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CD2 Regulates Responsiveness of Activated T Cells to Interleukin 12

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Summary

Interleukin (IL) 12 is a 70-kD heterodimeric cytokine produced by antigen-presenting cells (APCs) such as macrophages in response to infectious pathogens and interferon (IFN) γ . The varied immunomodulatory effects of IL-12 include the stimulation of proliferation and IFN- γ production by T cells, and it also has a central role in the development of the T helper cell type 1 immune phenotype. We undertook the production of antibodies capable of modulating the response of T cells to IL-12, and in the process we discovered two antibodies that inhibited the ability of IL-12 to stimulate T cell proliferation. In this report, we demonstrate that these antibodies recognize CD2, and we show how antibodies directed toward either the adhesion domain of CD2 or its ligand, CD58, specifically inhibit IL-12-induced proliferation and IFN- γ production by phytohemagglutinin-activated T cells, leaving the response to IL-2 unaffected. A three- to fourfold reduction in proliferation and IFN- γ production was observed at IL-12 concentrations as high as 1 nM, with complete inhibition occurring at ≤ 1 pM. This novel effect is not directly mediated at the level of the IL-12 receptor, as shown by the inability of these antibodies to block IL-12 binding to activated T cells. Furthermore, by using activating pairs of CD2 antibodies, we show that CD2 stimulation strongly synergizes with IL-12, even at 0.1 pM, in inducing both T cell proliferation and IFN- γ production. Cytolytic T lymphocyte-associated antigen 4-immunoglobulin-mediated inhibition of the B7/CD28 interaction did not affect the T cell response to either IL-12 or IL-2, but the removal of APCs selectively diminished the proliferative response to IL-12. Based on this data, we hypothesize that CD2 has a central role in an IL-12/IFN- γ positive feedback loop between T cell and APC, providing the key functional link via a CD2/CD58 interaction that controls T cell responsiveness to IL-12. This model provides a basis for future investigations aimed at defining the signaling mechanisms that mediate this cytokine-specific regulatory effect of CD2, and it offers insight into how a cytokine receptor and distinct adhesion molecule can interact to modulate responsiveness to that cytokine. In addition, it underscores the possibility that the clinical potential of an immunomodulatory drug like IL-12 may be governed by the presence or absence of specific costimulation.

IL-12 is a disulfide-linked, 70-kD heterodimeric cytokine, composed of a 35-kD and a 40-kD subunit (1). Its production was first noted in EBV-transformed B cell lines stimulated by phorbol esters, but, in addition to B cells, a primary source is macrophages stimulated by bacterial products, intracellular parasites, and IFN- γ (2). IL-12 has a variety of immunomodulatory effects on T and NK cells, most notably involving the stimulation of IFN- γ production (3), proliferation (4), and cytolytic activity (5, 6). In addition, IL-12 plays a central role in the development of the Th1 immune response (7), characterized by IFN- γ and IL-2 production by T cells, and in the subsequent enhancement of cellular immunity directed in particular toward intracellular pathogens (8, 9).

Apart from the unique effect on Th1 development, there is considerable overlap between the functional effects of IL-12 and IL-2 on T and NK cells, although important differences do exist. For example, the proliferative effect of IL-12 on T and NK cells is weaker when compared with IL-2 (1, 4), while IL-12 is a more potent inducer of IFN- γ (3). In addition, NK cell cytolytic activity induced by IL-2 is greater than that observed with IL-12 (5). While these differences most likely reflect, at least in part, differences in signaling through the IL-12R and IL-2R, they may also, from a physiologic standpoint, be artificial, in that optimal stimulation by either cytokine may require specific costimulatory signals that can be absent from *in vitro* assays. This possibility was illustrated

in a recent report demonstrating that the B7/CD28 interaction augments, in a synergistic fashion, the response (including proliferation and IFN- γ production) of resting and activated T cells to IL-12 (10). Through this synergy, T cell proliferation exceeded the maximal proliferation induced by IL-2. This suggested that the optimal physiologic response of T cells to IL-12 was in part dependent on costimulatory signals delivered through an interaction with APCs. In theory, therefore, specific costimulation could be the key factor in determining to what extent and under which circumstances a T or NK cell will respond to cytokines with similar properties, such as IL-12 and IL-2.

CD2 (T11) is a 50-kD surface glycoprotein present on T and NK cells. Although much is known concerning the structure and expression of CD2, its functional significance has not been fully delineated. CD58 has been identified as a ligand for CD2, interacting with the adhesion domain of CD2 (11, 12). Cellular adhesion between T cells and APCs, mediated in part through the CD2/CD58 interaction, facilitates antigen recognition and subsequent T cell activation (13). NK cell cytolytic activity is also, in some instances, partially dependent on the CD2/CD58-mediated adhesion between NK cell and its target (14). The CD2R epitope appears primarily on activated T cells (15) and, to a much lesser degree, on activated NK cells. There is no known ligand for CD2R. Although CD58 does not interact directly with CD2R, anergized T cells can regain responsiveness to alloantigen plus CD58 costimulation when expression of the CD2R epitope, down-regulated during anergy, is restored after prolonged culture in IL-2 (16). A more direct role for CD2 in lymphocyte activation has been suggested by the observation that the pair of anti-CD2 plus anti-CD2R mAbs or CD58 plus anti-CD2R can activate T cells, resulting in IL-2 production, up-regulation of the IL-2R, and proliferation (15). This stimulation reduces the threshold for T cell activation through the T cell receptor (17) and can activate the cytolytic pathway in T and NK cells (18). In addition, antibodies to CD2 have been reported to diminish the proliferation of activated T cells (19).

In the course of developing antibodies capable of modulating the functional effects of IL-12, we discovered two antibodies that individually had a potent, selective inhibitory effect on the IL-12-induced proliferation of activated T cells. These antibodies were found to recognize the adhesion domain of CD2 and CD2R. By examining the effects of these and other CD2 mAbs on IL-12- and IL-2-induced proliferation and IFN- γ production by T cells, we show that the response of T cells to IL-12 and IL-2 is differentially regulated by CD2.

Materials and Methods

Isolation and Culture of T Cells. PHA-activated T cells were prepared from heparinized blood obtained from normal volunteer donors by first isolating PBMC on Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) gradients. Whole PBMC were then cultured at a starting concentration of 1×10^6 cells/ml in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 15% FCS (Proteins Antibodies Antigens Laboratories, Newport

Beach, CA), 2% L-glutamine (BioWhittaker, Inc., Walkersville, MD), 1% sodium pyruvate (Sigma Chemical Co.), 1% penicillin-streptomycin (BioWhittaker, Inc.), 1% gentamicin (Life Technologies, Inc., Grand Island, NY), and 2.5 μ g/ml PHA (Murex, Dartford, UK). Cells grown in this manner were 90–95% CD3⁺CD56⁻, 0% CD56⁺CD3⁻, and 1–2% CD14⁺CD3⁻ when analyzed by flow cytometry on days 2–4. For the preparation of sorted, PHA-activated T cells, whole PBMC were stained with CD5-PE and Ia-FITC and sorted for CD5⁺Ia⁻ cells on a flow cytometer (EPICS 752; Coulter Corp., Hialeah, FL). Before sorting, cells were 8% CD3⁻Ia⁺ and 92% CD3⁺Ia⁻. After sorting, 99.8% were CD3⁺Ia⁻ and <0.2% CD3⁻Ia⁺. These sorted cells were cultured in PHA in the same manner as whole PBMC.

Production of CD2 mAbs. BALB/c mice were immunized with day 2–4 PHA-activated T cells. Fusions using BALB/c splenocytes and the NS-1 myeloma cell line were carried out as previously described (20), and hybridomas were screened in proliferation assays using day 4–5 PHA-activated T cells stimulated with 1 pM IL-12 in the presence of a 1:4 dilution of each tested supernatant (performed in duplicate). Two hybridomas, later designated CD2E1 (IgG1) and CD2E3 (IgM), were selected for their ability to inhibit IL-12-induced proliferation by >50%. These were each subcloned twice by limiting dilution.

Antibodies. Purified unconjugated and FITC- or PE-conjugated murine mAbs obtained from Coulter Corp. include T1 (CD5, IgG2a), T3 (CD3, IgG1), My4 (CD14, IgG2b), I2 (Ia), and NKH1 (CD56, IgG1). Anti-T11-1 (CD2, IgG2b) was used as a dilution of ascites, while purified anti-T11-2 (CD2, IgG2), anti-T11-3 (CD2R, IgG3), and CD2E1 (IgG1) mAbs were isolated from ascites by affinity chromatography using either protein A or protein G (Pharmacia LKB) columns. The anti-T11-1 and anti-T11-3 mAbs were conjugated to fluorescein as previously described (21). Purified CD2E3 (IgM) and 8C12 (CD18, IgM) mAbs were obtained by ammonium sulfate precipitation of ascites followed by dialysis against PBS. TS 2/9 (CD58, IgG1) was kindly provided by Dr. T. Springer (Center for Blood Research, Boston, MA) and was used as a dilution of ascites. Purified cytolytic T lymphocyte-associated antigen-4-Ig¹ and an isotype-matched control were kindly provided by Dr. Vassiliki Boussiotis (Dana-Farber Cancer Institute, Boston, MA).

Cell Lines. The control (C33) and CD2-expressing (W33) T cell lines were generated by infecting the murine T cell hybridoma MLV (23) containing either TKneo vector (24) alone or TKneo-CD2 sequence, respectively. The latter contains the full length wild-type human CD2 cDNA as previously described (11). C33 and W33 were selected by culturing under 1 μ g/ml neomycin growth medium (RPMI 1640 with 10% FCS, 1% penicillin-streptomycin, and 1% L-glutamine). CD2 expression on W33 was further maximized by three anti-CD2 mAb dynabead separations after neomycin selection. The surface expression of CD2 on W33 was confirmed by indirect immunofluorescence. Once established, both cell lines were maintained in 0.5 mg/ml neomycin growth medium.

Cytokines. All cytokines were recombinant human proteins. IL-12 (specific activity 5.26×10^6 U/mg) was kindly provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). IL-2 (3.9×10^6 U/ml) was kindly provided by Amgen Biologicals (Thousand Oaks, CA).

Immunofluorescence Analysis. Samples of cells were stained directly or indirectly with FITC- or PE-conjugated mAb, washed,

¹ Abbreviations used in this paper: CAM, cellular adhesion molecule; CTLA-4, cytolytic T lymphocyte-associated antigen 4; E-rosetting, erythrocyte rosetting; FGFR, fibroblast growth factor receptor; RAM, rabbit anti-mouse.

fixed in 1% formaldehyde, and analyzed by flow cytometry as described previously (5).

Proliferation Assays. Day 3–5 T cells preactivated with PHA were plated at 20,000 cells per well in 96-well U-bottom microtiter plates (Flow Laboratories, Inc., McLean, VA) in medium alone or medium containing cytokine and/or mAb(s) as indicated. Purified anti-T11-2, anti-T11-3, CD2E1, CD2E3, and anti-CD18 mAbs were used at effective final concentrations ranging from 5–15 $\mu\text{g}/\text{ml}$, while anti-T11-1 and anti-CD58 mAbs were used as ascites at a final dilution of 1:400. Purified CTLA-4-Ig was used at a final concentration of 5 $\mu\text{g}/\text{ml}$. Cells were not preincubated with mAbs before adding cytokine. Medium consisted of RPMI 1640 plus 15% FCS. Samples were cultured in triplicate in a total volume of 200 μl per well at 37°C in 5% CO₂ for 48 h. During the last 9 h of the 48-h culture period, cells were incubated with 1 μCi of [³H]thymidine (DuPont, Wilmington, DE). The cells were harvested onto glass fiber filters using a harvester (96 Mach II; Tomtec, Orange, CT), and the radioactivity on the dried filters was measured in a liquid scintillation counter (1205 Betaplate; Pharmacia LKB, Uppsala, Sweden). Results represent the mean of triplicate wells and are expressed as counts per min of [³H]thymidine incorporation.

IFN- γ Assays. Day 3–5 T cells preactivated with PHA were plated at 30,000 cells per well in 96-well U-bottom microtiter plates using conditions identical to those used in the proliferation assays except that samples were cultured in duplicate. Supernatants were harvested after a 72-h incubation and assayed by ELISA (Endogen, Inc., Boston, MA) for production of human IFN- γ .

Erythrocyte Rosetting (E-Rosetting) Assay. SRBC were preincubated for 15 min at 37°C with aminoethyl isothiuronium bromide (Sigma Chemical Co.), as previously described (13). 1×10^5 W33 cells (CD2 transfectants) were preincubated with medium alone or medium plus CD2 mAb (used as ascites at 1:50 dilution) in a total volume of 100 μl for 0.5 h at 4°C, followed by the addition of 10 μl of the aminoethyl isothiuronium bromide-treated SRBC suspension. Tubes were incubated at 37°C for 5 min, spun down for 5 min at 800 rpm, and incubated for 45 min at 4°C. The cell pellet of each tube was gently resuspended, and 100 cells were counted under a light microscope (American Optical Scientific Instruments, Buffalo, NY). W33 cells bound to at least five SRBC were counted as rosette positive.

¹²⁵I-IL-12 Binding Assays. IL-12 was ¹²⁵I labeled to a specific activity of 1.4×10^6 dpm/pmol using the Iodo-Bead method (Pierce, Rockford, IL). Successful radiiodination was confirmed by subjecting the labeled product to SDS-PAGE analysis (7.5% gel, reduced and nonreduced conditions), and the ¹²⁵I-IL-12 was quantitated using a protein assay reagent kit (Micro BCA; Pierce, Rockford, IL). The preservation of bioactivity after iodination was confirmed by using the ¹²⁵I-IL-12 in a proliferation assay with PHA-activated T cells and comparing the result with cold IL-12 used in the same assay. For binding assays, $1\text{--}1.5 \times 10^6$ cells were incubated at 4°C for 2 h with ¹²⁵I-IL-12 at serial dilutions ranging from 16 pM to 8 nM in binding medium containing RPMI 1640 plus 1% BSA, 25 mM HEPES, and 0.1% sodium azide, pH 7.4. Nonspecific binding was determined in the presence of a 150-fold excess of unlabeled IL-12. The effects of CD2E1, CD2E3, and CD18 mAbs on IL-12 binding were examined by incubating cells with 25 $\mu\text{g}/\text{ml}$ of each mAb for 0.5 h at 4°C before adding the ¹²⁵I-IL-12. IL-12 binding was analyzed by Scatchard plot and by the Ligand program (Biosoft, Ferguson, MO) (25).

Immunoprecipitation. Day 3–4 PHA-activated T cells (5×10^6 cells per lane) were surface labeled with ¹²⁵I using the lactoperoxidase method. Radiolabeled cells were washed and solubilized in 1 ml lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 8.15, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 2 $\mu\text{g}/\text{ml}$ pepstatin A, 10 $\mu\text{g}/\text{ml}$

aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin). Lysates were precleared with fixed *Staphylococcus aureus* cowan I (Calbiochem, La Jolla, CA) and rabbit anti-mouse Ig (RAM)-coupled protein A beads, followed by incubation with specific mAbs for 4 h and incubation with RAM-coupled protein A beads for an additional 4 h. Immunoprecipitates were washed extensively and subjected to SDS-PAGE analysis on a 7.5% gel.

Results

Development of Two Antibodies Modulating the Proliferative Response of T Cells to IL-12; CD2E1 and CD2E3 Recognize CD2 and CD2R. To investigate the mechanisms by which IL-12 exerts its heterogeneous immunomodulatory effects on T cells, we devised a strategy to produce antibodies against structures expressed on the surface of activated T cells that would be capable of modulating the T cell response to IL-12. Since PHA-activated T cells up-regulate expression of the IL-12R and respond to IL-12 (26), they were used to immunize mice in preparation for the development of hybridomas. Hybridoma supernatants were then screened for their ability to inhibit the proliferative response of PHA-activated T cells to IL-12. 1,200 hybridomas were screened, and, as shown in Fig. 1 A, two (later designated CD2E1 and CD2E3) were selected for their ability to inhibit IL-12-induced proliferation by 40–50%. After subcloning, immunoprecipitations were performed with both mAbs using surface-labeled PHA-activated T cells, and each precipitated a single band of ~ 50 kD (data not shown). CD2E1 stained resting T cells strongly and was further up-regulated with PHA activation, whereas CD2E3 exhibited only weak staining with resting T cells but was strongly up-regulated with activation (data not shown). These results together suggested that CD2 was the protein being recognized by both mAbs, and this was confirmed by staining a CD2 transfectant (Fig. 1 B). The staining pattern on resting and activated T cells, along with the observation that CD2E1 but not CD2E3 blocks E-rosetting (data not shown), indicated that CD2E1 and CD2E3 recognized the adhesion domain of CD2 and CD2R, respectively. As demonstrated in Fig. 1 C, CD2E1 and CD2E3 were only able to partially block the staining of a CD2 transfectant with FITC-conjugated anti-T11-1 and anti-T11-3, respectively, showing that the epitopes recognized by these new mAbs are unique.

CD2E1 and CD2E3 Selectively Inhibit IL-12-induced T Cell Proliferation and IFN- γ Production. After identifying CD2E1 and CD2E3 as being specific for different CD2 epitopes, we investigated whether the inhibitory effect on proliferation was specific for IL-12 and whether IFN- γ production was affected in a similar manner. Since IL-12 and IL-2 elicit similar biologic effects from preactivated T cells, we tested purified preparations of these antibodies against a wide range of concentrations of both cytokines. Fig. 2 A shows that both antibodies had a potent, selective inhibitory effect on the IL-12-induced proliferation of PHA-activated T cells. Even at an IL-12 concentration of 1 nM, there was nearly a fourfold reduction in proliferation, with complete inhibition observed at ≤ 1 pM. The maximal effect of the antibodies was reached at a concentration of 5–10 $\mu\text{g}/\text{ml}$ (data not shown). An identical, IL-12-specific inhibitory effect was observed with regard to

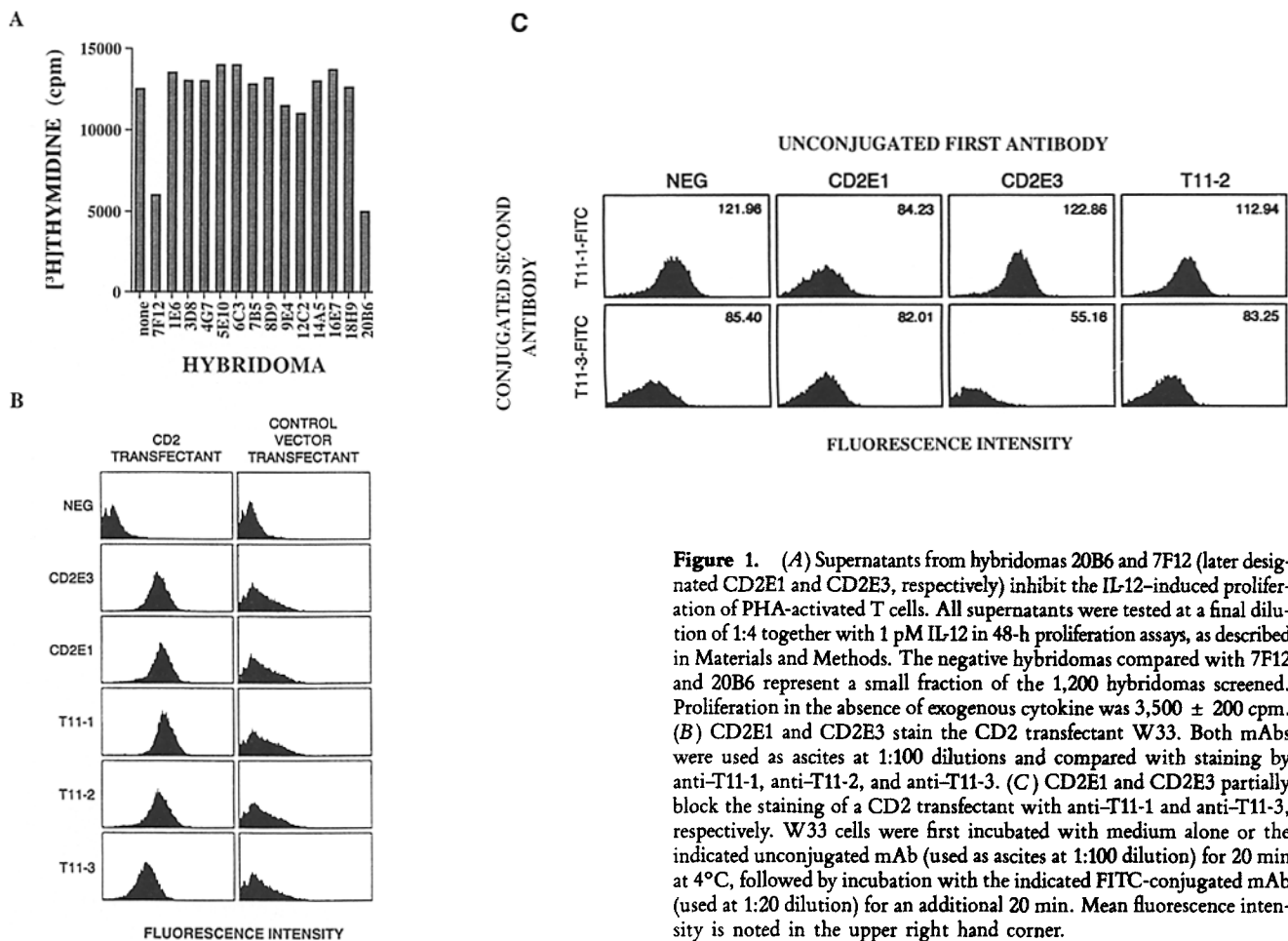


Figure 1. (A) Supernatants from hybridomas 20B6 and 7F12 (later designated CD2E1 and CD2E3, respectively) inhibit the IL-12-induced proliferation of PHA-activated T cells. All supernatants were tested at a final dilution of 1:4 together with 1 pM IL-12 in 48-h proliferation assays, as described in Materials and Methods. The negative hybridomas compared with 7F12 and 20B6 represent a small fraction of the 1,200 hybridomas screened. Proliferation in the absence of exogenous cytokine was $3,500 \pm 200$ cpm. (B) CD2E1 and CD2E3 stain the CD2 transfectant W33. Both mAbs were used as ascites at 1:100 dilutions and compared with staining by anti-T11-1, anti-T11-2, and anti-T11-3. (C) CD2E1 and CD2E3 partially block the staining of a CD2 transfectant with anti-T11-1 and anti-T11-3, respectively. W33 cells were first incubated with medium alone or the indicated unconjugated mAb (used as ascites at 1:100 dilution) for 20 min at 4°C, followed by incubation with the indicated FITC-conjugated mAb (used at 1:20 dilution) for an additional 20 min. Mean fluorescence intensity is noted in the upper right hand corner.

IFN- γ production, with both mAbs causing a threefold reduction at a concentration of 100 pM and complete inhibition at 1 pM (Fig. 2B). As shown in Fig. 2, there was no significant effect on IL-2-induced proliferation or IFN- γ production.

Individual Antibodies to the First Domain of CD2 and to CD2R Differentially Affect IL-12-induced T Cell Proliferation and IFN- γ Production. After observing the novel effect of CD2E1 and CD2E3 on the response of T cells to IL-12, we examined whether mAbs to other CD2 epitopes behaved in a similar manner. Fig. 3A demonstrates that, while anti-T11-1 (which recognizes an adhesion site within the first domain of CD2 and blocks E-rosetting) had the same IL-12-specific inhibitory effect on proliferation as CD2E1 and CD2E3, anti-T11-2 (which interacts with a region orthogonal to the AGFCC' face of the adhesion domain) did not inhibit the response to either IL-12 or IL-2. In sharp contrast, anti-T11-3 stimulated proliferation. As shown in Fig. 3B, maximal stimulation with anti-T11-3 was in some instances independent of exogenous cytokine, although the proliferative effect of anti-T11-3 alone varied between assays from three to nine times baseline proliferation. Fig. 3B also demonstrates that CD2E1, CD2E3, and anti-T11-1 all inhibited the baseline prolifer-

ation of preactivated T cells by 50–80%. Anti-T11-1 and anti-T11-2 were also individually tested for their effect on cytokine-induced IFN- γ production, and the results once again mirrored those obtained in the proliferation assays (Fig. 4).

CD2E1 and CD2E3 Do Not Block IL-12 Binding to T Cells. Because these two CD2 mAbs selectively blocked two major functional effects of IL-12, we investigated whether they were interfering with the binding of IL-12 to its receptor. Binding studies using radiolabeled IL-12 were performed on day 3–4 PHA-activated T cells, and two binding affinities were detected: (a) a low affinity site with a dissociation constant of ~ 2 nM, 1,000 sites per cell, and (b) a high affinity site, with a dissociation constant of ~ 100 pM, 350 sites per cell (Fig. 5A). Compared with a control antibody, CD2E3 had no significant effect on high or low affinity IL-12 binding (Fig. 5B). CD2E1 also had no measurable effect on binding (data not shown). In addition to demonstrating that IL-12 binding is not affected by our antibodies, we observed that radiolabeled IL-12 does not bind to a CD2 transfectant (data not shown), further supporting our conclusion that CD2 is unlikely to be a component of the IL-12R.

Anti-CD58 Selectively Inhibits IL-12-induced T Cell Prolifer-

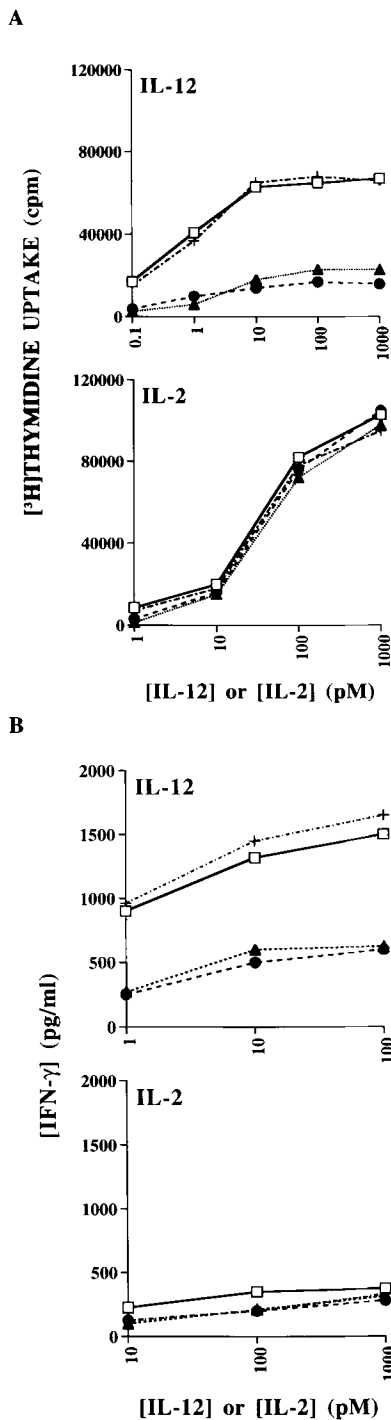


Figure 2. (A) CD2E1 and CD2E3 selectively inhibit the IL-12-induced proliferation of PHA-activated T cells. After 4 d of culture in medium containing PHA at 2.5 $\mu\text{g}/\text{ml}$, cells were incubated with IL-12 or IL-2, alone or together with the indicated purified mAb at 5–15 $\mu\text{g}/\text{ml}$, in a 48-h proliferation assay. Anti-CD18 served as a negative control. Proliferation in the absence of cytokine or mAb was $9,000 \pm 1,000$ cpm. Results are representative of four experiments. (B) CD2E1 and CD2E3 selectively inhibit the IL-12-induced production of IFN- γ by PHA-activated T cells. Purified mAbs were used at 5–15 $\mu\text{g}/\text{ml}$. IFN- γ concentration was measured with an ELISA assay after a 72-h incubation. The sensitivity of the assay is <5 pg/ml. The baseline production of IFN- γ in the absence of exogenous cytokine was 225 pg/ml. Results are representative of two experiments. \square —, no mAb; \cdots + \cdots , anti-CD18; \bullet — \bullet , CD2E3; \cdots \blacktriangle \cdots , CD2E1.

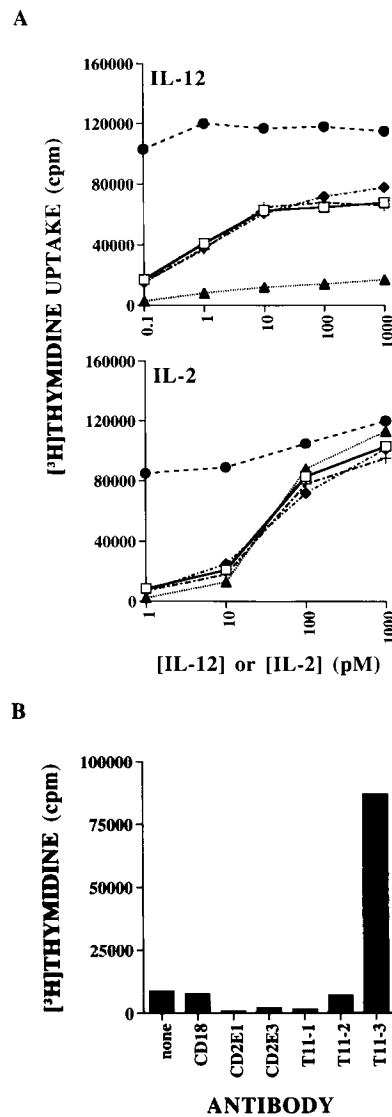


Figure 3. (A) Antibodies recognizing CD2 and CD2R differentially affect the IL-12 and IL-2-induced proliferation of PHA-activated T cells. All mAbs were used as purified protein at 5–15 $\mu\text{g}/\text{ml}$ except for anti-T11-1, which was used as ascites at 1:400 dilution. Cells were incubated with IL-2 or IL-12, alone or together with the indicated mAb, in a 48-h proliferation assay. Results are representative of four experiments. \square —, no mAb; \cdots + \cdots , anti-CD18; \blacktriangle \cdots , T11-1; \cdots \blacklozenge \cdots , T11-2; \bullet — \bullet , T11-3. (B) CD2 and CD2R mAbs affect the proliferation of PHA-activated T cells in the absence of exogenous cytokine. Cells were incubated alone or together with the indicated mAb in a 48-h proliferation assay. Results are representative of four experiments, although the degree of stimulation observed with anti-T11-3 alone varied between assays from three to nine times baseline proliferation.

ation and IFN- γ Production. Having established that the mechanism governing the IL-12-specific inhibitory effect of antibodies directed at the adhesion domain of CD2 and one of our anti-CD2R mAbs is not related to interference with IL-12 binding, we investigated whether interference with the interaction between CD2 and its known ligand, CD58, was the critical factor. This explanation would be plausible pri-

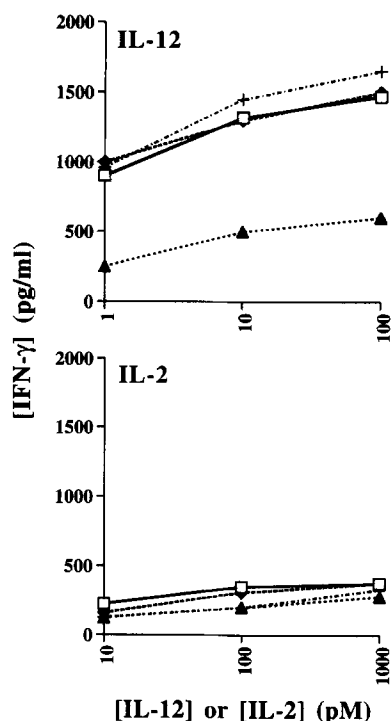


Figure 4. Anti-T11-1, but not anti-T11-2, selectively inhibits IL-12-induced IFN- γ production by PHA-activated T cells. Results are representative of two experiments. \square —, no mAb; $\cdots\blacktriangle\cdots$, anti-CD18; $\cdots\blacktriangle\cdots$, T11-1; $\cdots\blacklozenge\cdots$, T11-2.

marily for CD2E1 and for anti-T11-1, both of which block E-rosetting, a phenomenon reliant upon adhesion mediated via CD2/CD58. Using TS 2/9, an anti-CD58 antibody which blocks the CD2/CD58 interaction (13), we demonstrated the same potent IL-12-specific inhibition of proliferation and IFN- γ production that was observed with CD2E1, anti-T11-1, and CD2E3 (Fig. 6).

CTLA-4-Ig Does Not Inhibit IL-12-induced T Cell Proliferation. Having established that antibodies interfering with the CD2/CD58 interaction specifically inhibit the stimulation of activated T cells by IL-12, we investigated whether blocking the CD28/B7 interaction had a similar effect. A recent report demonstrated how CD28 antibodies and B7 transfectants can synergize with IL-12 in inducing T cell proliferation and IFN- γ production (10) but did not show whether the response of activated T cells to IL-12 is abrogated by inhibiting the binding of CD28 to B7. Since CTLA-4, a second ligand for B7, blocks the CD28/B7 interaction when used in its soluble form (CTLA-4-Ig) (27), we tested whether it was capable of affecting IL-12- and/or IL-2-induced T cell proliferation. As shown in Fig. 7, CTLA-4-Ig had no effect on the ability of IL-12 or IL-2 to stimulate the proliferation of activated T cells.

Anti-T11-1 Plus Anti-T11-3 Synergizes with IL-12 in Inducing T Cell Proliferation. We have already shown that anti-T11-3, distinct from CD2E3, can stimulate proliferation to near-maximal levels without exogenous cytokine. However, T cell

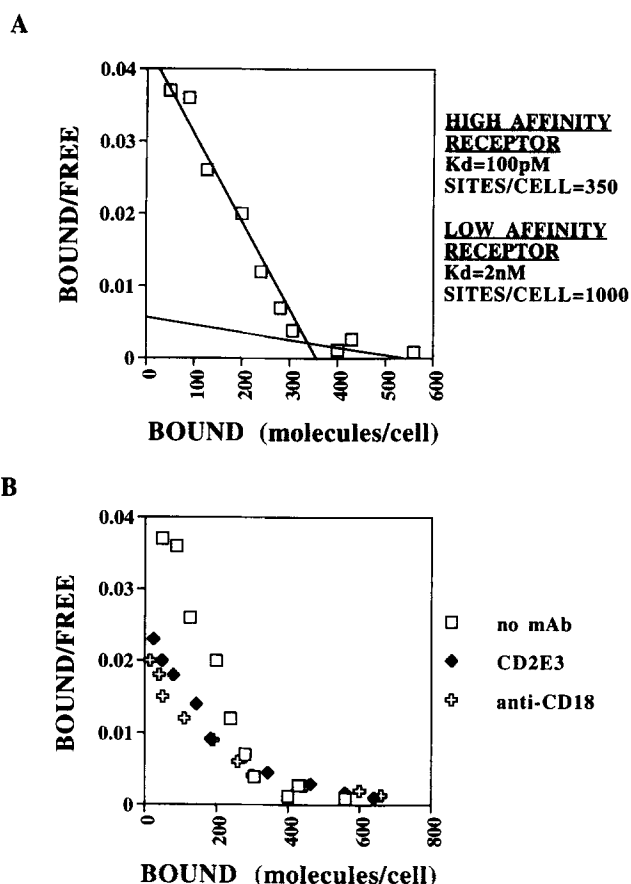


Figure 5. (A) Scatchard analysis of ^{125}I -IL-12 binding to day 3 PHA-activated T cells. 1×10^6 cells were incubated with ^{125}I -IL-12 at concentrations ranging from 8 nM to 16 pM. Nonspecific binding was determined in the presence of 140 nM cold IL-12 and used to calculate specific bound IL-12 (*x-axis*). Results are representative of three experiments. (B) The CD2E3 mAb does not affect the binding of IL-12 to day 3 PHA-activated T cells. Purified CD2E3 and anti-CD18 were used at 50 $\mu\text{g}/\text{ml}$. Results are representative of two experiments.

activation through CD2 has been attained in previous studies either through pairs of anti-CD2R plus anti-CD2 mAbs or through anti-CD2R combined with CD58. Since our results to this point implicated CD2 as being pivotal in determining T cell responsiveness to IL-12, we investigated the manner in which stimulatory pairs of CD2 antibodies interacted with IL-12 and IL-2. When used without exogenous cytokine, the anti-T11-2 plus anti-T11-3 pair was a strong stimulator of proliferation (data not shown). CD2E1 plus CD2E3 also stimulated proliferation, as did CD2E1 plus anti-T11-3 (Fig. 8 A). However, anti-T11-1, whether combined with CD2E3 or with anti-T11-3, either had no effect or had a mild inhibitory effect on baseline proliferation. Therefore, while CD2E1 and anti-T11-1 had identical effects on proliferation individually but different effects when part of a stimulatory pair, the converse was true for CD2E3 and anti-T11-3. When IL-12 or IL-2 was added either to the anti-T11-2 plus anti-T11-3 or to the CD2E1 plus CD2E3 pair, little to no further aug-

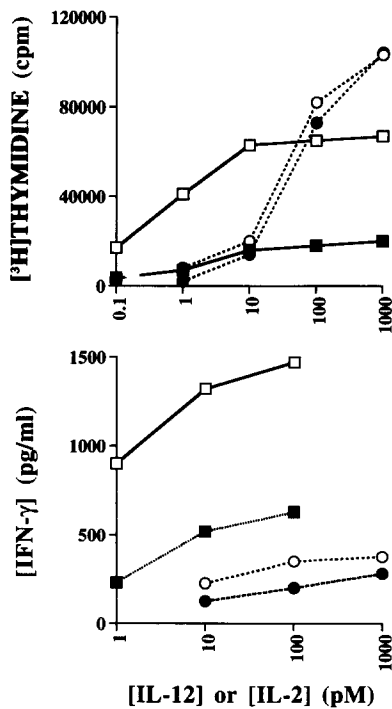


Figure 6. Anti-CD58 selectively inhibits IL-12-induced proliferation and IFN- γ production by PHA-activated T cells. Anti-CD58 was used as a 1:400 dilution of ascites. Results are representative of three experiments for proliferation and two experiments for IFN- γ production. \square —, IL-12; \blacksquare —, IL-12 plus anti-CD58; \circ —, IL-2; \bullet —, IL-2 plus anti-CD58.

mentation was observed (data not shown). However, when used in combination with cytokine, the anti-T11-1 plus anti-T11-3 pair synergized strongly with IL-12 at concentrations ranging from 0.1 to 1 nM (Fig. 8 B). Maximal stimulation achieved by IL-12 closely approximated that obtained

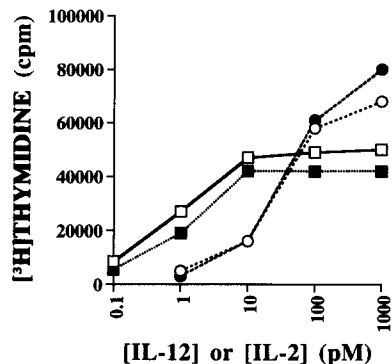


Figure 7. CTLA-4-Ig does not inhibit IL-12- or IL-2-induced proliferation of activated T cells. Purified CTLA-4-Ig was used at 5 $\mu\text{g}/\text{ml}$. Results with an isotype-matched control did not differ significantly from CTLA-4-Ig (data not shown). Results are representative of two experiments. \square —, IL-12; \blacksquare —, IL-12 plus CTLA-4-Ig; \circ —, IL-2; \bullet —, IL-2 plus CTLA-4-Ig.

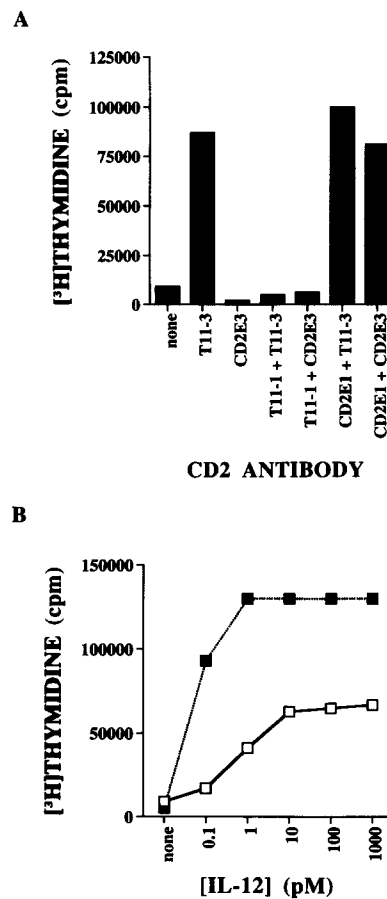


Figure 8. (A) CD2R antibodies can stimulate proliferation of T cells when paired with CD2E1, but fail to stimulate proliferation when paired with anti-T11-1. Day 4 PHA-activated T cells were incubated with the indicated mAbs without the addition of IL-12 or IL-2 in a 48-h proliferation assay. Data with the CD2R antibodies used alone are compared with that obtained with each CD2R mAb paired with anti-T11-1 or CD2E1. Results are representative of three experiments. (B) Anti-T11-1 plus anti-T11-3 synergizes with IL-12 in inducing the proliferation of PHA-activated T cells. Results are representative of three experiments. \square —, no mAb; \blacksquare —, T11-1 plus T11-3.

with IL-2, though 100–1,000-fold less IL-12 was required. No significant synergy was observed with IL-2 (data not shown).

Anti-T11-2 Plus Anti-T11-3 Synergizes with IL-12 in Inducing T Cell IFN- γ Production. After observing the synergy between a specific pair of CD2 antibodies and IL-12 with regard to proliferation, we investigated whether such synergy could be shown for IFN- γ production as well. Anti-T11-2 plus anti-T11-3, when used without cytokine, stimulated T cell IFN- γ production to a level approximating maximal stimulation with IL-12 (Fig. 9). However, it strongly synergized with IL-12, augmenting maximal IFN- γ production 20-fold at an IL-12 concentration of 1 pM and 40-fold at ≥ 10 pM. The effect observed with IL-2 was largely additive, with relatively weak synergy apparent only at a concentration of 1 nM.

The Proliferative Response of T Cells to IL-12 Is Diminished in the Absence of APCs. The observation that CD2 anti-

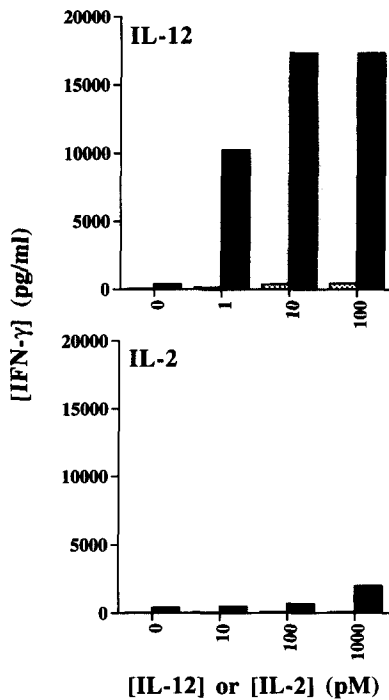


Figure 9. Anti-T11-2 plus anti-T11-3 synergizes with IL-12 in inducing the production of IFN- γ by PHA-activated T cells. Baseline IFN- γ production in the absence of exogenous cytokine was 50 μ g/ml. Maximal IFN- γ production was 450 μ g/ml for IL-12 alone and 70 μ g for IL-2 alone. Results are representative of two experiments. Stippled bar, no mAb; shaded bar, T11-2 plus T11-3.

bodies that block the CD2/CD58 interaction interfere with T cell responsiveness to IL-12 suggested that a cell-cell interaction between activated T cells and a second cell type bearing CD58 might be central to IL-12-mediated T cell stimulation. While this CD2/CD58 interaction could occur between T cells, we hypothesized that, *in vivo*, it would most likely be occurring between T cell and APC (either macrophages or B cells), since the latter expresses CD58 and is the primary source of IL-12 (2). To test this hypothesis, we investigated whether the removal of APCs from PBMC before activation with PHA affected the ability of PHA-activated T cells to respond to IL-12. Since both macrophages and B cells, but not resting T cells, express MHC class II molecules, we rigorously depleted PBMC of APCs by sorting for CD5⁺Ia⁻ cells and subsequently activated these APC-depleted T cells with PHA in the same manner used for whole PBMC. As shown in Fig. 10 A, the proliferative response of APC-depleted T cells to IL-12 was greatly diminished when compared with whole PBMC obtained from the same donor and preactivated in the same manner. Even at an IL-12 concentration of 1 nM, there was a fivefold reduction in proliferation. Notably, the proliferative response to IL-2 was not significantly altered in the absence of APCs. CD2E1, anti-T11-1, and CD2E3 were able to further inhibit IL-12-induced proliferation without affecting the response to IL-2 (data not shown). As shown in Fig. 10 B, when anti-T11-1 plus anti-T11-3 was added to the APC-depleted cells, the prolifer-

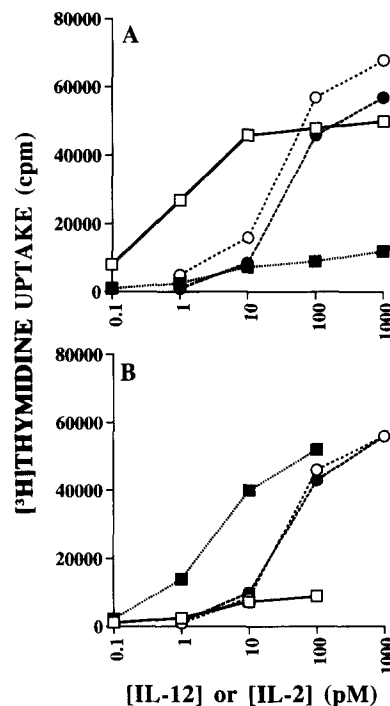


Figure 10. (A) T cell response to IL-12 is diminished in the absence of APCs. Sorted CD5⁺Ia⁻ T cells were activated with PHA for 4 d before incubation with IL-12 or IL-2 at the indicated concentrations in a 48-h proliferation assay. The results are compared with an assay performed under identical conditions using PHA-activated whole PBMC from the same donor. Proliferation in the absence of exogenous cytokine was 800 \pm 150 cpm for the sorted T cells and 5,000 \pm 450 cpm for unsorted T cells. Results are representative of two experiments. \square —, IL-12 plus APCs; \blacksquare —, IL-12 minus APCs; \circ —, IL-2 plus APCs; \bullet —, IL-2 minus APCs. (B) Anti-T11-1 plus anti-T11-3 restores T cell responsiveness to IL-12 in the absence of APCs. Sorted CD5⁺Ia⁻ T cells were incubated with IL-12 or IL-2 in the presence or absence of the indicated CD2 mAbs in a 48-h proliferation assay. Anti-T11-1 was used as ascites at 1:400 dilution, and purified anti-T11-3 was used at 10 μ g/ml. Proliferation with anti-T11-1 plus anti-T11-3, in the absence of exogenous cytokine, was 1,500 \pm 200 cpm. Results are representative of two experiments. \square —, IL-12; \blacksquare —, IL-12 plus T11-1 plus T11-3; \circ —, IL-2; \bullet —, IL-2 plus T11-1 plus T11-3.

ative response to IL-12 was augmented in a synergistic fashion, with maximal proliferation equaling that observed with IL-2. This antibody pair had no effect on IL-2-induced proliferation.

Discussion

In this report, we have used various mAbs specific for CD2 to demonstrate that this surface glycoprotein plays a central role in the response of T cells to IL-12. Although resting T cells are capable of being weakly stimulated by IL-12 to produce IFN- γ , preactivation with a mitogen such as PHA is necessary for up-regulation of the IL-12R (26), leading then to proliferation and augmented IFN- γ production in response to IL-12. The most notable finding in this current study is the dependence of preactivated T cells on the interaction of CD2 with at least one ligand, CD58, for IL-12-induced

proliferation and IFN- γ production. No such dependence was observed for IL-2. The selective inhibitory effect of our antibodies recognizing the adhesion domain of CD2, coupled with the observation that anti-CD58 has an identical effect, is compelling evidence that the CD2/CD58 interaction governs the response of T cells to IL-12. The lack of inhibition by anti-T11-2, an antibody which does not block E-rosetting, further supports the hypothesis that a specific CD2/CD58 interaction is required for T cell responsiveness to IL-12. In contrast to the IL-12-specific inhibitory effect of CD2 antibodies, interference with the CD28/B7 interaction did not alter the response to either IL-12 or IL-2.

The IL-12-specific inhibition observed with CD2E3, our new antibody which recognizes a unique CD2R epitope and does not interfere with E-rosetting, raises the possibility that the CD2/CD58 interaction is not the sole determinant of T cell responsiveness to IL-12. One explanation for the functional effect of CD2E3 is that, by binding to CD2, it prevents an essential conformational change in CD2 normally induced by the CD2/CD58 interaction. Because CD2E3 is an IgM antibody, its large size could be the key factor in interfering with a change in conformation. Anti-T11-3, on the other hand, may induce an activating conformational change upon binding to CD2, accounting for its ability to stimulate T cells when used alone. A second plausible interpretation is that CD2 may interact with a second, unidentified ligand X through CD2R, and this then cooperates with the CD2/CD58 interaction in stimulating a preactivated T cell and priming it for responsiveness to IL-12. In this model, CD2E3 would be a ligand X antagonist, blocking the CD2R/X interaction that would ordinarily be cooperating with the CD2/CD58 interaction. Anti-T11-3, however, would be a ligand X agonist, stimulating CD2 in a manner that cooperates with CD2/CD58 and results in proliferation.

In addition to demonstrating that individual antibodies to CD2 and CD2R can inhibit IL-12 activation, we showed how pairs of CD2R plus CD2 antibodies can synergize strongly with IL-12 in inducing proliferation and IFN- γ production. Some synergy could also be detected with IL-2, but this was far weaker and occurred over a narrower concentration range. As we have demonstrated, different pairs of CD2 plus CD2R mAbs varied in their ability to augment proliferation in the absence of exogenous cytokine. It is not clear why anti-T11-1 failed to overtly stimulate cells in the absence of added cytokine when paired with CD2R mAbs, but this further underscores the point that CD2E1 and anti-T11-1 (like CD2E3 and anti-T11-3) recognize two unique epitopes of CD2. With regard to proliferation, strong synergy was observed with anti-T11-1 plus anti-T11-3, perhaps indicating that CD2 perturbation by these antibodies can still activate cells in a manner that primes them for responsiveness to IL-12 without overtly stimulating them in the absence of cytokine. For IFN- γ production, synergy with IL-12 was demonstrable with anti-T11-2 plus anti-T11-3, because this particular function, unlike proliferation, was not maximally stimulated by these antibodies alone. Taken together with the IL-12-specific inhibitory effect of single antibodies, the data favor the hypothesis

that CD2 activation not only influences whether or not a T cell can respond to IL-12 but also determines the magnitude of the response.

Since we hypothesize that CD2 stimulation, mediated through its interaction with CD58, is required for T cells to respond to IL-12, it is important to know which cell bearing the appropriate ligand interacts with T cells in this capacity. Although many different hematopoietic cell types express CD58 (28), including T cells, we believe that the APC is a good candidate based on our data demonstrating a marked decrease in T cell responsiveness to IL-12 when APCs are removed before PHA activation. In particular, the macrophage would fit this role well based on its ability to present antigen and augment its production of IL-12 in response to IFN- γ (29). Although a T cell, after interacting with an APC bearing antigen, B7, and CD58, is able to perpetuate activation through IL-2 on its own through an autocrine pathway (30), this is not the case for IL-12-mediated activation. Since T cells cannot produce IL-12 (2), they are reliant on the macrophage for IL-12-mediated activation through a paracrine pathway. As illustrated in Fig. 11, we hypothesize from our data that CD2 may assume a central position in an IL-12/IFN- γ positive feedback loop between activated T cell and APC. In this model, stimulation through CD2, occurring when the adhesion domain of CD2 engages CD58 on an APC, confers on the T cell responsiveness to IL-12. Although in our assays T cells were preactivated with PHA, in vivo this preactivation would most likely result from antigen recognition through the T cell receptor along with B7/CD28 costimulation. Once this CD2-mediated functional link between activated T cell and APC is established, APC-derived IL-12 can act on the CD2-primed T cell to induce maximal IFN- γ production and proliferation. The T cell-derived IFN- γ would then, in turn, stimulate the APC to kill its intracellular pathogen and produce more IL-12. By ensuring that the T cell responds to IL-12 only when intimately associated with the source, this would be an efficient mechanism by which a T cell could perpetuate its activation by IL-12 and most effectively deliver IFN- γ to its primary target, the macrophage.

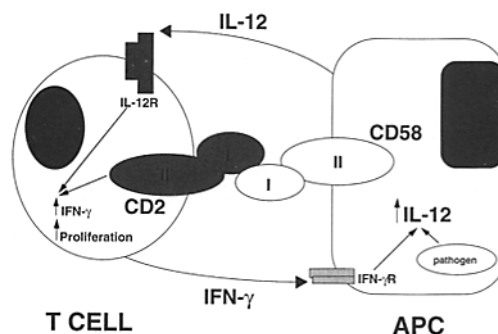


Figure 11. CD2 provides the key functional link between T cell and APC required for T cell responsiveness to IL-12. This hypothetical model shows how the interaction of CD2 with CD58 would play a key role in the generation of an IL-12/IFN- γ positive feedback loop between T cell and APC.

A salient feature of the data presented in this report is the apparent specificity the CD2/CD58 interaction has in controlling the T cell response to IL-12, as contrasted with IL-2. This functional pairing between a cytokine receptor and a distinct adhesion molecule may allow a hematopoietic cell to modulate its responsiveness to a particular cytokine based on an ability to discriminate between a microenvironment that can and one that cannot optimally support and benefit from such a response. The molecular basis by which the CD2/CD58 interaction controls IL-12 responsiveness now needs to be defined. Whatever the mechanism, the CD2/CD58 interaction will most likely be involved, along with IL-12, in shifting the immune response along a Th1 pathway.

Several interesting parallels to the IL-12/CD2-CD58 interaction exist outside of the hematopoietic system. For example, in a human pancreatic cell line, the interaction of a specific class of integrins ($\alpha_v\beta_3$) with its ligand, vitronectin, enhances the growth response to insulin (31). At the molecular level, stimulation through the insulin receptor results in the association of this integrin with tyrosine-phosphorylated insulin receptor substrate 1 and several other signaling molecules (31), suggesting a cooperative interaction between a growth factor receptor and adhesion molecule at the level of signal transduction. In rat cerebellar neurons, it has been demonstrated that neurite outgrowth stimulated by the homophilic binding of neuronal cell adhesion molecules (CAMs) to N-CAM, N-cadherin, or L1 involves activation of the fibroblast growth factor receptor (FGFR) (32). In this system, it appears as though a direct physical interaction between specific CAMs and the FGFR can result in FGFR-mediated signal transduction, even in the absence of soluble FGF (32).

In summary, we have demonstrated that antibodies directed at specific regions of CD2 influence the response of T cells to IL-12. To explain this novel effect, we have proposed a model in which CD2 provides a critical functional link for an IL-12/IFN- γ positive feedback loop between T cell and APC. Along with providing a new direction for investigating the

functional significance of CD2, our findings shed light on how the functional effect of a particular cytokine may be influenced not only by the presence or absence of a specific receptor, but also by specific, cooperative cell-cell interactions designed to maximize and perpetuate a particular immune response. Since our model is centered on a cooperative interaction between CD2 and the IL-12R, future investigations of the molecular basis governing this proposed interaction may yield valuable information regarding the signaling pathways of both CD2 and the IL-12R and how they differ from signaling via the IL-2R. From a clinical standpoint, since IL-2 is capable of activating T cells, NK cells, and monocytes in the absence of costimulation, it has been used with the assumption that parenteral administration would systemically activate those components of the immune system bearing receptors for IL-2. That this indeed occurs has been demonstrated both by in vitro studies of lymphocytes derived from patients receiving IL-2 (33, 34) and by the side effects (which can include fever, hypotension, myalgias, and capillary leak syndrome) reflecting IL-2-induced cytokine production (34, 35). On the other hand, if IL-12 is active on T cells primarily in the presence of CD2 stimulation, as our findings suggest, then the effectiveness of systemic administration will depend heavily on the microenvironment, with T cells responding to IL-12 only if tumor antigens or antigens from infectious pathogens are presented along with the appropriate ligand(s) for CD2. Furthermore, the side effects of IL-12 might be considerably less compared with IL-2 if IL-12-induced cytokine production is not systemically stimulated. Whether NK cells also require CD2 stimulation to respond to IL-12 remains to be investigated, although it seems less likely since an interaction with APCs is not required for antigen recognition. As IL-12 holds considerable promise, based on animal studies, for the treatment of both oncologic disorders (36) and infectious diseases (29), a better knowledge of those cellular interactions cooperating with IL-12 will help in translating the observed immunomodulatory effects of IL-12 into a clinically beneficial treatment modality.

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