

Differing Roles for B7 and Intercellular Adhesion Molecule-1 in Negative Selection of Thymocytes

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Summary

To ensure self tolerance, immature thymocytes with high binding affinity for self peptides linked to major histocompatibility complex (MHC) molecules are eliminated in situ via apoptosis (negative selection). The roles of two costimulatory molecules, B7-1 and intercellular adhesion molecule-1 (ICAM-1), in negative selection was examined by studying apoptosis of T cell receptor transgenic CD4⁺8⁺ thymocytes cultured with specific peptides presented by MHC class I-transfected *Drosophila* cells. When coexpressed on these cells, B7-1 and ICAM-1 act synergistically and cause strong class I-restricted negative selection of thymocytes. When expressed separately, however, B7-1 and ICAM-1 display opposite functions: negative selection is augmented by B7-1, but is inhibited by ICAM-1. It is notable that B7-1 is expressed selectively in the thymic medulla, whereas ICAM-1 is expressed throughout the thymus. Because of this distribution, the differing functions of B7-1 and ICAM-1 may dictate the sites of positive and negative selection. Thus, in the cortex, the presence of ICAM-1, but not B7-1, on the cortical epithelium may preclude or reduce negative selection and thereby promote positive selection. Conversely, the combined expression of B7-1 and ICAM-1 may define the medulla as the principal site of negative selection.

Stimulation of mature T cells is controlled by bone marrow-derived APC and requires TCR contact with peptide/MHC complexes plus interaction with costimulatory molecules such as B7 (B7-1 or B7-2) and intercellular adhesion molecule-1 (ICAM-1¹; 1–3). This requirement for joint contact with peptide/MHC complexes and costimulatory molecules also applies to negative selection (deletion) of immature T cells in the thymus (4–10). It is uncertain, however, which particular costimulatory molecules are required for negative selection.

Using a TCR transgenic mouse model and transfected *Drosophila* cells as APC, we show here that the importance of costimulation for negative selection is variable and is influenced by the binding affinities involved in TCR/peptide/MHC interaction. In addition, evidence is presented that two costimulatory molecules, B7-1 and ICAM-1, can play opposite roles in negative selection.

Materials and Methods

Mice. 2C TCR transgenic mice were originally obtained from Dr. D. Loh (Nippon Roche Research Center, Kamakura-shi, Ja-

pan) (11), and were bred, back-crossed to $\beta 2$ microglobulin^{-/-} ($\beta 2m^{-/-}$) mice, and maintained at The Scripps Research Institute.

Antibodies. Antibodies specific for CD8 (3.163.8, rat IgM) and CD3 (C363.29B, rat IgG) were previously described (12). Rabbit anti-mouse Bcl-X₁ and Bax were kindly provided by Dr. John Reed (La Jolla Cancer Research Foundation, La Jolla, CA) (13, 14). The following mAbs were purchased from PharMingen (San Diego, CA); anti-Bcl-2 (3F11, hamster IgG), anti-lymphocyte function antigen-1 (LFA-1) (M17/4, rat IgG), anti-CD43 (S7, rat IgG), and anti-ICAM-1 (3E2, hamster IgG). FITC- or Red613-conjugated anti-Thy 1.2 (30H12, rat IgG) and Red613-conjugated anti-CD8 (53-6.7, rat IgG) mAbs were purchased from GIBCO BRL (Gaithersburg, MD). Goat anti-rat Ig and FITC-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Peptides. Two peptides p2Ca (sequence: LSPFPFDL) and QL9 (QLSPFPFDL) were used in this study (15–19). Peptides were synthesized on a synthesizer (model 431 A; Applied Biosystems, Inc., Foster City, CA), and were purified with C18 reverse-phase high performance liquid chromatography.

Cell Lines and Cell Purification. *Drosophila* APC were constructed by transfecting Schneider SC2 cells with cDNA for L^d, $\beta 2$ -microglobulin ($\beta 2m$), B7-1, and/or ICAM-1 as described elsewhere (reference 20 and Cai, Z., A. Brunmark, M.R. Jackson, P.A. Peterson, and J. Sprent, manuscript submitted for publication); control cells were transfected with cDNA for D^b and ICAM-1. Cell-surface expression was moderate and no higher than on normal spleen cells. For purification of CD4⁺8⁺ (double-positive

¹Abbreviations used in this paper: DP, double positive; ICAM-1, intercellular adhesion molecule-1; LFA-1, leukocyte function antigen-1.

[DP]) thymocytes, thymocytes from $\beta 2m^{-/-}$ 2C TCR transgenic mice were depleted of contaminating TCR^{high} single-positive cells by treatment with anti-CD3 mAb and guinea pig C for 45 min at 37°C, followed by positive panning on anti-CD8 mAb-coated plates (21); the resulting TCR^{low} DP cells rapidly upregulate TCR expression when cultured in vitro and behave indistinguishably from unseparated $\beta 2m^{-/-}$ 2C thymocytes. Since contamination with TCR^{high} CD4⁻8⁺ cells in $\beta 2m^{-/-}$ 2C thymocytes is minimal, unseparated $\beta 2m^{-/-}$ 2C thymocytes were used in some experiments. The results obtained with these cells were essentially the same as for purified DP cells.

Culture Conditions. 2C DP thymocytes were cultured for 20 h at 5×10^5 /well with 2×10^5 transfected *Drosophila* cells in 0.2 ml of RPMI medium supplemented with 5×10^{-5} M 2-ME, L-glutamine, and 10% FCS; peptides were added at 10 μ M. For the studies involving soluble anti-LFA-1 and anti-CD43 mAbs, 2C DP thymocytes were pretreated with 10 μ g/ml of either anti-LFA-1 or anti-CD43 or both mAbs for 30 min, washed, and then cultured with L^d.ICAM APC plus QL9. For soluble anti-ICAM mAb, L^d.ICAM or L^d APC were pretreated with 10 μ g/ml of anti-ICAM-1 mAb for 30 min, washed, and then added to culture with 2C DP thymocytes plus QL9. For the studies involving cross-linked antibodies, cultures containing antibody-pretreated DP cells or APC were supplemented with 5 μ g/ml of goat anti-rat Ig; this antibody, which cross-reacts with hamster Ig, was left in the cultures until TUNEL staining.

Staining for Cell Surface and Intracellular Molecules. For cell-surface staining, cells were incubated with FITC-conjugated anti-CD69 and Red613-conjugated anti-Thy1.2 mAbs, and fixed with 70% ethanol and 1% paraformaldehyde in PBS. For Bcl-2, Bcl-X_L, and Bax staining, cells were first stained with Red613-conjugated anti-Thy1.2 mAb, fixed with 1% paraformaldehyde in PBS containing 0.1% glutaraldehyde, 0.02% Tween 20, then TUNEL stained. Thereafter, the cells were incubated with either anti-Bcl-2, rabbit anti-mouse-Bcl-X_L, or rabbit anti-mouse-Bax antibodies, and then stained with FITC-conjugated goat anti-hamster Ig. Cells were analyzed on a FACScan[®] (Becton Dickinson & Co., Mountain View, CA), gating on Thy1.2⁺ and TUNEL⁻ cells.

TUNEL Assay and DNA Staining. TUNEL assay and DNA staining by propidium iodide were described previously (21, 22).

Results and Discussion

The requirements for inducing negative selection of thymocytes were investigated with the aid of the MHC class I-restricted 2C transgenic line as a source of thymocytes, defined peptides as antigen, and MHC class I-transfected *Drosophila* cells as APC. *Drosophila* cells have two advantages for studying APC function. First, as expected from their distant relationship to mammalian T cells, the native cell-surface molecules on *Drosophila* cells do not display costimulatory function for mature T cells (Cai, Z., A. Brunmark, M.R. Jackson, P.A. Peterson, and J. Sprent, manuscript submitted for publication. Second, *Drosophila* cells lack TAP-1,2 peptide transporters (20, 23), which means that, after transfection, the class I molecules on the surface of these cells can be loaded with high concentrations of exogenous peptides (20).

Drosophila cells were transfected with MHC class I (L^d),

B7-1 (B7), or ICAM-1 (ICAM) genes linked to the metallothionein promoter (20) (Materials and Methods). These cells were then tested for their capacity to present peptide antigens to immature thymocytes taken from 2C TCR transgenic mice (11). The 2C line displays strong reactivity to L^d molecules complexed with an 8-mer peptide, p2Ca, derived from a Krebs cycle enzyme (15, 16). This natural peptide has intermediate affinity for soluble L^d molecules (4×10^6 M⁻¹) and (when complexed to L^d) high affinity for 2C TCR molecules (2×10^6 M⁻¹ to 1×10^7 M⁻¹) (17–19). A closely related 9-mer peptide, QL9, has even higher affinity for these molecules (2×10^8 M⁻¹ for L^d and 2×10^7 M⁻¹ for 2C TCR); QL9 is derived from the same self protein as p2Ca and, except for one additional amino acid, QL9 has the same sequence as p2Ca (16).

To study negative selection, purified CD4⁺8⁺ thymocytes from 2C mice were incubated in vitro with p2Ca or QL9 peptides presented by a panel of transfected *Drosophila* cells expressing L^d alone (L^d APC), L^d plus B7-1 (L^d.B7 APC), L^d plus ICAM-1 (L^d.ICAM APC), or L^d plus B7-1 and ICAM-1 (L^d.B7.ICAM APC). Negative selection was measured by quantitating T cell apoptosis using TUNEL staining and FACS[®] (Becton Dickinson & Co.) analysis (21); this method relies on the fact that through DNA strand breaks, apoptotic cells incorporate exogenous nucleotides (dUTP) in the presence of terminal deoxynucleotidyl transferase (22). As expected, incubating CD4⁺8⁺ 2C thymocytes with QL9 peptide and L^d-transfected *Drosophila* cells coexpressing both B7 and ICAM causes strong negative selection (apoptosis) relative to control cells (transfected *Drosophila* cells not induced with CuSO₄; Fig. 1 a).

Data on negative selection and CD69 expression induced by the panel of L^d-transfected *Drosophila* cells and p2Ca vs QL9 peptides (10 μ M) are shown in Fig. 1 b; the data are the mean of three separate experiments, and the level of background apoptosis found with uninduced *Drosophila* cells has been subtracted. The data make four points. First, with APC expressing L^d alone, the strong QL9 peptide induces a low but significant level of negative selection (apoptosis) associated with upregulation of CD69 on the surviving (TUNEL⁻) cells; by contrast, the weaker p2Ca peptide induced neither CD69 expression nor negative selection. Second, with L^d.B7 APC, the presence of B7-1 augments negative selection to QL9 and reveals low but significant negative selection to p2Ca; both peptides induce strong CD69 expression. Third, L^d.ICAM APC paradoxically induce strong CD69 expression but fail to cause negative selection, either to QL9 or to p2Ca. Fourth, with L^d.B7.ICAM APC, negative selection to both peptides is stronger than with L^d.B7 APC.

Two aspects of the above data are surprising: (a) the capacity of L^d APC to elicit apoptosis to the strong QL9 peptide; and (b) the lack of apoptosis found with L^d.ICAM APC. Further data on these two points are discussed below.

The low but significant level of apoptosis induced by L^d APC plus QL9 is associated with weak upregulation of Bcl-2 (contrasting with strong CD69 expression) and no change

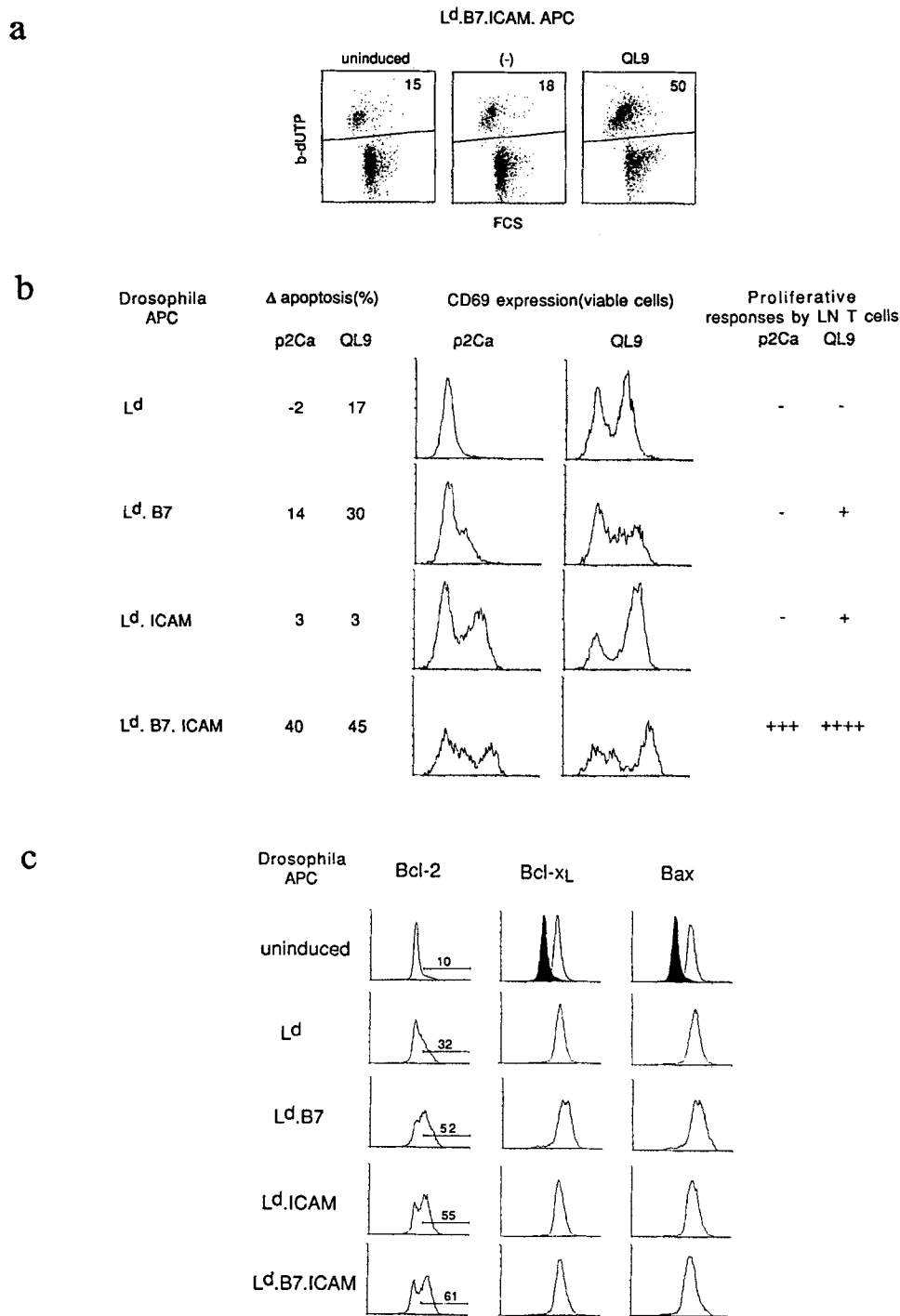
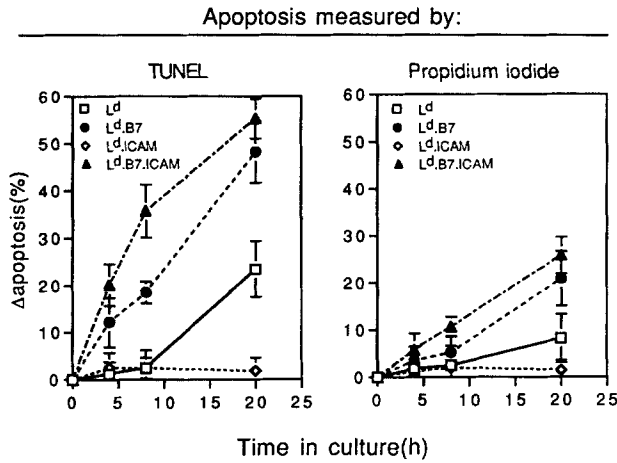


Figure 1. Negative selection of immature 2C thymocytes by transfected *Drosophila* cells. Purified CD4⁺8⁺ thymocytes were prepared from (β2m^{-/-} 2C mice and cultured in vitro overnight (16–24 h) with transfected *Drosophila* cells in the presence of the peptides (10 μM) shown (Materials and Methods); apoptosis was measured by TUNEL staining and FACS® analysis. (a) An example of thymocyte apoptosis (TUNEL staining) induced by culturing 2C thymocytes with L^d.B7.ICAM *Drosophila* cells overnight with or without QL9 peptide. *Drosophila* cells not induced with CuSO₄ were used as a negative control; in other experiments, background apoptosis of thymocytes cultured overnight with uninduced *Drosophila* cells was generally a little higher, i.e., 20–25%. (b) Comparison of apoptosis and CD69 expression induced by culturing 2C thymocytes with transfected *Drosophila* cells with p2Ca vs. QL9 peptides. For apoptosis, the data represent the mean of three separate experiments; background apoptosis observed with uninduced *Drosophila* cells has been subtracted. CD69 expression is shown for viable (TUNEL⁻) cells. The data at the right summarize the capacity of transfected *Drosophila* APC to induce purified CD8⁺ LN 2C cells to mount proliferative responses in vitro to p2Ca and QL9 peptides in the absence of added cytokines. (c) Expression of Bcl-2, Bcl-X_L, and Bax on 2C thymocytes after culture with the *Drosophila* APC shown plus QL9 peptide (10 μM). The data show staining of viable (TUNEL⁻) cells. For Bcl-2, the proportion of stained cells relative to background staining with normal hamster Ig is shown; for Bcl-X_L and Bax, control staining with normal rabbit serum is shown in the filled histograms.

in the expression of Bcl-X_L or Bax (Fig. 1 c); these three members of the Bcl-2 family are known to inhibit or augment apoptosis in other systems (24). QL9-induced apoptosis with L^d APC is most apparent late in culture (Fig. 2 a, left), and is more easily detectable by TUNEL staining (Fig. 2 a, left) than with the less-sensitive propidium iodide technique (which detects cells with subdiploid DNA) (Fig. 2 a, right). Apoptosis elicited by L^d APC is only seen with relatively high doses of QL9 peptide, i.e., ≥10 nM (10⁸ M⁻¹)

(Fig. 2 b), and is undetectable with up to 10 μM of the weaker p2Ca peptide (Fig. 1 b); these findings contrast with L^d.B7.ICAM APC, where apoptosis to QL9 is apparent with as low as 10 fM (10¹⁴ M⁻¹) (Fig. 2 b). Apoptosis with L^d APC is thus only seen with high doses of an extremely high affinity peptide. The implication therefore is that the requirement for costimulation in negative selection can be partly overcome by very strong signaling through the TCR. This differs from mature T cells where 2C LN

a



b

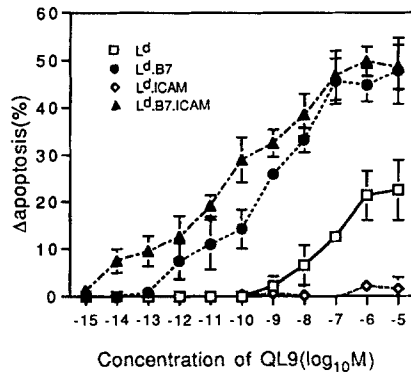


Figure 2. Kinetics and dose of QL9 peptide required for negative selection of 2C CD4⁺8⁺ thymocytes with *Drosophila* APC. (a) Kinetics of apoptosis induced by 10 mM QL9 peptide as measured by TUNEL staining (left) and staining for subdiploid DNA with propidium iodide (right). (b) Concentration of QL9 peptide required to induce apoptosis as measured by TUNEL staining at 20 h.

CD8⁺ cells cannot be activated by L^d APC plus QL9; data on the capacity of the panel of transfected *Drosophila* cells to stimulate mature 2C T cells (Cai, Z., A. Brunmark, M.R. Jackson, P.A. Peterson, and J. Sprent, manuscript submitted for publication) are summarized in Fig. 1 b (right).

With regard to L^d.ICAM APC, the data in Figs. 1 and 2 indicate that B7-1 and ICAM-1 have distinctly different roles in negative selection. When coexpressed, these two costimulatory molecules act synergistically to induce strong negative selection to L^d plus peptides. However, when expressed individually, ICAM-1 protects against negative selection to L^d plus peptides, whereas B7-1 augments negative selection. These differences are not apparent at the level of mature T cells: thus L^d.B7 and L^d.ICAM APC both induce low but significant proliferative responses of LN CD8⁺ cells to QL9 (but not to p2Ca) in the absence of added cytokines (Fig. 1 b, right). For negative selection, the opposing functions of B7-1 and ICAM-1 are not associated with differences in CD69 expression (see above, Fig. 1 b)

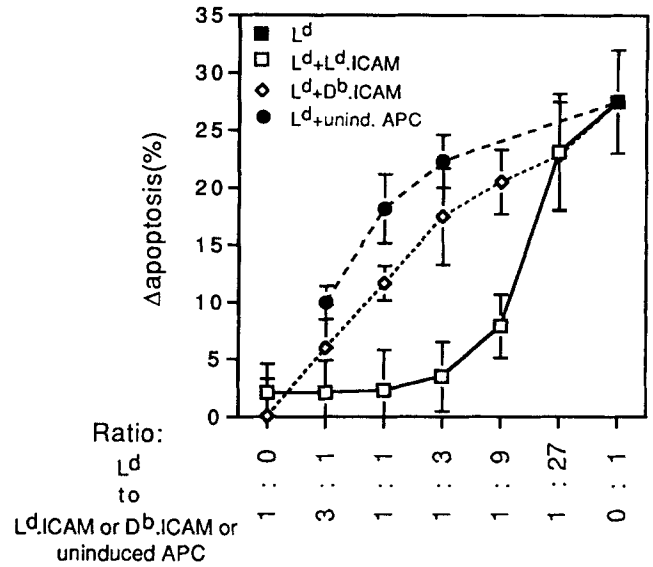


Figure 3. Capacity of L^d.ICAM APC to inhibit apoptosis induced by L^d APC. Using a fixed total dose of 2×10^5 *Drosophila* APC/well, 2C CD4⁺8⁺ thymocytes (5×10^5 /well) were cultured with QL9 peptide (10 μM), and various numbers of L^d APC supplemented with L^d.ICAM, D^b.ICAM, or uninduced APC. Apoptosis was measured by TUNEL staining at 20 h. It should be noted that QL9 peptide has no detectable capacity to bind to D^b molecules.

or in expression of Bcl-2 and related molecules; thus, L^d.ICAM and L^d.B7 APC both cause equivalent and moderately strong upregulation of Bcl-2 on TUNEL⁻ cells and no change in Bcl-X_L or Bax expression (Fig. 1 c).

The capacity of L^d.ICAM APC to protect against apoptosis is apparent in mixing experiments with L^d APC. Thus, apoptosis induced by L^d APC plus QL9 is reduced to near-background levels by addition of small numbers of L^d.ICAM APC (Fig. 3). This effect is less apparent with “bystander” D^b.ICAM APC (Fig. 3, see legend), implying that inhibition of apoptosis is most efficient when L^d and ICAM-1 are coexpressed on the same APC. (It could be argued that 2C DP cells bind preferentially to L^d.ICAM APC plus peptide, thus depriving T cells from contact with L^d APC. This possibility is unlikely because conjugate formation in the cultures is low, and is no higher with L^d.ICAM APC than with L^d APC).

The inhibition of apoptosis induced by L^d.ICAM APC presumably reflects recognition of ICAM-1 by the T cell counterreceptors for ICAM-1, i.e., by LFA-1 (25) or CD43 (26). In support of this notion, the failure of L^d.ICAM APC to induce apoptosis to QL9 can be overcome by adding soluble mAbs to ICAM-1, LFA-1, or CD43 (Fig. 4 a); antibodies to LFA-1 and CD43 are more effective as a mixture than when added separately. The restoration of apoptosis induced by soluble anti-ICAM mAb also applies when this antibody is cross-linked with anti-rat Ig (Fig. 4 b). Interestingly, cross-linking anti-LFA-1 or anti-CD43 mAbs with anti-RIg has the opposite effect and reduces apoptosis to low levels (Fig. 4 b). The simplest ex-

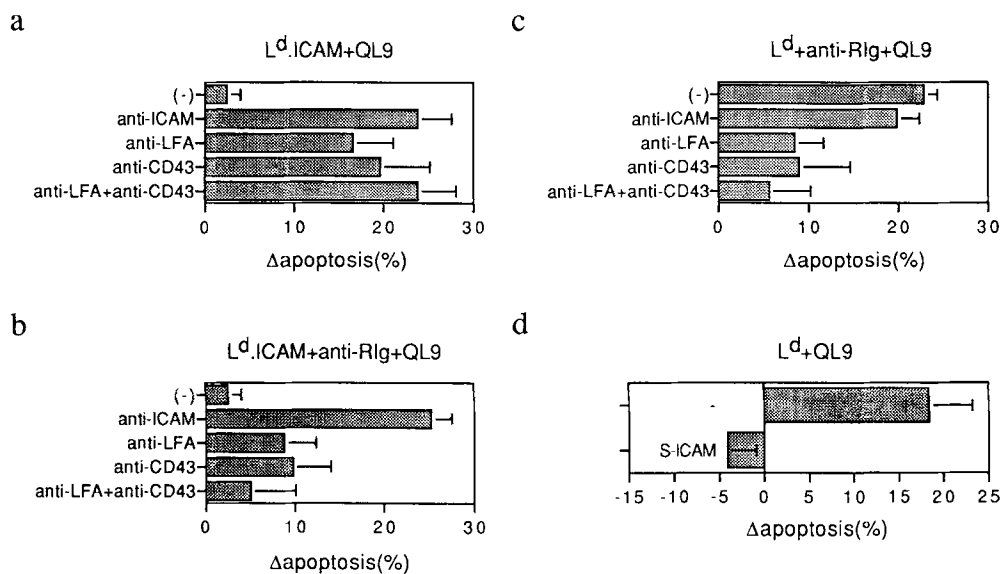


Figure 4. Protective role of ICAM-1 in negative selection to QL9 peptide. 2C CD4⁺8⁺ thymocytes (5×10^5 /well) were cultured with L^d.ICAM or L^d APC (2×10^5 /well) plus QL9 peptide (10 μ M) in the presence of the reagents shown and then TUNEL stained after 20 h. (a and b) Apoptosis induced by L^d.ICAM APC plus QL9 in the presence of soluble (a) or cross-linked (b) antibodies specific for ICAM-1, LFA-1, or CD43. (c and d) Apoptosis induced by L^d APC plus QL9 in the presence of cross-linked antibodies specific for ICAM-1, LFA-1, or CD43 (c) or in the presence of soluble ICAM-1 (S-ICAM) (d).

planation for this finding is that cross-linking LFA-1 or CD43 molecules on T cells leads to active signaling and thus creates the same protective signal received from ICAM-1 on L^d.ICAM APC. If so, the apoptosis induced by L^d APC plus QL9 should be inhibited (a) by a mixture of cross-linked anti-LFA-1 and anti-CD43 mAbs; or (b) by soluble ICAM-1. This is indeed the case (Fig. 4, c and d).

In considering the significance of the different functions of ICAM-1 and B7-1 in negative selection, it is of interest that ICAM-1 is expressed throughout the thymus, whereas B7-1 is expressed selectively in the medulla (27–30). The implication therefore is that when CD4⁺8⁺ cells undergo positive selection in the cortex through contact with epithelial cells, immature T cells are exposed to MHC/peptides in conjunction with ICAM-1, but not B7-1. Such contact may reduce negative selection and thereby promote positive selection. When the maturing T cells enter the medulla, however, the combined expression of B7-1 and ICAM-1 on marrow-derived APC is conducive to strong negative selection and thus ensures self-tolerance induction.

The capacity of ICAM-1 (without B7-1) to protect against TCR-mediated apoptosis of thymocytes has a precedent in the finding that LFA-1/ICAM-1 interaction inhibits IgM-mediated apoptosis of germinal center B cells and B lymphoma cells (31, 32). How signaling via LFA-1 prevents apoptosis is unclear. In the case of thymocytes, it is

of interest that combined signaling via CD3 and LFA-1 inhibits glucocorticoid-induced apoptosis (33). Hence, in addition to inhibiting negative selection, ICAM-1 expression on cortical epithelium may facilitate positive selection by inducing steroid resistance and thus prevent “death by neglect” from endogenous steroids.

It should be emphasized that the capacity of B7-1 or B7-1 plus ICAM-1 to cause strong negative selection of thymocytes in vitro does not necessarily indicate that these molecules play a mandatory role in negative selection under normal physiological conditions in vivo. Other molecules such as Fas (34), CD40 (8), CD30 (35), and their respective ligands may play an equally (or more) important role under in vivo conditions. Since the range of costimulatory molecules expressed in the thymus is considerable, many different costimulatory molecules may participate in negative selection. If so, it is perhaps not surprising that eliminating individual molecules, e.g., CD28 (the counter-receptor for B7), fails to demonstrably impair negative selection in vivo (36). Because of this problem of “redundancy,” reaching a clear appreciation of negative selection in vivo may be difficult and could hinge on determining which particular costimulatory molecules can induce or augment negative selection under defined conditions in vitro. Testing the function of a variety of different costimulatory molecules with the *Drosophila* system described here could thus be informative.

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