T Cell Receptor-independent CD2 Signal Transduction in FcR$^+$ Cells

By Antonio R. N. Arulanandam,* Shigeo Koyasu,* and Ellis L. Reinherz‡

*From the Laboratory of Immunobiology, Dana-Farber Cancer Institute, and the Departments of *Pathology and ‡Medicine, Harvard Medical School, Boston, Massachusetts 02115*

Summary

CD2 subserves both adhesion and signal transduction functions in T cells, thymocytes, and natural killer (NK) cells. In mature T lymphocytes, CD2-mediated signaling function apparently requires surface expression of T cell receptors (TCRs). In contrast, in CD2$^+$CD3$^-$ NK cells and thymocytes, signal transduction through CD2 is TCR independent. To resolve this paradox and characterize TCR-independent triggering mechanisms, we transfected a human CD2 cDNA into a murine mast cell line, C1.MC/57 (FcεRI$^+$, FcγRII$^+$, FcγRIII$^+$), which is known to produce interleukin 6 (IL-6) as well as release histamine in response to crosslinking of FcεRI. In the CD2 transfectant, a combination of anti-T11$_2$ + anti-T11$_3$ monoclonal antibodies (mAbs) induced a rise in intracellular free calcium ([Ca^{2+}]$_i$), IL-6 production, and histamine release. As expected, no activation was mediated by the same mAbs in C1.MC/57. F(ab)'$_2$ fragments of the activatory combination of anti-T11$_2$ + anti-T11$_3$ mAbs induced IL-6 in the CD2-transfected mast cells, demonstrating an Fcγ receptor ectodomain-independent triggering mechanism. In addition, either intact anti-T11$_2$ or anti-T11$_3$ IgG alone, which failed to induce [Ca^{2+}]$_i$ mobilization in the transfectant, was able to induce IL-6 production. A mAb directed against both FcγRII (previously denoted as FcγRIIb) and FcγRIII (previously denoted as FcγRIIa) inhibits this induction. These results indicate that: (*a*) Ca^{2+} mobilization is not essential for IL-6 production; and (*b*) crosslinking of CD2 and Fcγ receptors via intact anti-CD2 IgG stimulates IL-6 production. Thus, CD2-mediated IL-6 production occurs by both Fc receptor ectodomain-independent as well as Fc receptor ectodomain-dependent mechanisms in these nonlymphoid cells. Northern blot analysis demonstrates that although the mast cells do not express CD3ζ or CD3η mRNA, they express FcεRIγ mRNA. The latter is a known component of FcγRIII as well as FcεRI, has significant homology to CD3ζ/η, and is thought to have a signal transduction function. In these mast cells, CD2 signaling machinery does not require CD3ζ/η and may be linked to the FcεRIγ subunit. We predict that this subunit or a related structure may confer a TCR-independent signal transduction pathway upon CD2 in CD3$^-$ NK cells, thymocytes, and certain B lymphocytes.

The CD2 (T11 structure) has been shown to play an important role in the function of both thymus-derived T cells and NK cells (1, 2). mAbs directed against an epitope (T11$_1$) within the NH_2-terminal adhesion domain of CD2 block the ability of T cells (3–5), thymocytes (6), and NK cells (4) to interact with the LFA-3-expressing cognate partners. Predictably, therefore, helper and cytotoxic T cell responses, thymocyte-epithelial conjugate formation, and lytic effector function of NK cells are reduced or eliminated by such mAbs. Also, the adhesion function of CD2 molecule has been shown to directly contribute to the T cell recognition of nominal antigen (7, 8). In addition to the adhesion function of CD2, mAbs directed at a second adhesion domain epitope (T11$_2$), in concert with a mAb specific for an epitope (T11$_3$) within the membrane proximal domain, activate T lymphocytes (3, 4), thymocytes (9), and NK cells (4). Moreover, the cellular CD2 ligand LFA-3 itself, in conjunction with anti-T11$_3$ mAb, can activate T cell responses (10). Collectively, these findings indicate an important role for CD2, not only in functioning as an adhesion structure, but also as an activation receptor.

The signal transduction function of CD2 is dependent on its CD2 cytoplasmic tail (11–13). Truncation analysis and site-directed mutagenesis have pinpointed a region between amino acid residues 253 and 287 that is necessary for IL-2 gene induction and increase in intracellular free calcium ([Ca^{2+}]$_i$)[1] (11). Disruption of a single histidine and arginine at positions 264 and 265 within the first of two tandem PPPGHR repeat sequences can abrogate or dramatically reduce T cell activation (14). The activation of T lineage cells via CD2 is also dependent on other cellular structures. In this respect, studies conducted on a TCR$^-$ variant of the human T cell

[1] *Abbreviations used in this paper:* [Ca^{2+}]$_i$, intracellular free calcium; PTK, protein tyrosine kinase.

tumor line Jurkat lacking Ti β transcripts showed that such cells could not be stimulated via CD2 (15). Reconstitution of TCR surface expression in these Jurkat mutants by Ti β gene transfection restored CD2 triggering function. These findings directly demonstrate that the CD2-mediated signal transduction pathway is dependent on the expression of TCR and that both TCR and CD2 activation pathways in T cells are interconnected. Two indirect lines of evidence also support this notion in T lymphocytes. First, prior crosslinking of TCR inhibits subsequent CD2-mediated activation (16–18). Second, submitogenic concentrations of anti-TCR mAbs and anti-CD2 mAbs, which independently fail to activate T cells, in combination induce T cell proliferation (19). The importance of TCR expression for CD2 signal transduction function has been further emphasized by the failure of CD2 to trigger activation events when transfected and expressed in SF9 (*Spodoptera frugiperda*) worm gut epithelial cells (20) or murine fibroblasts (21).

Despite the dependence of CD2 function on surface TCR expression in T lymphocytes, both thymocytes and NK cells that lack surface TCR can be triggered upon CD2 stimulation to increase $[Ca^{2+}]_i$ and mediate cytotoxic activity, respectively (4, 9). The basis of this seemingly paradoxical TCR-independent CD2 signal transduction function is unknown, although we have speculated that it may result from the coupling of CD2 to other transduction elements in NK cells (1). A likely candidate that might functionally interact with the CD2 pathway in these $CD2^+CD3^-$ NK cells is FcγRIII (CD16). CD16 has recently been shown to be physically associated with CD3ζ (22, 23) and is also thought to associate with the γ subunit of FcεRI (FcεRIγ) (24). The latter is a component of the tetrameric high affinity IgE receptor expressed on mast cells and basophils (25), whose broader tissue distribution is only now becoming apparent (26). Interestingly, CD3ζ and FcεRIγ belong to the same gene family located on murine chromosome 1 and are homologous at the primary sequence level (27, 28). Thus, TCR-independent CD2 signal transduction might be linked to either CD3ζ or FcεRIγ or both.

To investigate these possibilities, we transfected a human CD2 cDNA into murine mast cells that express FcεRIγ as a component of their FcεRI as well as FcγRIII (previously denoted as FcγRIIa in mouse) and examined the ability of CD2 to trigger $[Ca^{2+}]_i$ increase and IL-6 production. The results demonstrate that CD2 is competent to transduce signals in mast cells, hence proving that TCR expression and/or lymphoid phenotype is not required for CD2 function. Moreover, because the mast cell line lacks both CD3ζ and CD3η mRNA, neither CD3ζ nor CD3η is required for CD2 signaling.

Materials and Methods

Cell Lines and Transfection of Human CD2. A φ-2 helper-free retrovirus packing cell line, which has been transfected with the expression vector DOL carrying both the neomycin resistance gene and a cDNA of human CD2, was used to generate virus stocks for the infection of mast cells (11). A growth factor–independent mast cell line, C1.MC/57 (denoted as MC/57 in the text below), derived from C57BL/6J mouse bone marrow was a kind gift from S. Galli (Beth Israel Hospital, Boston, MA) (29). MC/57 mast cells were maintained in DME (Whittaker MA Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated FCS (Sigma Chemical Co., St. Louis, MO), 5×10^{-5} M 2-ME (Sigma Chemical Co.), 2 mM L-glutamine, and 1% penicillin-streptomycin (Gibco Laboratories, Grand Island, NY). Procedures for the growth of φ-2 cells, harvesting of virus, and infection of MC/57 mast cells were performed as described (11). The MC/57 mast cell line was infected with freshly derived virus stocks in the presence of 8 μg/ml polybrene (Aldrich Chemical Co., Milwaukee, WI), and selection was initiated 48 h later in 2 mg/ml of G418 (geneticin; Gibco Laboratories). The G418-resistant colonies were screened by indirect immunofluorescence as described below. Additional cell lines used in this study, 2B4.11 and MA5.8, were provided by J.D. Ashwell (NIH, Bethesda, MD), and MH60.BSF2 was a gift from T. Hirano and T. Kishimoto (Institute for Molecular & Cellular Biology, Osaka University, Osaka, Japan) (30).

Flow Cytometric Analysis. Phenotypic analyses were performed using indirect immunofluorescence assays. 10^4 cells were analyzed in each sample on an Epics V cell sorter, and results were expressed as histograms displaying number of cells vs. fluorescence intensity on a log scale. A 1:400 dilution of ascites containing IgE anti-DNP mAbs (31) (H1-DNP-E-26, provided by S. Galli), a 1:200 dilution of ascites containing either anti-$T11_1$ (3Pt2H9, IgG1) (3), anti-$T11_2$ (1old24C1, IgG2a) (3), or anti-$T11_3$ (1mono2A6, IgG3) (3) mAbs, and culture supernatant containing monoclonal anti-H-$2D^b$ (AF6-88.5.3, IgG2a, kindly provided by K. Rock, Dana-Farber Cancer Institute) (32) were used in this study. Saturating amounts of an anticlonotypic mAb, 11C5 (IgG1 derived in our lab; 1:200 dilution of ascites), was used as a control to subtract background Fcγ receptor binding. Cells incubated with the above mAbs for 30 min at 4°C were washed with HBSS containing 2% FCS. The antibody bound was detected with a 1:40 dilution of fluorescein-conjugated goat anti–mouse IgG (H + L) second antibody (Whittaker MA Bioproducts) by using the Epics V cell sorter. CD2 transfectants were sorted for higher levels of CD2 expression on the Epics V cell sorter by staining up to 5×10^6 cells as described above.

Determination of Cytosolic-free Ca^{2+} by Indo-1 Fluorescence. $[Ca^{2+}]_i$ was determined according to Grynkiewicz et al. (33). Briefly, 2×10^6 cells were suspended in 200 μl of DMEM containing 10% FCS and 2 μg/ml acetoxymethylester of Indo-1 (Molecular Probes, Junction City, OR) for 30 min at 37°C. Cells were then diluted up to 1 ml with DME containing 10% FCS and kept at 37°C before analysis on the Epics V cell sorter. For determination of triggering through FcεRI, cells were incubated with a 1:400 dilution of ascites containing IgE anti-DNP mAb for 30 min at room temperature and washed twice as described above before loading with Indo-1. The ratio of Indo-1 fluorescence at 410 nm to that at 480 nm was recorded in real time and expressed in arbitrary units, each of which represents ~200 nM $[Ca^{2+}]_i$. Samples were analyzed at room temperature by running the Indo-1-loaded cells on the Epics V cell sorter for 1 min to establish a baseline before adding the following stimuli: 100 ng/ml DNP-BSA (Sigma Chemical Co.), a 1:100 dilution of anti-$T11_2$ + anti-$T11_3$, or a 1:100 dilution of anti-$T11_2$ + control mAb (anti-T8, 1mono2E7, an IgG3 isotype-matched mAb for anti-$T11_3$). Cells that did not show $[Ca^{2+}]_i$ mobilization received 1 μg/ml of calcium ionophore A23187 (Sigma Chemical Co.). All concentrations indicated above are final concentrations of the respective stimuli.

IL-6 Production Assays. Quantitation of IL-6 secreted into the culture supernatant after stimulation of mast cells was analyzed by bioassay. 10^6 mast cells were plated in 24-well plates and in-

cubated at 37°C, 5% CO_2, for 24 h with the following panel of stimuli: anti-$T11_2$ + anti-$T11_3$, anti-$T11_2$ + control IgG3 (anti-T8, 1mono2E7, a control irrelevant isotype-matched mAb for anti-$T11_3$), anti-$T11_2$, anti-$T11_3$, control IgG2a (anti-T1, 24T6G12, a control irrelevant isotype-matched mAb for anti-$T11_2$), control IgG3, DNP-BSA, PMA, and media. All of the above-mentioned antibodies were protein A purified and incubated at a final concentration of 10 μg/ml. DNP-BSA and PMA were used at a final concentration of 100 ng/ml. Mast cells that were triggered through FcεRI were incubated with IgE anti-DNP mAb for 30 min at room temperature and washed twice as described above before stimulation with DNP-BSA. For studies conducted on CD2 activation after Fcγ receptor blockade, a mAb, 2.4G2, that recognizes both mouse FcγRII and FcγRIII (34) (kindly provided by J.-P. Kinet, NIH) was used at 5 μg/ml either alone or in the presence of anti-$T11_2$ mAb. In other experiments, anti-$T11_2$ mAb stimulation was tested in the presence of 10–100 μg/ml of control IgG2a isotype-matched mAb. Supernatants harvested after 24 h were titrated in triplicate in serial fivefold dilutions for their ability to support the growth of an IL-6-dependent murine hybridoma clone MH60.BSF2 (30). Simultaneously, triplicates of twofold serial dilutions of rIL-6 (a kind gift of Ajinomoto Co. Inc., Tokyo, Japan) were prepared. MH60.BSF2 cells cultured in RPMI containing 10% FCS and 200 pg/ml rIL-6 were washed and then cultured for 6 h before the assay in the absence of IL-6. Subsequently, 10^4 MH60.BSF2 cells were added to each well of 96-well plates (flat-bottomed) containing either dilutions of samples or rIL-6 and cultured for 44 h at 37°C, 5% CO_2, and pulsed with 1 μCi/well [^{3}H]thymidine (ICN Biomedicals Inc., Irvine, CA) during the last 7 h of incubation. The cells were harvested on glass fiber filter papers and incorporation of [^{3}H]thymidine was determined by a liquid scintillation counter. Results are expressed as rIL-6 equivalent (ng/ml/10^6 cells) production as determined from the standard titration curve of rIL-6 in triplicate samples.

Generation of F(ab)'$_2$ Fragments. F(ab)'$_2$ fragments for anti-$T11_2$ and anti-$T11_3$ mAbs were produced using the Immunopure F(ab)'$_2$ preparation kit as recommended by the manufacturer (Pierce Chemical Co., Rockford, IL). 2 mg of protein A–purified anti-T11 mAbs (6 mg/ml) that were dialyzed against 20 mM sodium acetate buffer, pH 4.5 were incubated with 450 μl of the appropriate digestion buffer containing 150 μl of immobilized pepsin in a shaker water bath at 37°C. The anti-$T11_2$ (IgG2a) and anti-$T11_3$ (IgG3) mAbs were digested for either 60 min in digestion buffer, pH 4.1, or for 15 min in digestion buffer, pH 4.3, respectively. The digested F(ab)'$_2$, Fc fragments and undigested IgG were recovered from immobilized pepsin and, subsequently, the Fc fragments and undigested IgG were depleted on a protein A column as outlined in the protocol for the kit. Briefly, each anti-$T11_2$ and anti-$T11_3$ crude digest was added to separate immobilized protein A columns equilibrated with binding buffer and incubated for 1 h. Thereafter, the columns were washed with binding buffer and the flow through fractions containing the F(ab)'$_2$ fragments were collected and dialyzed against PBS, pH 7.4, in dialysis tubing with a molecular weight cut off of 50,000 to remove any small Fc fragments that did not bind protein A. The purity of the protein A–purified F(ab)'$_2$ fragments was checked by nonreducing and reducing SDS-PAGE using Coomassie G-250 staining and was >95%.

Histamine Release. Histamine release into culture supernatants after stimulation of mast cells was analyzed using the histamine radioimmunoassay kit (Amac Inc., Westbrook, ME). 10^6 mast cells were stimulated for 10 and 60 min with either anti-$T11_2$ + anti-$T11_3$, anti-$T11_2$, or DNP-BSA. Mast cells triggered with DNP-BSA were incubated with monoclonal IgE anti-DNP-BSA and washed as described above before stimulation. Histamine released in culture supernatants after activation was analyzed according to the manufacturer's recommendations. Briefly, 100 μl of samples or histamine standards was added to acylation tubes containing acylating agent. The acylated histamine was subsequently added into tubes coated with mAb to acylated histamine in the presence of ^{125}I-labeled acylated histamine. After incubation for 18 h at 4°C, tubes were emptied and counted for 1 min in a gamma counter. Results are expressed as nM histamine per 10^6 cells/ml by comparison of the radioactivity bound to a standard curve run in parallel.

Northern Blot Analysis. Total RNA was isolated from cell lines using guanidine isothiocyanate (35). RNA concentrations were measured spectrophotometrically and 10 μg of total RNA was run per lane on a 1% agarose gel containing 2.2 M formaldehyde and 1× MOPS buffer (35). The RNA was then transferred to nitrocellulose in 20× SSC and the RNA blot hybridized to a CD3η-specific probe containing the 3' AvaII/EcoRI fragment of pBS17 (36). Hybridization was carried out in a solution containing 50% formamide, 5× SSC, 5× Denhardt's, 250 μg/ml denatured salmon sperm DNA, 50 mM $NaHPO_4$, pH 6.5, at 42°C for 16–20 h with 10^6 cpm/ml probe. Subsequently, the blot was washed in 2× SSC, 0.1% SDS for 15–30 min at room temperature and 0.1× SSC, 0.1% SDS at 50°C for 30 min, and was exposed to Kodak X Omat AR X-ray film at −70°C for the indicated time. The blot was then stripped by boiling in distilled water for 10 min and exposed for 12–14 h to insure that all signals had been removed. The filter was then hybridized to the CD3ζ-specific probe containing the 3' EcoNI/EcoRI fragment of pBS23 (37) using conditions as described for the CD3η-specific probe, washed, and autoradiographed. After stripping once again, the blot was hybridized to the rat FcεRIγ-specific cDNA probe containing the 3' BamHI/XbaI fragment (25) using the same conditions described above.

Results

Generation of Human CD2-expressing Murine Mast Cell Lines. To generate CD2 surface expression in mast cells, MC/57 cells were infected with the defective retrovirus vector DOL, which bears the human CD2 cDNA and neomycin-resistant gene (11), and subsequently, a stable mast cell transfectant was selected by flow cytometry as described in Materials and Methods. As shown in Fig. 1, whereas the parental mast cell line, MC/57, does not show any detectable level of human CD2 expression (Fig. 1 *a*), the human CD2 transfectant C1.MC/57/64.1 (denoted as MC/64.1 in the text below) expresses surface CD2 as judged by its reactivity with mAbs to the distinct CD2 epitopes $T11_1$, $T11_2$, and $T11_3$ (Fig. 1 *d–f*). The level of CD2 expressed in these cells is comparable with that of the endogenous H-2D^b molecules (Fig. 1, *c* and *h*). Both the parental and CD2-transfected mast cell lines express equivalent levels of FcεRI (Fig. 1, *b* and *g*). These cells also express equivalent levels of both FcγRII (previously denoted as FcγRIIb) and FcγRIII (previously denoted as FcγRIIa) (data not shown). Neither MC/57 nor MC/64.1 express TCR as judged by indirect immunofluorescence staining with antimurine CD3ε mAb, 145.2C11 (data not shown).

Human CD2-mediated $[Ca^{2+}]_i$ Changes in CD2-transfected Mast Cells. Next, the ability of CD2 to trigger a rise in $[Ca^{2+}]_i$ in the MC/64.1 mast cell transfectant was examined.

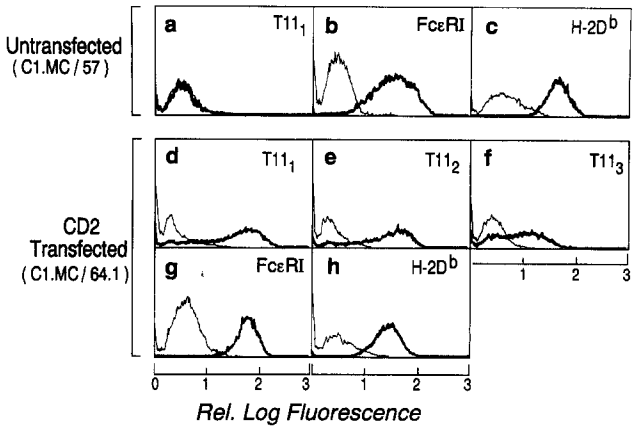

Figure 1. Flow cytometry analysis of human CD2-transfected and -untransfected mast cells. Human CD2-transfected MC/64.1 mast cells treated with anti-$T11_1$ (*d*), anti-$T11_2$ (*e*), or anti-$T11_3$ (*f*) (*bold lines*). Untransfected MC/57 mast cells treated with anti-$T11_1$ mAb (*a*) (*bold line*). CD2-transfected (*g*) and -untransfected (*b*) mast cells treated with IgE anti-DNP mAb (*bold lines*). CD2-transfected (*h*) and -untransfected (*c*) mast cells treated with anti-H-2D^b (*bold lines*). CD2-transfected and -untransfected mast cells treated with a control 11C5 antibody (*faint lines*). Antibody binding was detected by a fluorescein-conjugated goat anti-mouse IgG antibody. The ordinate denotes cell number.

To this end, changes in $[Ca^{2+}]_i$ triggered through FcεRI and CD2 were assessed in Indo-1-loaded cells after specific antibody addition and subsequent receptor crosslinking. As shown in Fig. 2, *a* and *c*, crosslinking of the FcεRI with monoclonal IgE anti-DNP and DNP-BSA (anti-DNP-IgE/DNP-BSA) resulted in a prompt increase in the level of $[Ca^{2+}]_i$ in both the parental MC/57 and CD2-transfected MC/64.1 mast cell lines, consistent with FcεRI-mediated activation of

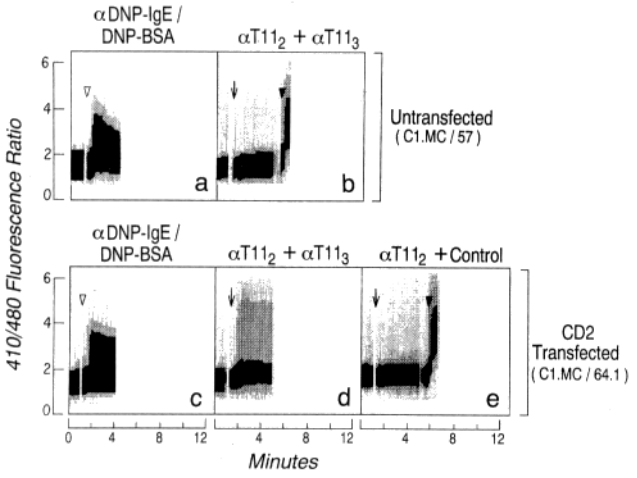

Figure 2. Increase in $[Ca^{2+}]_i$ after CD2 and FcεRI triggering. Changes in $[Ca^{2+}]_i$ were monitored in cells loaded with the Ca^{2+}-sensitive dye Indo-1. Cells were stimulated with either a combination of anti-$T11_2$ + anti-$T11_3$ or anti-$T11_2$ + control (an isotype-matched IgG3 mAb for anti-$T11_3$) (↓), DNP-BSA (▽), or calcium ionophore A23187 (▼). Results are expressed as ratio of Indo-1 fluorescence at 410 nm to that of 480 nm in arbitrary units (ordinate) vs. time in minutes (abscissa). One arbitrary unit represents ~200 nM $[Ca^{2+}]_i$.

Table 1. *IL-6 Induction in Mast Cells through Human CD2 or FcεRI*

	IL-6 produced by:		
	Parental mast cell line (C1.MC/57)	CD2-transfected mast cell line (C1.MC/64.1)	
Stimulus	Exp.1	Exp. 1	Exp. 2
	ng/ml/10⁶ cells		
Media	0.7	0.1	0.4
Anti-$T11_2$ + anti-$T11_3$	1.8	2,300	ND
Anti-$T11_2$ + control IgG3	ND	1,200	ND
Anti-$T11_2$	ND	1,500	2,700
Anti-$T11_3$	ND	1,100	ND
Control IgG2a	ND	1.1	ND
Control IgG3	ND	1.0	ND
Anti-H-2D^b	ND	8	ND
Anti-DNP-IgE/DNP-BSA	170	110	860
PMA	2,400	2,100	2,100

The amount of IL-6 present in culture supernatants was assessed from the titration of culture supernatants and rIL-6, as shown in Fig. 3, and is expressed in rIL-6 equivalents. Control IgG2a is a control mAb isotype matched for anti-$T11_2$. Control IgG3 is a control mAb isotype matched for anti-$T11_3$. SD of IL-6 concentrations were <20%. Results are representative of more than six experiments.

a functional signal transduction machinery in both cell types (38). In contrast, as shown in Fig. 2, stimulation of MC/64.1 cells (*d*) but not MC/57 cells (*b*) with anti-$T11_2$ + anti-$T11_3$ mAbs resulted in an increase in the level of $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ after addition of anti-$T11_2$ + anti-$T11_3$ mAbs is therefore attributable to CD2 expression on MC/64.1 cells. Note that a nonmitogenic combination of anti-$T11_2$ + control mAb (an irrelevant, IgG3 isotype equivalent to that of anti-$T11_3$ mAb) did not induce a measurable increase in the level of $[Ca^{2+}]_i$ in MC/64.1 cells (Fig. 2 *e*). Nevertheless, the MC/64.1 cells were efficiently loaded with Indo-1 as judged by the ability of the calcium ionophore A23187 to increase $[Ca^{2+}]_i$ (Fig. 2 *e*, *arrowhead*). These findings indicate that both $T11_2$ and $T11_3$ epitopes on the MC/64.1 mast cells need to be ligated by antibody in order to increase $[Ca^{2+}]_i$ mediated through CD2. Hence, these data are consistent with earlier studies in T cells, thymocytes, and NK cells, demonstrating that the addition of both anti-$T11_2$ + anti-$T11_3$ mAbs triggers a $[Ca^{2+}]_i$ flux (3–5, 9).

Induction of IL-6 Production in CD2-transfected Mast Cells with Anti-CD2 mAbs. Stimulation of bone marrow–derived mast cell lines including MC/57 through their FcεRI was previously shown to result in induction of a number of cytokine genes including IL-6 (38). To determine whether stimulation of CD2 would result in IL-6 production from the MC/64.1 transfectant, culture supernatants from either the parental

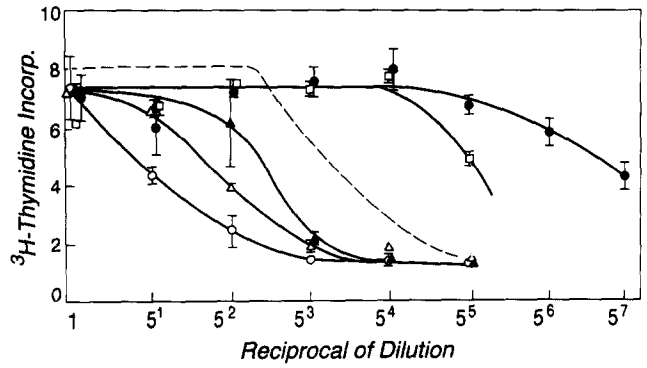

Figure 3. Titration of IL-6 induced in mast cells through either human CD2 or FcεRI. Fivefold dilutions of culture supernatants from CD2-transfected and -untransfected mast cells treated with the various stimuli were titrated on the IL-6-dependent MpH60.BSF2 B cell clone as described in Materials and Methods. The lines shown in the figure correspond to the following cells and stimuli: MC/64.1 media (○), MC/64.1 anti-T11$_2$ + anti-T11$_3$ (●), MC/57 media (△), MC/57 anti-T11$_2$ + anti-T11$_3$ (▲), MC/57 monoclonal anti-DNP IgE/DNP-BSA (□), and twofold dilutions of 1 ng/ml of rIL-6 (*dotted line*). 10% of the original culture supernatant corresponds to the first dilution. The SD of [^{3}H]thymidine incorporation is indicated at each point. The ordinate represents [^{3}H]thymidine incorporation (× 10^{-4}).

MC/57 or MC/64.1 lines treated with various stimuli were assayed for IL-6 activity. For comparison, IL-6 production stimulated via FcεRI was examined in parallel. As expected, the stimulation of both MC/57 and MC/64.1 lines through their FcεRI receptors with a combination of anti-DNP-IgE and DNP-BSA resulted in production of IL-6 (Table 1, Fig. 3). More importantly, the CD2-transfected MC/64.1 mast cell line produced IL-6 when stimulated with a combination of anti-T11$_2$ + anti-T11$_3$ mAbs. In contrast, treatment of the same cells with media or the parental MC/57 mast cells with anti-T11$_2$ + anti-T11$_3$ mAbs failed to induce IL-6 production (Fig. 3, Table 1). In fact, the amount of IL-6 produced by CD2 stimulation was greater than that after FcεRI stimulation in MC/64.1 cells by a factor of 20. Thus, signalling through CD2 with anti-T11$_2$ + anti-T11$_3$ mAbs in MC/64.1 mast cells results in both an increase in $[Ca^{2+}]_i$ and IL-6 production.

Somewhat unexpectedly, we observed that stimulation of MC/64.1 mast cells with either anti-T11$_2$ or anti-T11$_3$ mAbs alone also generated significant IL-6 production. As shown in Table 1, MC/64.1 mast cells stimulated with a combination of anti-T11$_2$ + control IgG3 (an irrelevant mAb isotype matched to anti-T11$_3$ mAb) produced a substantial quantity of IL-6 relative to MC/64.1 cells stimulated with media or parental MC/57 cells stimulated with a combination of anti-T11$_2$ + anti-T11$_3$ mAbs (1,200 ng/ml/10^6 cells vs. 0.1–1.8 ng/ml/10^6 cells). Similarly, as shown in Table 1 (Exp. 1), the stimulation of MC/64.1 mast cells with anti-T11$_2$ mAb alone induced the production of IL-6 comparable in magnitude with the amount produced by the combination of anti-T11$_2$ + anti-T11$_3$ (1,500 vs. 2,300 ng/ml/10^6 cells). Likewise, the anti-T11$_3$ mAb by itself was capable of inducing MC/64.1 cells to produce IL-6. Note that the stimulation of MC/64.1 mast cells with either control IgG2a or control IgG3 mAbs resulted in little or no IL-6 production. Thus, in the MC/64.1 mast cells, crosslinking of more than one T11 epitope on CD2 is not an absolute requirement for the generation of signals leading to production of IL-6.

In this context, it is known that mast cells express Fcγ receptors including FcγRIII (39) to which the above mAbs could bind. To investigate Fcγ receptor ectodomain-independent signal transduction via CD2, anti-T11$_2$ and anti-T11$_3$ F(ab)'$_2$ fragments were produced and MC/64.1 cells were cultured with a combination of anti-T11$_2$ F(ab)'$_2$ + anti-T11$_3$ F(ab)'$_2$. As shown in Table 2, this stimulation resulted in production of IL-6 (2,100–3,100 ng/ml/10^6 cells), comparable with that observed with intact anti-T11$_2$ + anti-T11$_3$ IgG (Table 1). This result indicates that MC/64.1 cells can be activated with a combination of anti-T11 mAbs independent of Fcγ receptor ectodomain binding. Table 2 also shows that reduced but clearly significant IL-6 production was triggered by addition of anti-T11$_3$ F(ab)'$_2$ alone and, to a lesser extent, by anti-T11$_2$ F(ab)'$_2$. The amount of IL-6 induced with either one of the anti-T11 F(ab)'$_2$ fragments (Table 2) was significantly less than the amount of IL-6 produced with any single intact anti-T11 mAb (Table 1). Thus, the binding of intact anti-T11 mAbs to FcγRII or FcγRIII as well as to CD2 in MC/64.1 cells apparently enhances induction of IL-6. In contrast, a mouse anti-H-2D^b mAb, which is of the same IgG2a isotype as anti-T11$_2$, failed to induce any IL-6 production in MC/64.1 (Table 1), indicating that the mere binding of antibody to both H-2D^b and Fcγ receptor molecules is not sufficient to trigger IL-6 production.

To more definitively examine the role of the Fcγ receptor in CD2-mediated IL-6 production, two types of blocking studies were performed. In the first experiment, mAb 2.4G2, which recognizes both FcγRII and FcγRIII (34), was added to the culture along with anti-T11$_2$ mAb. As shown in Table 3, 2.4G2 addition markedly reduced anti-T11$_2$-induced IL-6 production and was itself without any activatory effect. In a separate experiment, isotype-matched IgG2a control mAb used in 10-fold excess of anti-T11$_2$ mAb completely blocked IL-6 induction. Taken together, these findings show that the

Table 2. *Fcγ Receptor Ectodomain-independent IL-6 Induction through Human CD2*

	IL-6 produced by MC/64.1	
Stimulus	Exp. 1	Exp. 2
	ng/ml/10^6 cells	
Media	0.4	0.25
T11$_2$ F(ab)'$_2$	22	44
T11$_3$ F(ab)'$_2$	580	260
T11$_2$ F(ab)'$_2$ + T11$_3$ F(ab)'$_2$	2,100	3,100

The methods are as described in the legend of Table 1. Exp. 1 with anti-T11 F(ab)'$_2$ fragments was done in parallel with Exp. 2 of Table 1.

Table 3. *Fcγ Receptor Ectodomain-dependent IL-6 Induction through Human CD2*

Stimulus	IL-6 produced by MC/64.1
	ng/ml/10⁶ cells
Media	<0.5
Anti-FcγRII (2.4G2)	0.5
Anti-$T11_2$	440
Anti-$T11_2$ + anti-FcγRII (2.4G2)	33
Anti-$T11_2$ + control IgG2a (100 μg/ml)	1
Anti-$T11_2$ + control IgG2a (10 μg/ml)	690
Control IgG2a (100 μg/ml)	<0.5
Control IgG2a (10 μg/ml)	<0.5
PMA	1,100

10^6 MC/64.1 cells were incubated with either 5 μg/ml of mAb 2.4G2, which recognizes FcγRII and FcγRIII, or indicated concentrations of control IgG2a mAb for 45 min at room temperature. They were further incubated for 24 h in the presence or absence of 10 μg/ml of anti-$T11_2$ mAb.

CD2-mediated signal transduction pathway in MC/64.1 mast cells occurs via both Fcγ receptor ectodomain-binding independent and dependent mechanisms.

CD2 Couples to the Histamine Release Pathway in Mast Cell Transfectants. Stimulation of mast cells via the FcεRI receptor is known to induce histamine release. To determine whether CD2 stimulation couples to the histamine release pathway, MC/57.1 and MC/64.1 were stimulated with anti-$T11_2$ + anti-$T11_3$ mAbs or anti-$T11_2$ alone for 1 h and supernatants examined for histamine content by RIA. As shown in Table 4, a combination of anti-$T11_2$ + anti-$T11_3$ or anti-$T11_2$ alone showed stimulation to the same degree as stimulation by cross-linking of FcεRI by IgE/DNP-BSA in MC/64.1. Similar results were obtained when MC/64.1 was examined after 10 min of stimulation (data not shown). Note that, as expected, anti-CD2 mAbs had no effect on MC/57.1. These results in conjunction with the above studies demonstrate that CD2 couples to both IL-6 and histamine release pathways in mast cells.

Mast Cell Line MC/57 Does not Express CD3ζ or CD3η. Recent biochemical analysis of human FcγRIII (CD16) on human NK cells reveals that CD16 is associated with the TCR CD3ζ subunit (22, 23). In addition, it is thought that the FcεRIγ, which is homologous to CD3ζ, associates with CD16 in human NK cells (24), and FcγRIII in murine macrophages and mast cells (26). We thus examined if murine mast cells expressing FcεRI and FcγRIII also express CD3ζ or the related CD3η, since one or both might be involved in CD2-mediated signal transduction in the MC/64.1 mast cell line. To this end, Northern blot analysis was performed using specific cDNA probes for CD3ζ, CD3η, and FcεRIγ, with RNA from the MC/57 parental mast cell line, a murine T cell hybridoma 2B4.11 known to express both CD3ζ and CD3η and a 2B4.11 variant, termed MA5.8, that lacks CD3ζ and CD3η (37). As shown in Fig. 4, MC/57, like MA5.8, lacks transcripts corresponding to CD3ζ and CD3η. In contrast, and as expected, 2B4.11 contains transcripts of 2.0 and 1.8 kb, which correspond to CD3ζ and CD3η, respectively (Fig. 4, *b* and *c*). On the other hand, MC/57 mast cells but not 2B4.11 or MA5.8 express a transcript of ~0.5 kb, which corresponds to the mRNA of FcεRIγ (Fig. 4 *a*). These results indicate that the MC/57 mast cell line can transmit signals via the transfected CD2 gene product in the absence of CD3ζ or CD3η.

Table 4. *CD2-mediated Histamine Release in Mast Cell Transfectants*

Stimulus	Histamine release	
	MC/57	MC/64.1
	nM/10⁶ cells/ml	
Media	8.7	15.0
Anti-$T11_2$ + anti-$T11_3$	8.0	95.0
Anti-$T11_2$	–	75.0
Anti-DNP-IgE/DNP-BSA	63	96.0

Supernatants from 10^6 mast cells stimulated for 1 h under various conditions were tested for histamine content by RIA as described in Materials and Methods. Results are representative of two experiments.

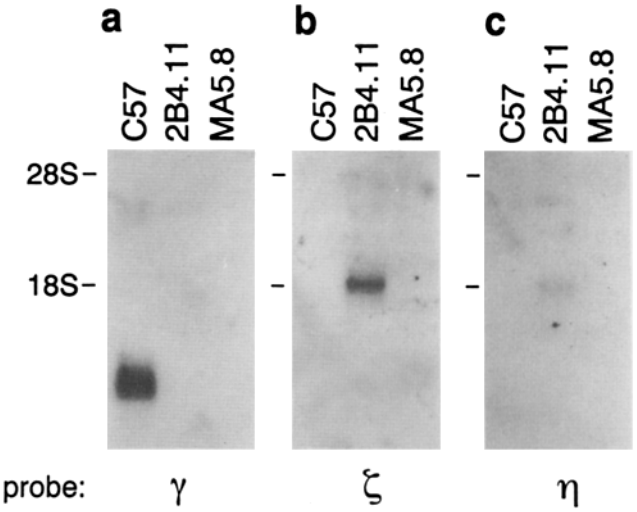

Figure 4. Northern blot analysis of CD3ζ, CD3η, and FcεRIγ subunit gene expression in MC/57 mast cells (C57). 10 μg of total RNA isolated from the indicated cells was size fractionated on a 1% agarose gel containing formaldehyde and transferred to nitrocellulose. Subsequently, the filter was hybridized with a CD3η-specific probe, washed, and exposed at −70°C for 1.5 d with an intensifying screen (*c*). The filter was then stripped, reprobed with a CD3ζ-specific probe, and exposed for 4 d (*b*). After stripping once more, the filter was hybridized with a probe for the FcεRIγ subunit and exposed for 2.5 d (*a*). Positions of ribosomal 28S and 18S RNAs are indicated.

Discussion

In an effort to better understand the nature of TCR-independent CD2 signal transduction, we performed CD2 transfection studies using mast cells as recipients. The latter were chosen based on the premise that Fc receptors might function in lieu of the TCR for transduction coupling to CD2 in some cells and to determine whether CD2 signal transduction might be operative in nonlymphoid cells. The present study provides new insight into both the signal transduction mechanism of CD2 and the pathway of IL-6 production and histamine release. Our results indicate that when human CD2 is expressed in mast cells, perturbation of the extracellular segment with a combination of anti-$T11_2$ + anti-$T11_3$ mAb leads to an increase in $[Ca^{2+}]_i$, IL-6 production, and histamine release. IL-6 production can be mediated independently of the extracellular segment of Fcγ receptor since $F(ab)'_2$ fragments of these same mAbs induced IL-6 production comparable with that obtained with intact mAbs. In addition, we observed that crosslinking of the CD2 with Fcγ receptor using a single anti-$T11_2$ or anti-$T11_3$ mAb could also stimulate IL-6 production. Under these latter circumstances, the crosslinking apparently results from the Fc portion of the mAb binding to Fcγ receptor and the antigen-binding sites of the same mAb ligating to CD2, thereby approximating CD2 and FCγ receptor structures.

Cytokine gene activation studies conducted on parental MC/57 mast cells by Burd et al. (38) with a panel of activating agents showed that the IL-6 gene activation can be induced independently of FcεRI. Activation by PMA or Con A, as well as through FcεRI, resulted in the induction of IL-6 mRNA as detected by Northern blot analysis. Interestingly, the calcium ionophore A23187 did not induce the IL-6 gene in these MC/57 cells, whereas translocation of protein kinase C by itself was sufficient for IL-6 gene induction, indicating that Ca^{2+} mobilization is not essential for IL-6 induction. In contrast to the failure of calcium ionophore to induce the IL-6 gene, it has been shown that a calcium ionophore is itself sufficient to mimic IgE-dependent histamine release in mast cells (40). These findings indicate that histamine release and IL-6 production utilize different signal transduction mechanisms in mast cells.

Additional evidence for a calcium-independent mechanism for IL-6 production was obtained from the present study. A single anti-$T11_2$ mAb that failed to induce Ca^{2+} mobilization in MC/64.1 cells was capable of inducing IL-6 in these same cells. On the other hand, the combination of anti-$T11_2$ + anti-$T11_3$ mAbs induces Ca^{2+} mobilization and IL-6. These results clearly indicate that IL-6 production is induced either in the presence or absence of Ca^{2+} mobilization. Although activation through CD2 produced less Ca^{2+} mobilization relative to activation via FcεRI (Fig. 2 *d* compared with 2, *a* and *c*), the amount of IL-6 produced upon CD2 stimulation was significantly higher than IL-6 produced after activation through FcεRI (Table 1). The dichotomy observed between Ca^{2+} mobilization and IL-6 induction through CD2 and FcεRI may be explicable if several transduction pathways operate in parallel in the mast cell. It is likely that a Ca^{2+}-independent pathway plays a significant role in the induction of IL-6 after activation via either CD2 or FcεRI. This pathway may include a Ca^{2+}-independent activation of protein tyrosine kinase (PTK), which has already been demonstrated in rat basophilic leukemia cells upon triggering through FcεRI (41). It is thus possible that the MC/64.1 mast cells may utilize a PTK pathway for IL-6 induction. Interestingly, it was shown that tyrosine phosphorylation (in the absence of extracellular calcium) is not sufficient to induce histamine release and required the presence of calcium in the extracellular media. This finding is consistent with the fact that calcium mobilization in mast cells can lead to histamine release (40). However, the ability of anti-$T11_2$ mAb to trigger histamine release (Table 4) without a detectable increase in $[Ca^{2+}]_i$ (Fig. 2) implies an additional complexity to the process of histamine release.

Our observations that an activatory combination of anti-$T11_2$ + anti-$T11_3$ mAbs causes calcium mobilization, production of IL-6, and histamine release on human CD2-transfected MC/64.1 mast cells in the absence of TCR expression (Fig. 2 *d* and Table 1) is somewhat analogous to the situation in $CD3^-$ thymocytes. Previous studies with thymocytes have shown that the same combination of anti-$T11_2$ + anti-$T11_3$ mAbs causes a mobilization of $[Ca^{2+}]_i$ and IL-2R expression (9, 9a). Although the activation requirements for IL-2R expression and IL-6 induction are not directly comparable, TCR-independent human CD2 signal transduction in MC/64.1 cells and thymocytes results in both an early change in the ionic milieu of the cell and activation of gene programs. Moreover, the observation that a single anti-$T11_2$ antibody is incapable of mobilizing $[Ca^{2+}]_i$ in these MC/64.1 mast cells (Fig. 2, *e*) is consistent with the activatory requirements of TCR-independent signaling in $CD3^-$ thymocytes (9).

The TCR-independent human CD2 signal transduction obtained with MC/64.1 cells is also reminiscent of the activation requirements in $CD2^+CD3^-$ NK cells. Anti-$T11_2$ + anti-$T11_3$ mAbs activate NK cells to lyse their target (4). Moreover, $F(ab)'_2$ fragments of anti-$T11_2$ + anti-$T11_3$ mAbs, which activate MC/64.1 cells to produce IL-6 (Table 2), have also been shown to activate NK cells to lyse target (42). Thus, an Fcγ receptor ectodomain-independent mechanism of human CD2 signal transduction is operative in both MC/64.1 cells and NK cells. On the other hand, it should be noted that human NK cells express FcγRIII (CD16), and single anti-CD2 mAbs activate NK cells in the same way that a single anti-CD2 mAb can induce IL-6 production in MC/64.1 cells. For example, in one report, a single anti-CD2 mAb CLB-T11 was shown to induce cytolytic activity of $CD3^-CD16^+$ NK cells (5). In a second independent study, Annasetti et al. (43) showed that the addition of an anti-CD2 mAb 9.1 but not its $F(ab)'_2$ fragment to $CD3^-CD16^+$ NK cell lines resulted in cytolysis of targets. The observation of Fcγ receptor ectodomain dependency in NK cell activation is consistent with our observation in mast cells that $F(ab)'_2$ fragments of either anti-$T11_2$ or anti-$T11_3$ mAbs were not as efficient as a single intact anti-$T11_2$ or anti-$T11_3$ mAb in inducing IL-6

production in MC/64.1 cells (Tables 1 and 2). Perhaps more importantly, a mAb that recognizes both FcγRII and FcγRIII or excess control IgG2a isotype–matched mAb blocks anti-CD2-mediated activation of IL-6 production by a single anti-CD2 mAb in CD2-transfected mast cells. Hence, it is likely that the ectodomain of FcγRIII, the murine homologue of human CD16 (44, 45), is involved in CD2 signal transduction when a single anti-CD2 mAb binds to CD2 in both mouse MC/64.1 and human NK cells. Crosslinking of H-2D^b molecules to Fcγ receptor failed to stimulate any significant amount of IL-6 (Table 1). Therefore, crosslinking of Fcγ receptors to other unrelated molecules is not necessarily capable of inducing activation. These results collectively indicate that activation pathways involving Fcγ receptor ectodomain-dependent and -independent mechanisms operate in TCR-independent activation through CD2.

Other studies have shown that Fc receptors on both mast cells and NK cells function as signal transduction molecules. Crosslinking of CD16 molecules (FcγRIII) by certain anti-CD16 mAbs activate human NK cells (46). Similarly, crosslinking of the FcεRI on both the parental MC/57 and human CD2 transfected MC/64.1 cells with a combination of IgE anti-DNP mAb and DNP-BSA resulted in mobilization of $[Ca^{2+}]_i$ and IL-6 production (Fig. 2, *a* and *c*, and Table 1) (38). With respect to the structure of the FcεRI and FcγRIII expressed on mast cells, it is known that they both share a common γ subunit, FcεRIγ (26). FcεRIγ has been shown to have substantial homology with CD3 subunits of the TCR including CD3ζ and CD3η subunits (36, 47, 48). Recent studies on human NK cells have revealed that the CD3ζ subunit is associated with CD16 in the absence of the other TCR subunits (22, 23, 49). In addition, studies conducted on the expression of CD16 suggested that FcεRIγ is associated with CD16 (24). Given the fact that there is evidence suggesting that the CD2-mediated activation of NK cells requires the coexpression of CD16 (46), it is possible that CD2 functionally couples to either the CD3ζ or FcεRIγ subunit or both. Our findings by Northern analysis that the CD2-transfected MC/64.1 mast cells do not express any CD3ζ or related CD3η indicate that these subunits are not essential for CD2 signalling (Fig. 4). In view of the aforementioned expression data, it is tempting to speculate that the FcεRIγ subunit is a structure involved in the functional coupling of CD2 and FcγRIII on MC/64.1 cells.

In addition to NK cells and thymocytes, murine B cells express CD2 in the absence of TCR (50). Although it is not known whether murine CD2 transmits activation signals, the fact that both murine and human CD2 are highly homologous and share a long cytoplasmic tail suggests that murine CD2 has a similar role in B cell activation (50, 51). In this respect, CD2 on murine B cells may also couple with signal transduction elements on Fcγ receptors or membrane immunoglobulins. The recent demonstration that the B cell antigen receptor of the IgM class is noncovalently associated with another subunit termed mb-1 (52), which has a cytoplasmic tail consisting of a motif shared between CD3 subunits and FcεRIγ (48, 53) (as described below), suggests that mb-1 may couple functionally to murine CD2 on B cells.

The FcεRIγ subunit of FcεRI also appears to be a component of murine FcγRIII (26) and human FcγRIII (CD16) (24), and bears significant homology to CD3ζ/η (36, 47, 48). The localization of FcεRIγ and CD3ζ/η subunit genes to mouse chromosome 1 (27, 28), their similar genomic and structural organization, and the sequence homologies between exon 2 encoding the transmembrane region of both genes and between the last two exons of the cytoplasmic domains suggest that they belong to a new family of genes that play a significant role in signal transduction (36, 47). The functional relevance of the primary sequence homology between CD3ζ and the FcεRIγ subunit has been provided by a recent experiment in *Xenopus* oocytes showing that CD3ζ can substitute for FcεRIγ in the assembly and surface expression of FcεRI (54). Moreover, a conserved amino acid motif (D [or E] x x Y x x L x x x x x x x Y x x L [or I]) is present in the cytoplasmic domains of receptor molecules such as FcεRIγ, CD3ζ, CD3η, and mb-1 (48). Amino acid residues composing the motif (upper case letters and underlined: mouse FcεRIγ [DavYtgLntrsqetYetL; aa 62–79], mouse CD3ζ [DglYaqLstatkdtYdaL; aa 118–135], mouse CD3ζ/CD3η [EgvYnaLqkdkmaeaYseI; aa 87–105], mouse mb-1 [EneYegLnlddcsmYedI; aa 179–196]) would be expected to lie on the same side of an α-helical barrel if the cytoplasmic sequence formed an α helix. As such, the residues could form a binding site for putative proteins involved in the generation of signals after receptor crosslinking. Therefore, it is possible that the FcεRIγ subunit shared between murine FcεRI and FcγRIII is involved in coupling with the CD2-mediated signal transduction pathway in MC/64.1 cells. It seems likely that the TCR-independent CD2 signalling machinery is linked to the FcεRIγ, CD3ζ, and/or CD3η and mb-1 subunits in some cells such as CD3$^-$ NK cells, thymocytes, and murine B cells. Further analysis, including reconstitution of CD2-mediated signal transduction in TCR$^-$ Jurkat variants by transfection with CD16 or other Fc receptors, could provide additional evidence for this hypothesis.

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Address correspondence to A.R.N. Arulanandam, Laboratory of Immunobiology, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 02115.

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Note added in proof: As expected, transfection of CD16 into TCR$^-$ Jurkat cells restores CD2 signaling function (Moingeon, P., et al., manuscript submitted for publication).

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