drosophila* APCs with or without QL9 for 1 h at 37°C. After incubation, cells were allowed to attach to poly-l-lysine–coated cover slips (35) by gravity for 30 min at 4°C. Cells were then sequentially fixed and permeabilized with 2% paraformaldehyde in PBS for 20

min at room temperature and 0.05% saponin in PBS for 10 min at room temperature (36). The fixed cells were stained with FITC-conjugated anti–B7-1 and Cy5-conjugated anti-CD8 mAbs. Alternatively, T cells were stained with the mAbs immediately after incubation with *Drosophila* APCs. The mAb-stained T cells were allowed to adhere to poly-l-lysine coated coverslips for 30 min at 4°C and the cells were then fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. The stained cells were observed under an Axiovert S100 TU™ inverted microscope (Zeiss), and LaserSharp™ (Bio-Rad) software was used for confocal microscope image analysis.

Flow Cytometric Analysis of T–APC Conjugate Formation. PMA and ionomycin–stimulated B6 $CD8^+$ T cells (1×10^5) were cultured in a final volume of 100 μl with *Drosophila* APCs (1×10^5). Cell mixtures were centrifuged briefly (10 s at 200 *g*) in 12 × 75 mm tubes (FALCON™ 2054; Becton Dickinson) and incubated at 37°C in a humidified CO_2 incubator for the indicated period of time. After incubation, anti-CD8 mAb (53-6.7, Cy5) was added for 20 min at 4°C. After staining, T–APC conjugates were FACS® analyzed. T cells and *Drosophila* APCs were easily distinguished on the basis of side scatter.

Results

T Cell Absorption of B7 Molecules from Drosophila APCs. As reported previously, primary responses of 2C TCR transgenic $CD8^+$ cells to H2-L^d–restricted QL9 peptide can be elicited by L^d-transfected *Drosophila* cells, but only when these cells are cotransfected with either B7 (B7-1 or B7-2) or ICAM-1 molecules (abbreviated L^d.B7 and L^d.ICAM-1 Dros. APCs, respectively [25]). With high concentrations of QL9 peptide, L^d.B7 and L^d.ICAM-1 Dros. APCs both elicit strong and equivalent 2C $CD8^+$ proliferative responses, even though only B7 and not ICAM-1 is viewed as a classic costimulatory molecule. At low concentrations of peptide, 2C proliferative responses are very low unless Ld Dros. APCs coexpress both B7 and ICAM-1, implying that these two molecules behave synergistically.

Using FACS® analysis, we examined CD28 and B7 expression on 2C cells after overnight culture with Dros. APCs plus QL9 peptide; exposure to a mixture of cross-linked anti-TCR plus anti-CD28 mAbs was used as a control. Before culture, cell surface expression on resting 2C cells was low but significant for CD28 and B7-2 and undetectable for B7-1 (Fig. 1 A).

After culture with L^d.ICAM-1 Dros. APCs plus QL9, expression of CD28 on 2C cells increased considerably, whereas expression of B7-1 and B7-2 remained as low as on naive 2C cells (Fig. 1 B); essentially similar findings applied after mAb-induced TCR/CD28 ligation. The results were quite different after culture with L^d.B7 APCs. With these APCs, expression of CD28 on 2C cells remained low or was reduced, whereas B7 expression increased to high levels. The striking finding was that incubation with L^d.B7-1 Dros. APCs led to marked upregulation of B7-1 on 2C cells but no change in B7-2. Conversely, only B7-2 and not B7-1 upregulation followed incubation of 2C cells with L^d.B7-2 Dros. APCs. As levels of B7-1 and B7-2 mRNA in 2C cells in these two situations were not elevated relative to resting 2C cells (Fig. 1 C), the appearance

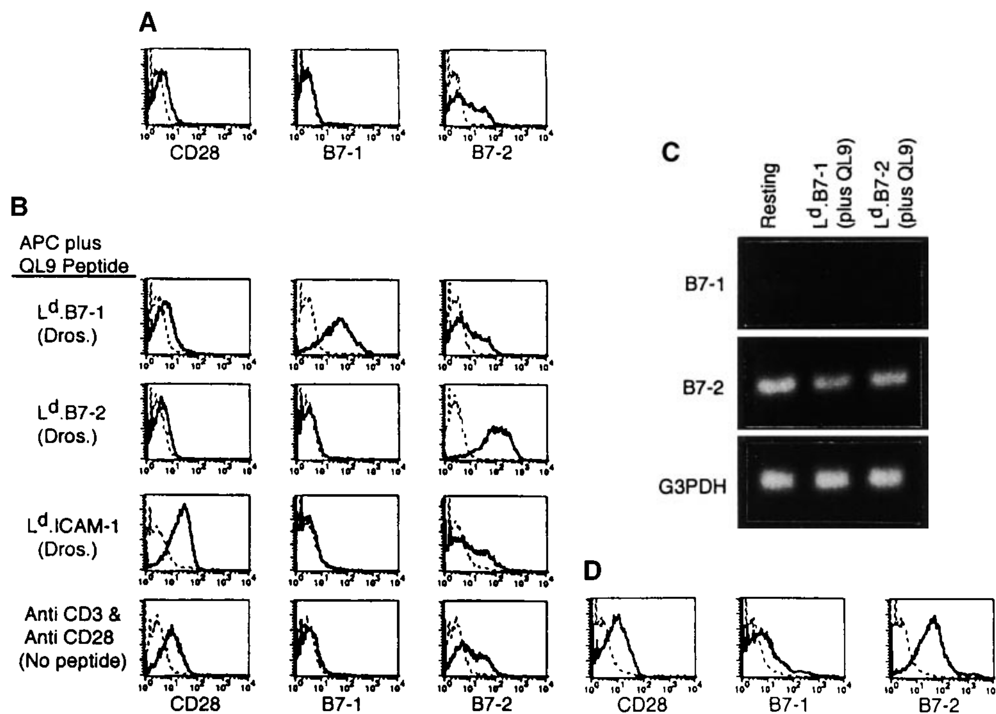

Figure 1. Expression of B7-1 and B7-2 on 2C $CD8^+$ cells cultured with Dros. APCs. (A) Surface staining of purified resting 2C $CD8^+$ T cells. Cells were stained with PE-conjugated anti-CD28, anti–B7-1, and anti–B7-2 specific mAbs. Isotype control staining is shown as a dotted line and staining with mAbs as a solid line. (B) Surface staining of 2C $CD8^+$ cells after overnight culture with the indicated Dros. APCs loaded with 1 μM QL9 peptide. After culture the cells were stained for CD8 and for the markers indicated. The data refer to staining of gated $CD8^+$ cells. Dros. APCs were gated out on the basis of their $CD8^-$ phenotype and their pattern of forward and side scatter. (C) Expression of B7-1 and B7-2 mRNA by 2C cells before and after culture with Dros. APCs plus QL9 peptide. After culture 2C cells were depleted of Dros. APCs by sequential Percoll and Ficoll gradient centrifugation. Relative levels of mRNA were measured by semiquantitative reverse transcription PCR analysis. (D) Surface staining of 2C T cells after overnight culture with LPS-stimulated BALB/c ($H2^d$) B cell blasts.

of B7 on 2C cells appeared to reflect absorption from the APCs rather than de novo synthesis. In agreement with this idea, culturing 2C cells with QL9 peptide plus L^{d+} LPS blasts, i.e., APCs expressing moderate amounts of both B7-1 and B7-2, led to enhanced staining of both molecules on 2C cells (Fig. 1 D). Three mechanisms could account for the apparent absorption of B7 from APCs: (1) direct binding of B7 to CD28, (2) TCR-mediated binding of L^d–QL9 complexes physically linked to B7, e.g., on membrane vesicles, and (3) nonspecific absorption.

Absorption Via CD28. To examine the role of CD28, normal $CD28^+$ versus $CD28^{-/-}$ T cells prepared from normal (nontransgenic) B6 mice were cultured with L^d.B7-1 Dros. APC (without peptide) for 1 h before staining and FACS® analysis. B7-1, rather than B7-2, transfectants were chosen because of the negligible background expression of B7-1 on normal T cells. With normal resting $CD28^+$ B6 T cells, culture with L^d.B7-1 Dros. APCs induced clear expression of B7-1 on the T cells accompanied by downregulation of CD28 (Fig. 2 A). Correlating with the higher initial levels of CD28 on $CD4^+$ cells than $CD8^+$ cells, "uptake" of B7-1 by resting T cells was higher on $CD4^+$ cells than $CD8^+$ cells. Significantly, in marked contrast to normal B6 T cells, incubating resting $CD28^{-/-}$ T cells with L^d.B7-1 Dros. APCs failed to cause B7-1 expression on either $CD4^+$ or $CD8^+$ cells (Fig. 2 A). Thus, T cell uptake of B7-1 was CD28 dependent.

Essentially similar findings applied to activated T cells, e.g., to B6 T cells precultured overnight with PMA plus ionomycin (Fig. 2 B). The main difference with these T blast cells was that as manifested by mean fluorescence intensity (MFI), uptake of B7-1 was substantially higher than with resting T cells, especially for $CD8^+$ cells; for $CD8^+$ cells, this finding correlated with higher CD28 expression on activated cells rather than resting $CD8^+$ cells. As with resting T cells, B7-1 uptake by $CD28^{-/-}$ activated T cells was minimal, though detectable; whether or not the residual uptake of B7-1 is mediated via CTL-associated antigen 4 has yet to be tested.

Uptake of L^d and other molecules from Dros. APCs is shown in Fig. 3. When activated $CD28^+$ B6 $CD8^+$ cells were incubated with B7-1$^+$ APCs (either L^d.B7-1 Dros. APCs [not shown] or L^d.B7-1.ICAM-1 Dros. APCs, Fig. 3 A, right), the T cells stained positive for both L^d and B7-1; by contrast, with $CD28^{-/-}$ B6 cells, the T cells were negative for both L^d and B7-1. Thus, the presence of CD28 on the T cells allowed these cells to absorb both L^d and B7-1, presumably in the form of membrane vesicles containing these two molecules. Significantly, the uptake of L^d by $CD28^+$ T cells only occurred when Dros. APCs coexpressed both L^d and B7 (B7-1). Thus, no uptake of L^d occurred when $CD28^+$ cells were incubated with $B7^-$ APCs, i.e., with Ld Dros. APCs (not shown) or L^d.ICAM-1 Dros. APCs (Fig. 3 A, left).

The above findings suggested that CD28 interaction with B7-1 on Dros. APCs might enable the T cells to absorb any molecule that was coexpressed on the APCs, i.e., any molecule that was physically associated with B7-1 on the APC

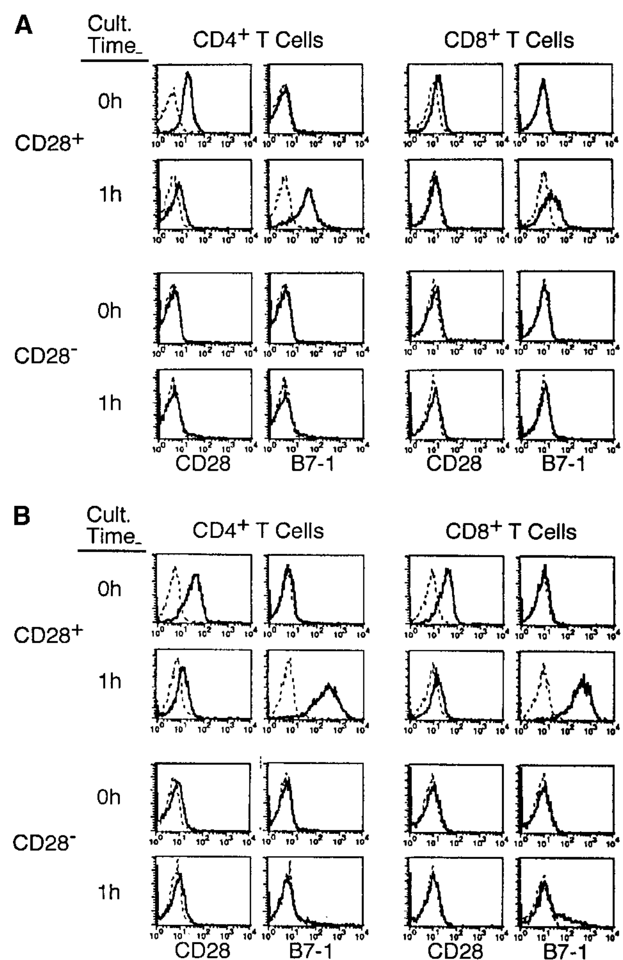

Figure 2. CD28-dependent absorption of B7-1 molecules by T cells after incubation with Dros. APCs. Resting (A) and preactivated (B) purified T cells prepared from normal B6 ($CD28^+$) or $CD28^{-/-}$ B6 mice were surface stained for CD28 and B7-1 before and after incubation for 1 h at 37°C with L^d.B7-1 Dros. APCs. Stained cells were examined by flow cytometry. The data show staining on gated $CD4^+$ and $CD8^+$ cells; contaminating Dros. APCs were excluded on the basis of forward and side scatter and by CD4 or CD8 mAb staining. Dashed lines refer to staining with an irrelevant isotype-matched control mAb.

cell membrane, e.g., ICAM-1 on L^d.B7-1.ICAM-1 Dros. APCs. Testing ICAM-1 uptake by normal T cells was not feasible because of the high constitutive expression of ICAM-1 on T cells (Fig. 3 A). However, activated T cells from ICAM-$1^{-/-}$ mice ($CD28^+$ICAM-$1^{-/-}$ T cells) became strongly ICAM-1^+ (and L^{d+}B7-1^+) after incubation with L^d.B7-1.ICAM-1 Dros. APCs (Fig. 3 B, right). Bearing in mind that T cells express LFA-1, the principal receptor for ICAM-1 (37, 38), it was notable that no T cell uptake of ICAM-1 occurred with L^d.ICAM-1 Dros. APCs (Fig. 3 B, left). Thus, the uptake of ICAM-1 from L^d.B7-1.ICAM-1 Dros. APCs appeared to be controlled solely by CD28–B7-1 interaction and not by LFA-1–ICAM-1 interaction. Nevertheless, uptake of ICAM-1 (and L^d) from L^d.B7-1.ICAM-1 Dros. APCs was partially blocked by anti–LFA-1 mAb (Fig. 3 B, right). Hence LFA-1–ICAM-1

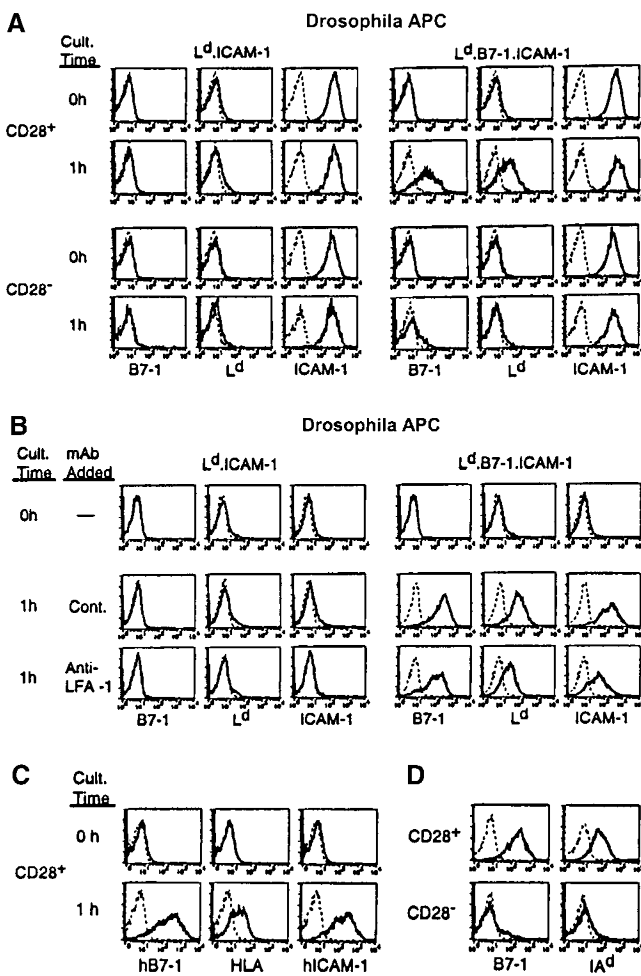

Figure 3. CD28-dependent T cell absorption of various cell surface molecules from Dros. APCs. Preactivated CD8$^+$ cells prepared from normal B6 (A, C, and D), CD28$^{-/-}$ B6 (A and D), or ICAM-1$^{-/-}$ B6 mice (B) were cultured with or without M17/4 anti–LFA-1 mAb (15 μg/ml) or an isotype-matched control (Cont.) mAb (anti-B220) with Dros. APCs expressing various mouse or human (h) cell surface molecules (L^d.ICAM-1 and L^d.B7-1.ICAM-1, A and B; HLA.hB7-1.hICAM-1, C; and IA^d.B7-1, for 1 h, D) and then stained for the markers indicated; staining of T cells before culture is shown as a control. (B) An anti-B220 mAb was used as a control. In other experiments, no inhibition was seen with mAbs specific for CD8, CD27, and TCR-β. The data show staining on gated CD8$^+$ cells.

interaction was perhaps too weak to cause direct binding and uptake of ICAM-1, but was nevertheless able to stabilize CD28-mediated binding of B7-1 plus ICAM-1.

Studies with Dros. APCs expressing human (h)ICAM-1 and hB7-1 showed that B7-dependent uptake of ICAM-1 by T cells was not a property restricted to ICAM-1$^{-/-}$ T cells. Thus, incubating activated normal CD28$^+$ B6 CD8$^+$ cells with HLA class I.hB7-1.hICAM-1 Dros. APCs caused T cell uptake of all three molecules from the APCs (Fig. 3 C). Class I expression on APCs was not essential for uptake because activated CD28$^+$, but not CD28$^{-/-}$, B6 T cells absorbed both MHC class II IA^d and B7-1 (mouse) from IA^d.B7-1 Dros. APCs (Fig. 3 D).

It should be emphasized that removal of cell surface molecules from Dros. APCs by T cells caused no apparent injury to the APCs. Thus, after the short-term culture was used, the Dros. APCs remained fully viable in terms of PI exclusion.

The above data indicate that CD28–B7 interaction can lead to T cell absorption of a wide range of molecules from B7$^+$ APCs. LFA-1–ICAM-1 interaction is less important, but can stabilize CD28-mediated absorption. The role of TCR–peptide–MHC interaction is discussed below.

Absorption Via the TCR. In the above experiments, the fact that normal polyclonal (nontransgenic) T cells were used made it unlikely that CD28-dependent absorption of B7 (and other molecules) involved the TCR. To seek direct evidence on the role of TCR molecules, 2C CD8$^+$ cells were incubated with L^d.B7-1 Dros. APCs with or without QL9 peptide for 1 h. To avoid CD28-dependent absorption, CD28$^{-/-}$ 2C cells were used. The key finding here was that after culture with L^d.B7-1 Dros. APCs plus QL9 peptide, CD28$^{-/-}$ 2C cells stained strongly for both L^d and B7-1 (Fig. 4 A). Uptake of these molecules applied to both resting and activated CD28$^{-/-}$ 2C cells, and was not seen in the absence of QL9 peptide. Significantly, staining for L^d and B7-1 on CD28$^{-/-}$ 2C cells was totally inhibited by adding anticlonotypic 1B2 mAb to the cultures. Addition of the Vβ8.2-specific F23.2 mAb to 2C (Vβ8.2$^+$) cells was ineffective. Absorption of L^d and B7-1 molecules was thus TCR (and peptide) dependent and CD28 independent.

With resting cells, TCR-mediated uptake of L^d and B7-1 molecules from Dros. APCs was also apparent with CD28$^+$ 2C cells (Fig. 4 B). With these cells, absorption of L^d and B7-1 from L^d.B7-1 APCs was low, although significant, in the absence of QL9 peptide (reflecting minimal CD28-mediated uptake), but prominent in the presence of QL9 peptide; as with CD28$^{-/-}$ 2C cells, peptide-dependent uptake of L^d and B7-1 molecules was blocked by 1B2 mAb. The results with activated CD28$^+$ 2C cells were less clear cut. With these cells, CD28-mediated uptake was prominent and led to strong staining for L^d and B7-1 after culture with Dros. APCs in the absence of QL9 peptide; significantly, this nonantigen-specific uptake was not inhibited by 1B2 mAb. With addition of QL9 peptide, uptake of L^d and B7-1 by activated CD28$^+$ 2C cells increased only slightly (relative to cells cultured with APCs without peptide) and was not efficiently blocked by 1B2 mAb.

Absorption at the APC Level. As expected, T cell absorption of B7 from Dros. APCs led to a significant (10–20%) reduction of B7 expression on viable (PI$^-$) APCs (data not shown). Significantly, staining of APCs revealed no evidence that B7$^+$ Dros. APCs could absorb CD28 from T cells (data not shown). Likewise, when cultured with QL9 peptide, L^{d+} Dros. APC failed to absorb TCR from 2C CD8$^+$ cells. Thus, for both CD28- and TCR-mediated absorption, the transfer of molecules from APCs to T cells appeared to be unidirectional.

Absorption from DCs. As with transfected Dros. cells, T cells were able to absorb B7 and other molecules from con-

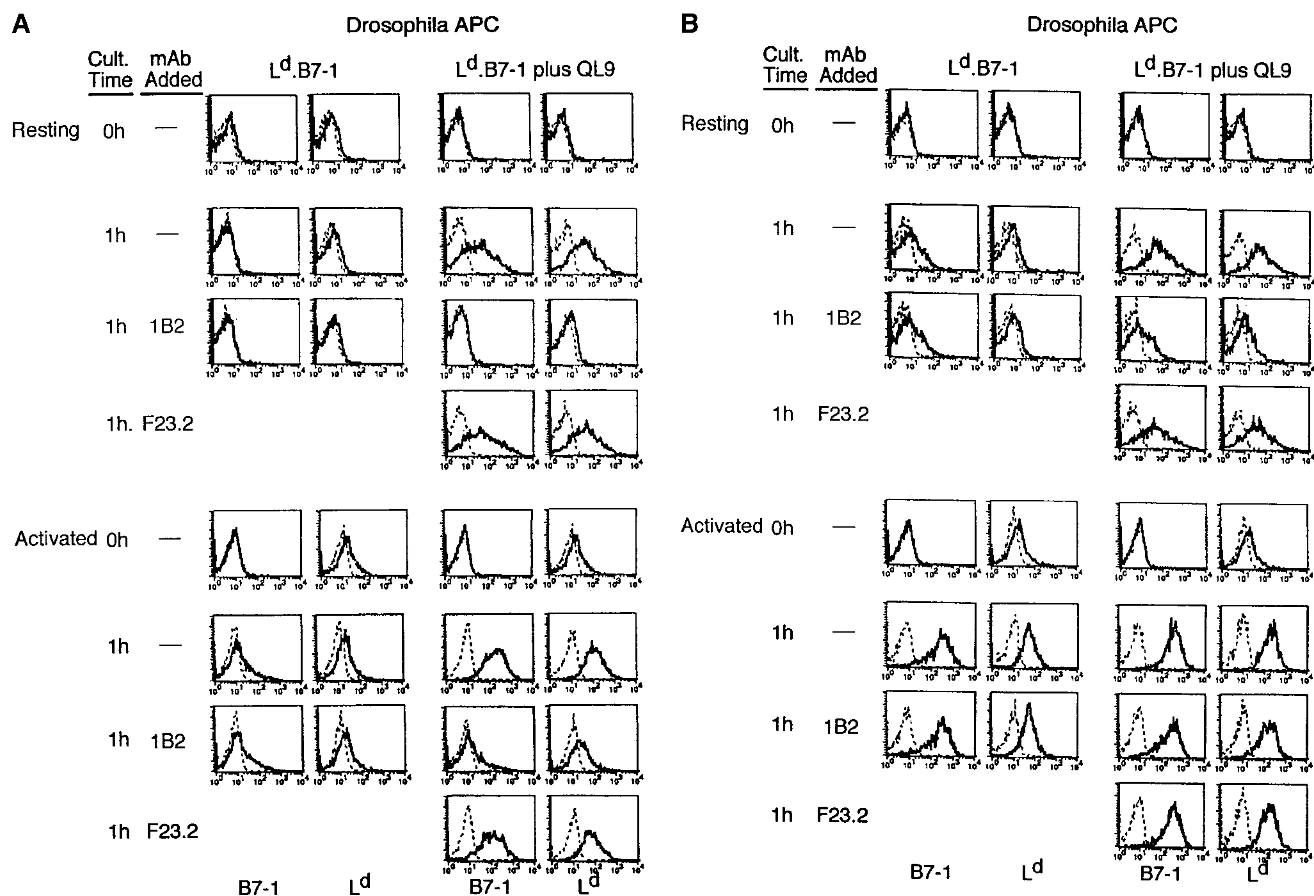

Figure 4. TCR-dependent uptake of B7-1 and L^d from Dros. APCs by CD28$^{-/-}$ (A) versus CD28$^+$ (B) 2C CD8$^+$ cells. Resting or preactivated CD8$^+$ 2C cells (B6 background) were incubated with L^d.B7-1 Dros. APCs with or without QL9 peptide (1 μM) and then stained for B7-1 and L^d expression. Some cultures were supplemented with 1B2 or F23.2 (anti-Vβ8.2) mAb (0.25% ascites fluid). Staining of T cells before culture is shown as a control. The data show staining on gated CD8$^+$ cells.

ventional APCs, i.e., from purified DCs. This applied to both CD28-dependent and TCR-dependent absorption (Fig. 5). CD28-dependent absorption by preactivated CD28$^+$ versus CD28$^{-/-}$ normal (nontransgenic) B6 CD8$^+$ cells after culture with syngeneic B6 (H2^b) DCs is illustrated in Fig. 5 A. Before culture with DCs, the activated T cells showed no detectable expression of B7-1 or MHC class II IA^b but strong expression of class I K^b. After culture for 1 h with DCs, CD28$^+$ T cells showed clearly significant expression of B7-1, and strong expression of IA^b; high K^b expression remained unchanged. However, with 1 h culture of CD28$^{-/-}$ T cells, B7-1 expression on these cells was barely detectable and IA^b expression was much reduced, i.e., ~10-fold lower than on CD28$^+$ T cells. These data confirm that B7 (and class II) absorption by activated T cells is largely a reflection of CD28–B7 interaction. Nevertheless, the finding that class II expression on DC-cultured CD28$^{-/-}$ T cells was low rather than absent suggests that class II absorption also involved other molecules.

To examine TCR-dependent absorption from DCs, we used B6 CD28$^{-/-}$ 2C CD8$^+$ cells and B6 DCs. Here, we used the fact that in addition to recognizing QL9/L^d, the 2C TCR also has specificity for SIYR peptide plus K^b (B6; see Materials and Methods). When resting CD28$^{-/-}$ 2C cells (B6, H2^b) were incubated for 1 h (not shown) or 3 h (Fig. 5 B) with syngeneic B6 DCs without exogenous peptide, the 2C cells stained negative for B7-1 (and also IA^d and L^d), but showed low, significant expression of IA^b (compared with undetectable IA^b expression on the cells before culture). However, after culture with B6 DCs in the presence of SIYR peptide, B7-1 expression on 2C cells was clearly apparent and IA^b expression increased by ~10-fold. Control staining for L^d and IA^d remained negative. These findings imply that culturing CD28$^{-/-}$ 2C cells with B6 DCs led to peptide-dependent uptake of both B7-1 and IA^b from the DCs. Thus, uptake of these molecules appeared to be TCR dependent and CD28 independent.

In Vivo Absorption. To examine whether T cell absorption of B7-1 (and other) molecules occurs under physiological conditions, we tested the capacity of rat T cells to acquire mouse B7-1 after transfer to irradiated SCID mice. The advantage of this rat to mouse system is that rat (r) and mouse (m) B7-1, 2 are serologically distinct, thus enabling us to detect whether rat T cells can absorb mB7. In prelimi-

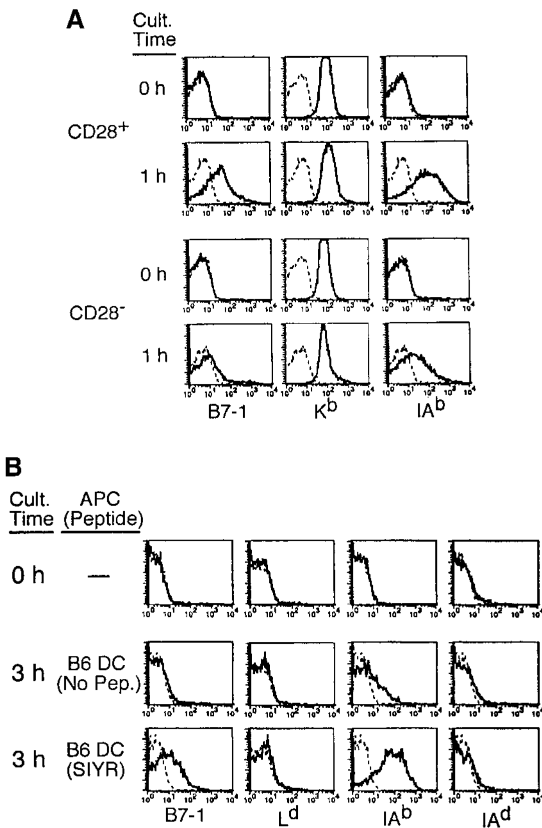

Figure 5. T cell absorption of surface molecules from DCs. (A) Preactivated CD28$^+$ or CD28$^{-/-}$ B6 CD8$^+$ cells were cultured for 1 h with purified normal B6 DCs, then treated with EDTA (1 mM) to disrupt T–DC contact and stained for expression of the markers shown. (B) Resting CD28$^{-/-}$ 2C CD8$^+$ cells were incubated for 3 h with B6 DCs with or without SIYR peptide (1 μM) and then stained for the markers indicated. As controls, T cells were stained before culture. The data show staining on gated CD8$^+$ cells.

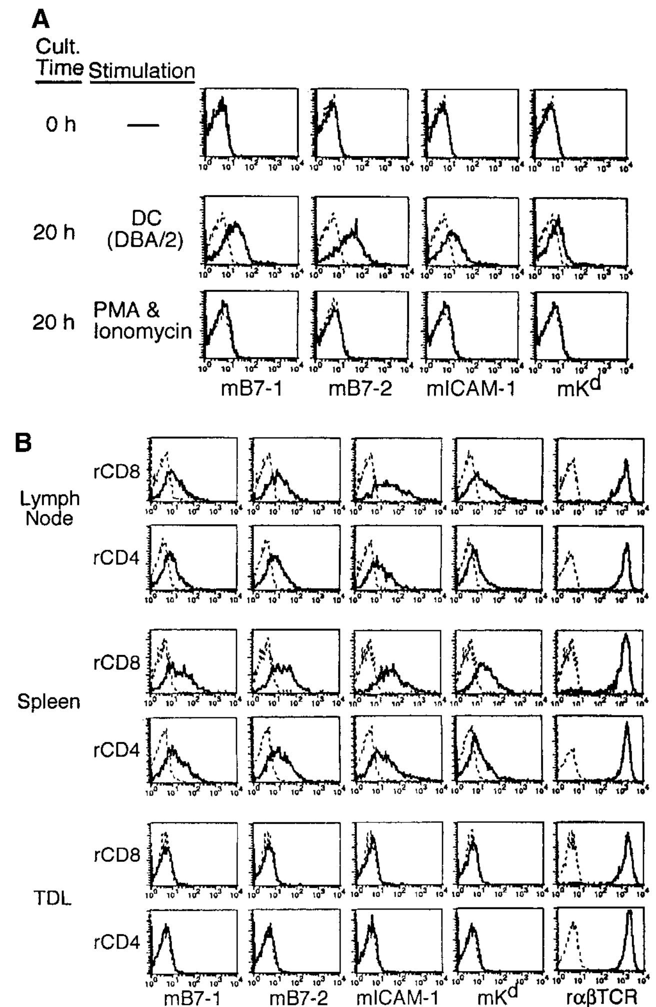

Figure 6. Transfer of cell surface molecules from mouse APCs to rat T cells. (A) Purified resting Lewis rat T cells were cultured for 20 h with purified DBA/2 (H2^d) DCs without peptide, treated with EDTA (see the legend to Fig. 5), and then stained for the markers shown. The data show staining on gated rCD8$^+$ cells; staining of fresh T cells and T cells cultured with PMA and ionomycin is shown as a control. (B) Purified rat T cells were transferred to irradiated SCID mice, recovered from spleen, LN, or thoracic duct lymph 3 d later, and stained for the markers indicated. These data show staining on gated rCD4$^+$ and rCD8$^+$ cells.

nary experiments, culturing purified naive Lewis rat T cells (a mixture of CD4$^+$ and CD8$^+$ cells) with mouse (DBA/2, H2^d) DCs for 3 h (not shown) or 20 h (Fig. 6 A) in vitro led to significant absorption of mB7-1, mB7-2, and mICAM-1 and marginal absorption of mK^d. Staining for these four molecules was negative when rat T cells were cultured alone or with PMA and ionomycin. Similar absorption of mouse molecules by rat T cells occurred when rat T cells were injected intravenously into irradiated (350 cGy) SCID (H2^d) mice (Fig. 6 B). Thus, when the rat cells were recovered from spleen or LNs of the SCID hosts 2–3 d later, CD4$^+$ and CD8$^+$ rat T cells (mostly blast cells) showed significant expression of mB7-1, mB7-2, mICAM-1, and mK^d. Interestingly, absorption of these molecules was undetectable on circulating cells, i.e., on rat T cells recovered from thoracic duct lymph.

Fate of Absorbed Molecules. In agreement with prior findings on transfected Chinese hamster ovary cells as APCs (39), culturing preactivated B6 CD28$^+$ mouse T cells with B7-1$^+$ Dros. APCs in the absence of peptide led to prominent conjugate formation within 10 min (Fig. 7 A, top). Significantly, conjugate formation was much lower with CD28$^{-/-}$ than CD28$^+$ T cells. With B7$^-$ APCs, e.g., L^d.ICAM-1 Dros. cells, conjugate formation was low with both CD28$^+$ and CD28$^{-/-}$ T cells (Fig. 7 A and data not shown). These findings imply that CD28-dependent uptake of B7 from APCs is a consequence of initial CD28–B7-dependent T–APC adhesion.

Cell-sorting experiments demonstrated that the absorbed B7-1 molecules found on T cells after short-term culture with B7-1$^+$ APCs in vitro disappeared rapidly from the cell surface. In the experiment shown in Fig. 7 A, bottom, preactivated normal B6 CD8$^+$ cells were first cultured at 37°C with L^d.B7-1 Dros. APCs (without peptide). After 1 h, CD8$^+$ cells in the cultures were purified by cell sorting and then cultured alone, i.e., without Dros. APCs. Staining ali-

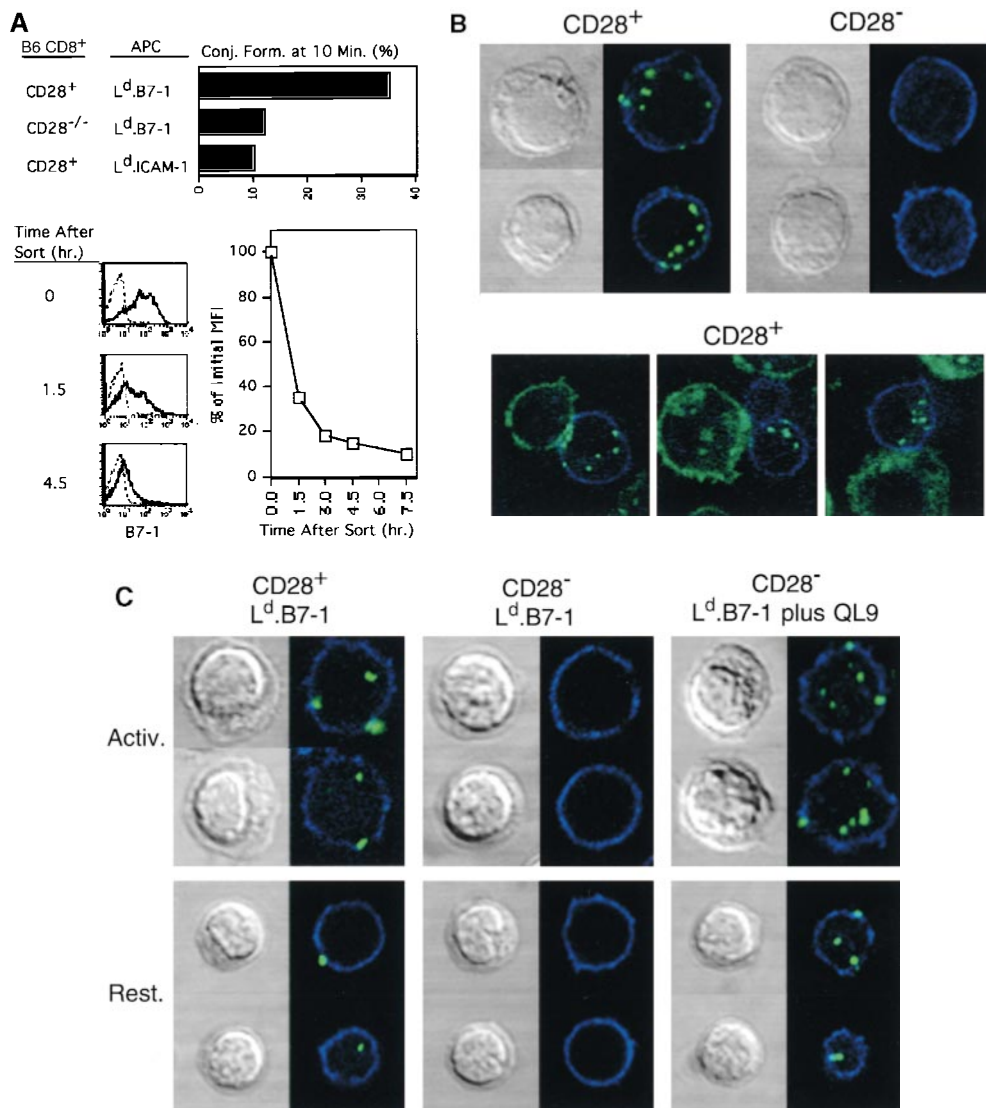

Figure 7. Fate of B7-1 molecules absorbed by T cells. (A) Top, Preactivated B6 CD8$^+$ cells were cultured with the indicated Dros. APCs in the absence of peptide for 10 min. T–APC conjugates were detected by FACS® analysis on the basis of combined CD8 expression and high side scatter (SSC). Percentage of CD8$^+$ cells forming the conjugates with Dros. APCs was plotted. Bottom, Preactivated B6 CD8$^+$ cells were cultured with L^d.B7-1 Dros. APCs for 1 h, then depleted of APCs by cell sorting for CD8 expression and cultured alone (at 37°C) for the intervals indicated before surface staining for B7-1. (B) Preactivated CD28$^+$ and CD28$^{-/-}$ B6 CD8$^+$ cells were incubated for 1 h with L^d.B7-1 Dros. APCs (without peptide), fixed, and stained for B7-1 (green, FITC) and CD8 (blue, Cy5). Two experiments are shown: (top) after culture in normal medium, cells were disrupted and then fixed and stained after adherence to poly-l-lysine–coated coverslips; (bottom) cells were cultured initially on poly-l-lysine–coated coverslips and then fixed and stained without disruption. (C) Preactivated or resting CD28$^+$ and CD28$^{-/-}$ CD8$^+$ 2C cells were incubated for 1 h with L^d.B7-1 Dros. APCs with or without QL9 peptide, stained for B7-1 and CD8 as in B, and then fixed. The data in the right panels show representative surface and intracellular staining seen in multiple slices of two representative cells as detected by confocal microscopy (see Materials and Methods). Nomarski images of the cells are shown on the left. In other experiments, staining the cells for B7 with two different fluorochromes showed that B7 staining before permeabilization was restricted to the cell membrane, whereas B7 staining after permeabilization was largely intracellular.

quots of these T cells at intervals showed that the MFI of B7-1 staining declined rapidly in culture and reached very low levels within 4–5 h (80–90% decrease in MFI).

The rapid disappearance of B7-1 from the cell surface could either reflect internalization or external shedding. To investigate the first possibility, the cells were examined by confocal microscopy. Here, preactivated normal CD28$^+$ versus CD28$^{-/-}$ CD8$^+$ cells were incubated with L^d.B7-1 Dros. APCs (without peptide) for 1 h, then allowed to bind to coverslips after fixation and staining for CD8 (blue) and B7-1 (green; Fig. 7 B, top). Nomarski images of the cells are shown in the left of each panel. For CD28$^+$ T cells, examining the stained cells by confocal microscopy (right) showed discrete punctate staining for B7-1 not only on the cell surface, but throughout the cytoplasm. Representative internal staining of two individual cells is shown; the pattern of ring staining for CD8 denotes the cell surface. In marked contrast, CD28$^{-/-}$ CD8$^+$ cells showed no staining for B7-1, either internally or on the cell surface. In other experiments, intracellular staining of nondisrupted conjugates formed between activated CD28$^+$ T cells and B7-1$^+$ Dros. APCs on coverslips showed conspicuous internal staining for B7-1 in T cells (Fig. 7 B, bottom). Hence, T cell internalization of B7-1 from APCs could occur under conditions where T cells and APCs were not physically disrupted.

The above data refer to CD28-mediated absorption of B7-1 by preactivated normal B6 CD8$^+$ cells. Essentially identical findings occurred with 2C CD8$^+$ cells (Fig. 7 C). Thus, with preactivated (top) and resting (bottom) 2C cells, incubation with L^d.B7-1 Dros. APCs without peptide led to clear surface and internal staining for B7-1 with CD28$^+$ cells but no staining with CD28$^{-/-}$ cells. However, when CD28$^{-/-}$ 2C cells were incubated with L^d.B7-1 Dros. APCs in the presence of QL9 peptide, both internal and ex-

ternal staining for B7-1 was clearly apparent. Hence internalization of absorbed B7-1 applied not only to CD28-dependent absorption but also to TCR-dependent uptake.

Discussion

As mentioned in the Introduction, there are several reports that T–APC interaction leads to the appearance of APC-derived molecules on T cells. Although this process was shown to be antigen specific, the mechanisms and receptor–ligand interactions involved were not resolved. As shown here, T cell uptake of molecules from APCs occurs very rapidly ($\leq$1 h), applies to a wide range of molecules on APCs, and is controlled by at least two different receptors on T cells, namely CD28 and TCRs.

The role of CD28 was defined by culturing normal (nontransgenic) $CD28^+$ versus $CD28^{-/-}$ T cells with $B7^+$ Dros. APCs or B6 DCs. With $CD28^+$ T cells, short-term culture with APCs in the absence of specific antigen led to rapid T cell uptake of B7 (B7-1) and other molecules from the APCs. By contrast, with $CD28^{-/-}$ T cells uptake of these molecules was undetectable with Dros. APCs and very low (though significant) with DCs. Hence, for Dros. APCs, uptake of B7 and other molecules by T cells appeared to be solely under the control of CD28.

The extent of T cell B7 absorption via CD28 correlated closely with the level of CD28 on T cells. Thus, with resting T cells, the stronger absorption of B7 by $CD4^+$ cells than $CD8^+$ cells correlated with substantially higher CD28 expression on $CD4^+$ cells than $CD8^+$ cells. Likewise, the marked increase in CD28 expression found on activated $CD4^+$ and $CD8^+$ cells led to a corresponding increase in B7 uptake from APCs. It is of interest that both for resting and activated T cells, uptake of B7 from APCs was associated with a marked decrease in CD28 staining. The simplest explanation for this finding is that the CD28 epitopes recognized by anti-CD28 mAb are occluded after binding of B7 to CD28.

The observation that nonantigen-specific T cell uptake of molecules from APCs is primarily under the control of CD28 is surprising because CD28 is generally viewed as a signaling molecule (3, 40–42). Evidence that CD28 can act as an adhesion molecule is sparse, although CD28-transfection of Chinese hamster ovary cells was shown to induce B7-specific adhesion to B cells (39). As cell–cell adhesion is thought to be largely under the control of integrins such as LFA-1 (43, 44), T cell uptake of APC-derived molecules would be expected to involve LFA-1–ICAM-1 interaction. However, this was not the case. Thus, even with activated T cells, uptake of ICAM-1 from Dros. APCs was undetectable unless these cells coexpressed B7. Nevertheless, it is of interest that CD28-dependent uptake of ICAM-1 from $B7^+$ APCs was partially reduced by anti–LFA-1 mAb. This finding implies that LFA-1 can augment or stabilize T cell uptake of ICAM-1 from APCs, but only when ICAM-1 is absorbed indirectly via CD28–B7 interaction. For uptake of molecules from APC, the affinity and/or avidity of LFA-1–ICAM-1 interaction thus appeared to be substantially weaker than for CD28–B7 interaction. The data on nonantigen-specific conjugate formation are consistent with this notion. Thus, with activated T cells, prominent conjugate formation was seen only with $CD28^+$ T cells and $B7^+$ APCs (Fig. 7 A); with $CD28^{-/-}$ ($LFA\text{-}1^+$) T cells, conjugate formation was low with both $B7^+$ $ICAM\text{-}1^-$ and $B7^-ICAM\text{-}1^+$ APCs, implying poor conjugate formation via LFA-1–ICAM-1 interaction.

It should be emphasized that CD28-mediated absorption of molecules from APCs also applied to DCs, i.e., to conventional APCs. However, with these APCs, absorption was not totally CD28 dependent. Thus, short-term culture of $CD28^{-/-}$ B6 $CD8^+$ cells with normal B6 DCs led to low but significant uptake of IA molecules. This uptake was ~10-fold lower than with $CD28^+$ $CD8^+$ cells. These findings with DCs indicate that nonantigen-specific uptake of APC-derived molecules by T cells is not an exclusive property of CD28–B7 interaction. Apparently other, as yet unknown, receptor–ligand interactions are also involved.

Demonstrating TCR-mediated uptake of molecules from APCs by TCR transgenic T cells was complicated by concomitant absorption via CD28–B7 interaction. This problem was avoided by using $CD28^{-/-}$ T cells. Thus, when either resting or activated $CD28^{-/-}$ 2C $CD8^+$ cells were cultured with L^d.B7-1 Dros. APCs, T cell uptake of these two molecules was conspicuous providing specific QL9 peptide was added to the cultures. By contrast, in the absence of peptide, uptake of these molecules by 2C cells was very low or undetectable. Confirming that uptake was TCR mediated, QL9-dependent 2C absorption of both L^d and B7-1 from Dros. APCs was almost totally inhibited by 1B2 anticlonotypic mAb. Curiously, no inhibition was seen with Vβ-specific F23.2 mAb, presumably indicating that the TCR epitopes detected by this mAb are distant from the sites involved in TCR–peptide–MHC interaction.

With $CD28^+$ 2C cells, TCR-mediated absorption from APCs by activated 2C cells was largely obscured by concomitant strong CD28-mediated absorption. However, this problem was much less noticeable with resting $CD28^+$ 2C cells. With these cells, CD28-mediated absorption was limited (reflecting the low level of CD28 on resting $CD8^+$ cells) and led to only low nonantigen-specific absorption when 2C cells were cultured with Dros. APCs in the absence of peptide. However, with addition of QL9 peptide, uptake of L^d and other molecules from APCs was conspicuous. As with $CD28^{-/-}$ 2C cells, peptide-dependent uptake of these molecules by resting $CD28^+$ 2C cells was blocked by 1B2 mAb.

It is notable that both for CD28–B7 and TCR–peptide–MHC interaction, T cell absorption from APCs applied not only to the particular ligand recognized, but to a spectrum of other molecules on APCs. As such binding applied with several types of APC (Dros. cells, DCs, and LPS blasts), and was also conspicuous under in vivo conditions (in rat to mouse chimeras), artifact seems unlikely. However, precisely how molecules are transferred from APCs to T cells is still unclear. Perhaps the simplest idea is that T

cells bind APC-derived molecules in the form of detached membrane fragments as vesicles. Against this idea we have seen only minimal CD28–B7-mediated uptake of APC-derived molecules when T cells and APCs are separated by a semipermeable (0.2 μM) membrane, or when T cells are incubated with either APC sonnicates or supernatants from live APCs (our unpublished data). These findings suggest that T cell absorption of molecules from APCs may require direct cell–cell contact (see below). Here it should be mentioned that we have observed only very limited absorption of B7 from paraformaldehyde-fixed APCs (our unpublished data), which suggests that B7 absorption involves a dynamic process and requires a normal APC membrane. An obvious concern is that T cell binding of APC-derived molecules reflects the sheer forces involved in physically separating T cells and APCs for FACS® analysis. This possibility is unlikely because at the end of the short-term culture period used (1 h), microscopic examination of T–APC cultures revealed surprisingly little evidence of aggregate formation, especially with resting T cells, and the cells were easily dispersed. It is also worth noting that at least for Dros. APCs, physically separating these cells from T cells did not obviously impair APC viability, implying that separating the cells did not induce "holes" in the APC membrane, even though contact with T cells removed 10–20% of B7 molecules from the cells.

With regard to the fate of the absorbed material, short-term culture of T cells with APCs followed by culture under APC-free conditions showed that most of the material absorbed by T cells disappeared from the cell surface within 3 h. Using confocal microscopy, the key observation was that at least for B7, the absorbed material was internalized, both for TCR- and CD28-mediated absorption. This internalization also occurred when T cells and APCs were not physically disrupted. With regard to other molecules, in recent studies we have observed peptide-dependent internalization of L^d molecules when 2C cells are incubated with Dros. APCs transfected with green-fluorescent protein–labeled L^d molecules (GFP.L^d; 45). As for B7-1, such internalization of L^d molecules by 2C cells is apparent under conditions where T–APC conjugates are not disrupted. These latter studies have shown that internalized L^d molecules are rapidly degraded in lysosomes. Whether the same applies to B7 has yet to be examined. For GFP.L^d, it is notable that T cell internalization is almost entirely restricted to T cells forming conjugates with APCs (our unpublished data). This finding provides further support for the view that transfer of molecules from APCs to T cells requires direct cell contact (see above).

With regard to biological significance, it is now well documented that T–APC interaction can lead to rapid internalization of the TCR (46, 47). This process is thought to allow TCR molecules to dissociate from MHC–peptide complexes, and after recycling to the cell membrane, allow TCR molecules to engage in serial triggering (47). However, our finding that TCR internalization is accompanied by internalization of MHC molecules (presumably as MHC–peptide complexes) and also of B7 and probably several other molecules suggests an alternative possibility. Thus, one purpose of internalization of TCR and various APC-derived molecules at the site of T–APC contact could be simply to terminate cell–cell interaction and allow the cells to dissociate. In favor of this idea, we have found that T–APC dissociation in culture occurs more slowly with paraformaldehyde-fixed APCs than with unfixed APCs (our unpublished data). This finding correlates with reduced T cell uptake of molecules from fixed APCs. For T cells, interrupting tight contact with APCs could be crucial for enabling activated T cells to find other APCs and eventually make their way into the circulation for dissemination as effector cells. Here, it is of interest that absorption of mouse molecules by rat T cells transferred to SCID mice was apparent only in the lymphoid tissues and not in thoracic duct lymph.

The notion that T cell internalization of APC-derived molecules is important for T–APC dissociation has yet to be proved and several other possibilities need to be considered. First, based on studies on eye development in insects (48, 49), it is conceivable that internalization of cell surface ligands may contribute to intracellular signaling. Whether this notion is applicable to mammalian cells is unknown. Second, ingestion of peptide–MHC complexes by T cells could serve to guard against overstimulation of T cells and tolerance via exhaustion; consistent with this idea, we have found that decreased T–APC dissociation observed with fixed APCs correlates with much higher T proliferative responses (our unpublished data). Finally, T cell uptake of peptide–MHC complexes from APCs could also have negative consequences. Thus, in recent studies we have found that T cells absorbing peptide–MHC complexes from APCs can become targets for CTLs (45). Such fratricidal killing of T cells may contribute to the massive elimination of T cells found in high dose viral infections (50).

In conclusion, the data in this paper demonstrate that T–APC interaction can lead T cells to absorb and ingest various cell surface molecules from APCs. This process is primarily under the control of CD28–B7 and TCR–peptide–MHC interactions. The biological significance of this phenomenon is still unclear and will have to await further investigation.

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Note added in proof. After the submission of this paper, other workers (Patel, D.M., P.Y. Arnold, G.A. White, J.P. Nardella, and M.D. Mannie. 1999. Class II MHC/peptide complexes are released from APC and are acquired by T cell responders during specific antigen recognition. *J. Immunol.* 163:5201–5210) have reported TCR-mediated transfer of various molecules from APC to the surfaces of T cells.

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