Interleukin-12 can replace CD28-dependent T-cell costimulation during nonspecific cytotoxic T lymphocyte induction by anti-CD3 antibody

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Abstract: Cytotoxic T lymphocyte (CTL) development is regulated closely by an intricate series of signals provided by the T-cell receptor/CD3 complex, cytokines, and costimulatory ligand/receptor systems. In this study, we have explored the role of interleukin (IL)-12 and CD28 in mouse CTL development. Activation of T cells with anti-CD3 monoclonal antibody (mAb) in the presence of anti-CD86 mAb, which prevents CD28-CD86 interaction, led to decreased production of type 1 (IL-2, interferon-γ) and type 2 (IL-4, IL-6, IL-10) cytokines, as well as diminished expression of granzyme B (Gzm B) and reduced cytotoxic effector function. Cytolytic activity in T-cell cultures that were activated in the presence of anti-CD86-blocking mAb alone or in combination with anti-CD80 mAb could be restored by the addition of exogenous IL-12 at initiation of culture. The ability of IL-12 to substitute for CD28-costimulatory signaling during CTL development was found to be dependent on the presence of IL-2 rather than interferon-γ. IL-2 is required for IL-12Rβ2 expression by T cells activated in the presence of anti-CD86 mAb. Moreover, IL-12Rβ2 expression by T cells activated in the presence of anti-CD86 mAb is enhanced by IL-12. We, therefore, conclude that the ability of IL-12 to substitute for CD28-costimulatory signaling during CTL development is a result of the interaction of IL-12 with IL-12Rβ2 induced by low levels of IL-2 synthesized by T cells activated in a CD28-independent manner. *J. Leukoc. Biol.* 69: 113–122; 2001.

Key Words: *CTL gene induction · cytotoxicity · costimulatory signaling · cytokines*

INTRODUCTION

T-cell activation requires signal transduction through several distinct cell-surface receptors [1]. The antigen-specific recognition signal is supplied by the T-cell receptor/CD3 complex following ligation by antigen in the context of class I or class II major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells. T cells also require a costimulatory signal provided by antigen-presenting cells to become fully activated and produce cytokines such as interleukin (IL)-2, which promote subsequent T-cell proliferation and differentiation [2]. The CD28-CD80/CD86 receptor/ligand system is arguably the most important and best-studied costimulatory pathway [3]. Ligation of CD28 by CD80 or CD86 on antigen-presenting cells results in enhanced production of IL-2 by T helper cells [4]. In addition, cytotoxic T lymphocyte (CTL) precursors are activated by target cells that bear CD28-binding ligands [5]. Once activated, these CTLs gain the ability to produce IL-2, which supports their proliferation and differentiation in an autocrine fashion. As a result, CTL can synthesize the cytotoxic-effector molecule granzyme (Gzm) B independently of cytokine secretion by T helper cells [5]. Perforin is another CTL granule-associated protein with cytotoxic function that is upregulated strongly by IL-2 [6]. CTL can use membranolytic mechanisms also such as CD95 ligand (CD95L), which interacts with CD95 on target cells to induce apoptosis and target-cell destruction [7]. Human and mouse CTLs kill target cells more efficiently if the targets express CD80 or CD86 proteins [8, 9]. However, many target cells do not express CD80 or CD86, thereby restricting the self-sufficiency of CTL induction as well as diminishing the overall cytotoxic potential of CTL generated under these conditions.

IL-12 is a cytokine, which, through its ability to upregulate Gzm B and perforin mRNA expression, plays an important role in the development of CTL with optimal cytotoxic activity [10]. For example, the cytotoxicity of human $CD8^+$ T cells collected from peripheral blood is increased ten- to 20-fold when these cells are activated with anti-CD3 monoclonal antibody (mAb) in the presence of IL-12 [11]. IL-12 is secreted by a wide variety of professional and nonprofessional antigen-presenting cells, including B cells [12], monocytes/macrophages [13], dendritic cells [14], Langerhans cells [15], and keratinocytes [16]. Macrophages, however, appear to be the major source of IL-12 [13]. IL-12 production by antigen-presenting cells is induced via CD40-CD40L interaction with activated T cells [17]. Bioactive IL-12 is a heterodimeric molecule composed of p40 and p35 chains [12]. Dimerized p40, although not bioactive, is able to bind to the IL-12 receptor (IL-12R) also and prevent the productive binding of the bioactive p70 het-

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erodimer [18]. The IL-12R, composed of IL-12Rβ1 and IL-12Rβ2 subunits, is expressed on $CD4^+$ and $CD8^+$ T cells, as well as on natural killer (NK) cells [19]. In addition to upregulating the cytotoxic activity of CTL and NK cells, IL-12 induces interferon (IFN)-γ production by T cells and NK cells [20, 21]. IL-12 signaling and CD28 costimulation have a synergistic-enhancing effect on T-cell proliferative responses and cytokine production [22]. In fact, costimulation with CD80, IL-6, and IL-12 is sufficient to induce tumor-specific mouse CTL *in vitro* [23]. Moreover, compared with tumor cells transfected with CD80 alone, transfection of tumor cells with IL-12 and CD80 enhances dramatically the ability of mice implanted with the transfected tumor cells to develop effective antitumor immunity [24].

In the present investigation, we have determined the effect of IL-12 on the development of MHC-unrestricted cytotoxicity in mouse T-cell cultures stimulated with anti-CD3 mAb in the presence of anti-CD86 mAb to block CD28-CD86 interaction and subsequent CD28-costimulatory signaling. We have shown previously that CD86 rather than CD80 is the principal costimulatory ligand for CD28 during anti-CD3-induced CTL induction [25]. Because CD86 blockade resulted in a dramatic reduction in cytokine synthesis, as well as a profound decrease in the cytotoxic activity of anti-CD3-induced CTL, we reasoned that CTL function in anti-CD86-treated T-cell cultures might be restored by the addition of IL-12, which has known cytotoxicity-promoting activity [10, 11]. Here, we describe the novel finding that IL-12 can substitute effectively for CD28 costimulation, leading to the generation of potent CTL with high levels of Gzm B expression and activity.

MATERIALS AND METHODS

Mice

Female 6- to 8-week-old C57BL/6 mice were purchased from Charles River Canada (Lasalle, Quebec). Mice were maintained on standard laboratory chow and water, supplied *ad libitum* in our animal care facilities.

Medium and reagents

RPMI 1640 medium (ICN Biomedicals Canada Ltd., Mississauga, Ontario), hereafter referred to as complete RPMI 1640 medium, was supplemented with 10 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all from ICN Biomedicals Canada), 5 mM HEPES buffer (Sigma Chemical Co., St. Louis, MO), pH 7.4, and 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (Life Technologies Ltd., Burlington, Ontario, Canada). Human recombinant IL-2 (active in the murine system) was obtained from Collaborative Biomedical Products (Becton Dickinson Labware, Bedford, MA). Specific activity is expressed as U/ml, where 1 U is defined as the reciprocal of the dilution required to cause 50% stimulation of mouse CTLL-2 cells. Mouse recombinant IFN-γ was purchased from Genzyme Diagnostics (Cambridge, MA). Specific activity is expressed as Genzyme U/ml. Mouse recombinant IL-12 was generously provided by Dr. J. Marshall (Dalhousie University, Halifax, Nova Scotia). The hybridoma (clone 145-2C11), which produces hamster anti-mouse CD3ε mAb [26], was kindly provided by Dr. J. Bluestone (University of Chicago, Chicago, IL). The hybridoma (clone GL1), which produces rat anti-mouse CD86 mAb [27], was a generous gift from Dr. K. Hathcock (National Cancer Institute, Bethesda, MD). The hybridoma (clone 16-10A1), which produces hamster anti-mouse CD80 mAb, was obtained from American Type Culture Collection (ATCC; Manassas, VA). Rat anti-mouse IL-2-neutralizing mAb was from Genzyme Diagnostics, and rat anti-mouse CD4 mAb, rat anti-mouse CD28 mAb, and rat anti-mouse CD25 mAb [fluorescein isothiocyanate (FITC)-conjugated and −unconjugated] were from Cedarlane Laboratories (Hornby, Ontario, Canada). Rat anti-mouse IFN-γR α chain mAb and rat anti-mouse CD80 mAb (clone 1G10) were from PharMingen Canada (Mississauga, Ontario), and rat anti-mouse IFN-γ-neutralizing mAb was from Upstate Biotechnology Inc. (Lake Placid, NY). Purified rat immunoglobulin G (IgG) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). All cytokines and mAb were aliquoted and stored at −70°C. P815 murine ($H\text{-}2^d$) mastocytoma cells were obtained from ATCC and maintained by *in vitro* passage in complete RPMI 1640 medium.

Generation of anti-CD3-activated CTL

C57BL/6 spleen-cell preparations were depleted of erythrocytes by osmotic shock and passaged once through nylon-wool columns (Cellular Products, Inc., Buffalo, NY) to remove most B cells and macrophages [28]. Nylon-wool nonadherent spleen cells were depleted of NK cells by treatment with anti-asialoGM1 rabbit polyclonal antiserum (Wako Chemicals, Richmond, VA) plus rabbit complement (Cedarlane Laboratories). The resulting T-cell-enriched preparation (typically ~90% $CD3^+$ and <0.1% $NK1.1^+$) [29] was adjusted to a concentration of 4×10^6 cells/ml in complete RPMI 1640 medium and seeded into wells of a 24-well, flat-bottom, tissue-culture plate. In some experiments, $CD8^+$ T cells (prepared by anti-CD4 mAb plus complement treatment) were used instead of unfractionated T cells. CTLs were induced as previously described [25] by stimulating T cells with soluble anti-CD3 mAb (1:20 dilution of hybridoma supernatant) in the presence or absence of additional mAb and/or cytokines. Cultures were maintained for 48 h at 37°C and 5% CO_2 in a 95% humidified atmosphere. Anti-CD3-activated CTL were then collected for use.

^{51}Cr-release assay

MHC-unrestricted CTLs induced with anti-CD3 mAb were washed extensively with phosphate-buffered saline, pH 7.2, resuspended in complete RPMI 1640 medium, and seeded into wells of a 96-well, V-bottom, microtitre plate in graded dilutions to obtain the desired effector:target (E:T) ratios. P815 mastocytoma cells were labeled with 100 μCi $Na_2\ ^{51}CrO_4$ (ICN Biomedicals Canada) for 1 h at 37°C, washed three times, resuspended in complete RPMI 1640 medium, and added to the microtitre plate at a concentration of 5×10^3 cells/well. The microtitre plate was then incubated for 4 h at 37°C and 5% CO_2 in a 95% humidified atmosphere. Following centrifugation of the microtitre plate, 100 μL supernatant was collected from each well, and ^{51}Cr release (in cpm) was determined by γ-counting. Percent lysis was determined by the following equation: % lysis = (E−S)/(M−S) × 100, where E is the release from experimental samples, S is the spontaneous release, and M is the maximum release upon lysis with 10% sodium dodecyl sulfate (SDS).

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in supernatants from 24 or 48 h cultures of anti-CD3-activated T cells were measured by sandwich ELISA using paired mAb, recombinant cytokines, and protocols supplied by PharMingen Canada (with the exception of the capture mAb for the IL-12 ELISA). The capture mAb for the IL-12 ELISA was hamster anti-mouse IL-12 p35 mAb (Genzyme Diagnostics), which recognizes mouse IL-12 p35 and p70 but not the p40 monomer or p40 homodimer.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from CTL using TRIzol reagent as recommended by the manufacturer (Life Technologies). To determine Gzm B mRNA expression, RNA was reverse-transcribed and amplified in a one-step reaction using RT-PCR beads (Pharmacia Biotech Inc., Baie D'Urfé, Quebec, Canada). The reaction was carried out in a 50 μL vol pyrogen-free water containing 1 μg random hexanucleotide primers, 0.5 μM each PCR primer, and 0.5 μg RNA. Each reaction mixture was overlaid with 100 μL mineral oil, and synthesis of cDNA was facilitated by sequential incubation at 42°C for 30 min and 95°C for 5 min. Gzm B mRNA expression was then determined by PCR (28 cycles) using the following amplification protocol: denaturation at 92°C for 30 sec, annealing at 57°C for 30 sec, and primer extension at 72°C for 2 min. To determine IL-2, IL-12Rβ2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression, cDNA was synthesized by reverse transcription

of ~1 μg RNA with 200 U Moloney murine leukemia virus in the presence of 1 μg random hexanucleotide primers and 0.5 mM dNTPs. Following incubation of the reaction mixture for 1 h at 37°C and for 10 min at 95°C, the vol was adjusted to 0.2 ml with pyrogen-free water. Each PCR used a 50 μL vol cDNA, 2.5 U Taq DNA polymerase (Life Technologies), 0.2 mM dNTPs, and 50 mM each primer pair in a 1:10 dilution of PCR buffer [2 M KCl, 1 M Tris-HCl, pH 8.4, 1 M $MgCl_2$, 1 mg/ml bovine serum albumin (BSA)]. PCR mixtures were overlaid with 100 μL mineral oil. The amplification protocol for GAPDH, IL-12Rβ2 (both 28 cycles), and IL-2 (32 cycles) was as follows: denaturation at 92°C (GAPDH and IL-12Rβ2) or 94°C (IL-2) for 30 sec, annealing at 57°C (IL-2 and GAPDH) or 59°C (IL-12Rβ2) for 30 sec, and primer extension at 72°C for 1 min (IL-2) or 1.5 min (GAPDH and IL-12Rβ2). The number of PCR cycles used was determined previously to generate PCR product during the exponential phase of amplification. All primers were designed to bind intron-bridging exons of the respective gene. GAPDH (F) 5′-ACTCACGGCAAATTCAACGGC-3′; GAPDH (R) 5′-ATCACAAACATGGGGGCATCG-3′ (product size: 247 bp); IL-12Rβ2 (F) 5′- GCACAGACTGTTAGAGAATGC-3′; IL-12Rβ2 (R) 5′-CCTTCCTGGACACATGATATG-3′ (product size: 443 bp); Gzm B (F) 5′-GCCCACAACATCAAAGAACAG-3′; GzmB (R) 5′-GAGAACACATCAGCAACTTGGG-3′ (product size: 889 bp); IL-2 (F) 5′-TGATGGACCTACAGGAGCTCCTGAG-3′; IL-2 (R) 5′-GAGTCAAATCCAGAACATGCCGCAG-3′ (product size: 170 bp). PCR products were visualized by electrophoresis across an ethidium bromide-stained 1.5% agarose gel. The quantity of RNA employed in the one-step RT-PCR reaction was electrophoresed also, and bands of 18S and 28S ribosomal RNA were used as a visual control for equal template loading. Alternatively, steady-state expression of GAPDH mRNA was used to control for equal product loading.

Colorimetric Gzm B assay

Gzm B activity in the cytosolic fraction of CTL was measured by colorimetric-enzyme assay as previously described [29] using the Gzm B-specific synthetic substrate Boc-Ala-Asp thiobenzyl ester (Enzyme Systems Products, Dublin, CA). Gzm B-specific esterolytic activity correlates with absorbance at 405 nM.

Flow cytometric analysis

The percentage of CD25-, CD80-, and CD86-positive cells in 48 h cultures of anti-CD3- activated T cells was determined by flow cytometric analysis using a standard protocol [29].

Statistical analysis

Statistical comparisons of data by Student's *t*-test were performed using the Instat statistics program (GraphPad Software, Inc., San Diego, CA). Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

CD86 blockade inhibits cytokine synthesis in anti-CD3-activated T-cell cultures

CD86 is the principal costimulatory ligand of CD28 during the induction of MHC-unrestricted mouse CTL by anti-CD3 mAb, because lack of CD86-dependent costimulation results in dramatically reduced cytotoxicity and Gzm B gene transcription, whereas blocking CD28-CD80 interaction fails to affect cytotoxicity substantially or Gzm B expression [25]. Given the importance of CD28 ligation by CD86 in promoting the synthesis of IL-2 and IFN-γ [9], which are important cytokines in CTL development [30], we wished to determine the effect of CD86 blockade on cytokine production in anti-CD3-stimulated T-cell cultures. T lymphocytes from C57BL/6 mice were cultured for 48 h in the presence of anti-CD3 mAb in combination with a saturating concentration of anti-CD86 mAb (~4 μg/ml) or an equivalent concentration of an irrelevant rat IgG. Culture supernatants were then collected, and cytokine levels were determined by cytokine-specific sandwich ELISA assays. As shown in **Table 1**, anti-CD3-activated T-cell cultures treated with anti-CD86 mAb contained reduced levels of IL-2, IL-4, IL-6, and IL-10, and IFN-γ synthesis was virtually ablated. In contrast, tumor necrosis factor α (TNF-α) production was not diminished by anti-CD86 mAb treatment. Interestingly, the IL-12 p70 heterodimer was not detectable in T lymphocyte cultures activated with anti-CD3 mAb.

IL-12 can substitute for CD28 costimulatory signaling during CTL induction

CD86 blockade had the most dramatic effect on IFN-γ production in anti-CD3-activated T-cell cultures (Table 1). Given the importance of IFN-γ in promoting the development of anti-CD3-induced CTL [29], we felt that diminished IFN-γ levels might be the cause of reduced cytotoxicity in anti-CD3-activated T-cell cultures performed in the presence of anti-CD86 mAb. IL-12 is a potent inducer of IFN-γ synthesis by T lymphocytes [20] and also enhances the development of cytotoxic-effector cells [31]. We, therefore, determined whether addition of IL-12 might be able to substitute for CD28-dependent costimulation in anti-CD3-activated T-cell cultures performed in the presence of anti-CD86 mAb. Recombinant IL-12 (25 ng/ml) was added at the start of culture to T-cell cultures containing anti-CD3 mAb alone or in combination with anti-CD86 mAb, and cytotoxic activity against P815 mastocytoma cells was measured after 48 h of culture by standard ^{51}Cr-release assay. Previous studies have established that mouse T cells activated with anti-CD3 mAb acquire potent MHC-unrestricted cytotoxic activity against a range of tumor target cells, including P815 mastocytoma cells, which peaks at 48 h of culture [32]. **Figure 1A** shows that cytotoxicity was dimin-

TABLE 1. Differential Effects of CD86 Blockade on Anti-CD3-Induced Cytokine Synthesis by T Cells

		Cytokine concentrations in culture supernatants (pg/mL)[b]						
Expt.	Additions to culture[a]	TNF-α	IFN-γ	IL-2	IL-4	IL-6	IL-10	IL-12 (p70)
1.	Anti-CD3 + rat IgG	27 ± 2	429 ± 8	22 ± 1	7 ± 1	243 ± 3	22 ± 1	BLD
	Anti-CD3 + anti-CD86	34 ± 5	1 ± 1*[c]	12 ± 1*	BLD[d]	56 ± 2*	4 ± 1*	BLD
2.	Anti-CD3 + rat IgG	32 ± 1	1400 ± 280	24 ± 1	7 ± 1	743 ± 130	48 ± 1	BLD
	Anti-CD3 + anti-CD86	38 ± 2	22 ± 2*	11 ± 1*	3 ± 1*	186 ± 4*	35 ± 1*	BLD

[a] T cells were stimulated with anti-CD3 mAb in the presence of rat IgG or anti-CD86 mAb (~4 μg/mL). [b] Cytokine levels in culture supernatants after 24 h of culture were evaluated by cytokine-specific sandwich ELISA (SD from triplicate samples is indicated). [c] Asterisk indicates a statistically significant difference compared with the control as determined by Student's *t*-test. [d] BLD, below limits of detection.

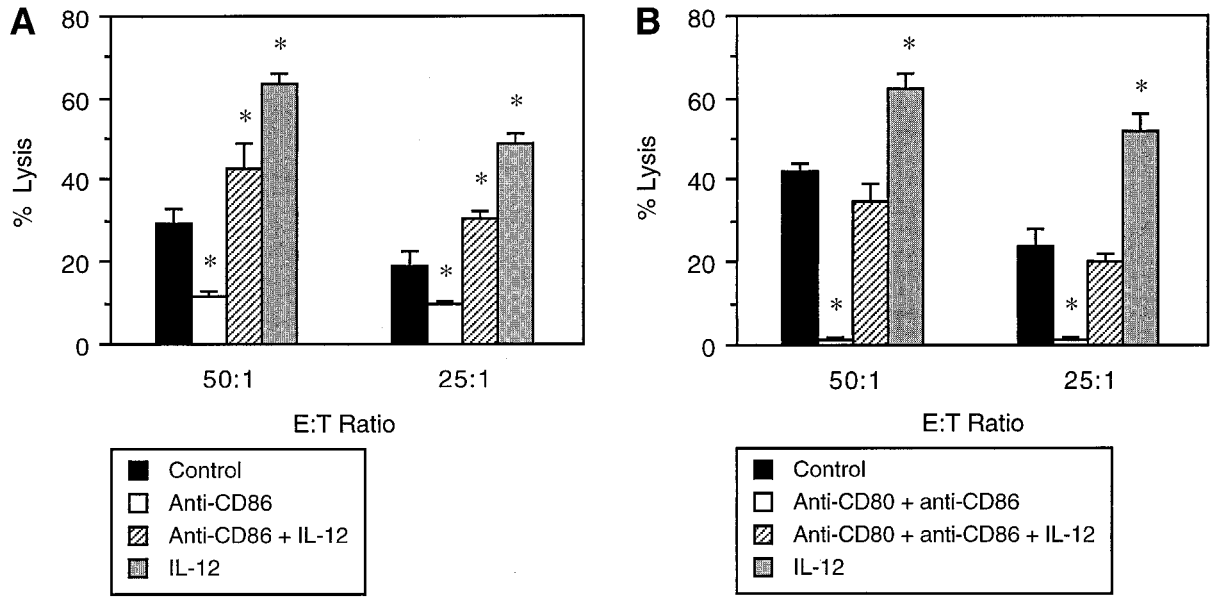

Fig. 1. Restorative effect of exogenous IL-12 on CTL induction in the absence of (A) CD28- CD86 or (B) CD28-CD80/CD86 interaction. T cells were stimulated with anti-CD3 mAb in the presence of rat IgG or optimal-blocking concentrations of anti-CD86 mAb (~4 μg/ml) plus or minus anti-CD80 mAb (~0.2 μg/ml), with or without exogenous IL-12 (25 ng/ml). Following 48 h of culture, cytotoxicity against P815 target cells at E:T ratios of 50:1 and 25:1 was determined by ^{51}Cr-release assay. Results are expressed as mean percent lysis (±SD) and are representative of three independent experiments. Asterisks denote a statistically significant (as determined by Student's *t*-test) change in cytotoxic activity compared with the untreated control.

ished greatly in anti-CD3-activated T-cell cultures containing anti-CD86 mAb alone but was restored to control levels in cultures that contained IL-12 also. Furthermore, the ability of IL-12 to compensate for a lack of CD28-CD86 interaction during CTL development did not involve the ligation of CD28 by CD80 (expressed at a minimal level in these cultures) [25], because IL-12 was equally effective in restoring cytotoxicity when CTLs were induced in the presence of optimal blocking concentations of anti-CD80 and anti-CD86 mAb (Fig. 1B). Although anti-CD3-activated T-cell cultures contain $CD4^+$ and $CD8^+$ T cells, similar results were obtained when IL-12 was added to anti-CD3- activated $CD8^+$ T-cell cultures containing anti-CD80 and anti-CD86 mAb (**Table 2**), indicating that IL-12 is able to act directly on precursor CTL. Consistent with an earlier study [31], IL-12 alone did not elicit substantial cytotoxicity in mouse T-cell cultures (unpublished results).

IL-12 substitutes for CD28-costimulatory signaling during CTL development via an IFN-γ-independent mechanism

We determined next whether IL-12 was substituting for costimulatory signaling through CD28 via an IFN-γ-dependent mechanism. To neutralize completely the bioactivity of any IL-12- induced IFN-γ in anti-CD3-activated T-cell cultures treated with anti-CD86 mAb plus rIL-12, we added anti-IFN-γ-neutralizing mAb in combination with anti-IFN-γR-blocking mAb at initiation of culture. Surprisingly, cytotoxicity in 48 h cultures of T cells activated in the presence of anti-CD86 mAb was largely restored to control levels upon addition of rIL-12, despite neutralization of IFN-γ bioactivity in these cultures (**Fig. 2A**). Furthermore, the addition of exogenous IFN-γ (100 U/ml) at initiation of culture failed to reverse the inhibitory effect of CD86 blockade on CTL induction (Fig. 2B). Taken together, these data indicate that the ability of IL-12 to compensate for a lack of CD28-CD86 interaction in anti-CD3-activated T-cell cultures containing anti-CD86 mAb does not involve IFN-γ.

IL-12 substitutes for CD28-costimulatory signaling during CTL induction through an IL-2-dependent mechanism

Because IL-2 is a critical cytokine for CTL development [30], we next considered the possibility that IL-12 might be acting through an IL-2-dependent mechanism to restore normal levels

TABLE 2. IL-12 Causes $CD8^+$ T Cells to Develop Cytotoxic Activity in the Absence of CD28-Costimulatory Signaling

	% Lysis P815		
Additions to culture[a]	E:T 50:1	25:1	Significance[b]
Anti-CD3 + hamster/rat IgG	27 ± 1	14 ± 4	
Anti-CD3 + IL-12	68 ± 11	68 ± 13	$P < 0.001$
Anti-CD3 + anti-CD80 + anti-CD86	3 ± 1	3 ± 1	$P < 0.001$
Anti-CD3 + anti-CD86 + anti-CD86 + IL-12	30 ± 2	13 ± 5	NS

[a] $CD8^+$ T cells were stimulated with anti-CD3 mAb in the presence of hamster plus rat IgG, IL-12 (10 ng/mL), or anti-CD80 plus anti-CD86 mAb (~0.2 and 4 μg/mL, respectively) with or without IL-12. [b] Statistical significance compared with the control was determined by Student's *t*-test. NS, not significant.

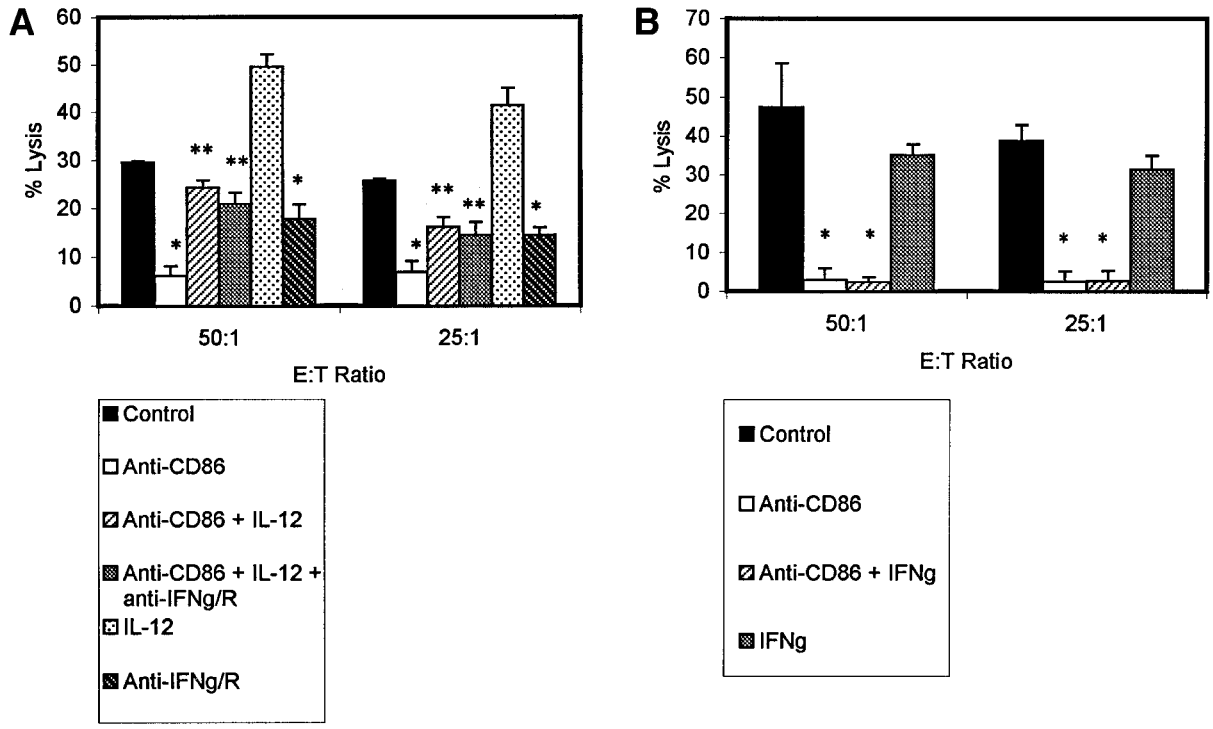

Fig. 2. IL-12 substitutes for CD86-dependent costimulation of CTL via an IFN-γ-independent mechanism. (A) T cells were stimulated with anti-CD3 mAb in the presence of rat IgG or anti-CD86 mAb (~4 μg/ml final concentration) with or without IL-12 (10 ng/ml), anti-IFN-γ plus anti- IFN-γR mAb (both at 10 μg/ml), or IL-12 in combination with anti-IFN-γ plus anti-IFN-γR mAb. (B) T cells were stimulated with anti-CD3 mAb in the presence of rat IgG or anti-CD86 mAb (~4 μg/ml final concentration) with or without IFN-γ (100 μ/ml). Following 48 h of culture, cytotoxicity against P815 target cells at the indicated E:T ratios was determined by ^{51}Cr-release assay. Results are expressed as mean percent lysis (±SD) and are representative of at least two independent experiments. A single asterisk denotes a statistically significant (as determined by Student's *t*-test) reduction in cytotoxic activity compared with the control, and a double asterisk denotes a statistically significant difference compared with anti-CD86-treated cells.

of cytotoxicity in anti-CD3-activated T-cell cultures performed in the presence of blocking anti-CD86 mAb. To test this hypothesis, T cells were activated with anti-CD3 mAb in the presence of anti-CD86 mAb and IL-12, with or without anti-IL-2-neutralizing mAb in combination with an IL-2R-blocking (anti-CD25) mAb. **Figure 3** shows that the resulting abrogation of IL-2 bioactivity resulted in the failure of IL-12 to restore cytotoxicity in T-cell cultures activated in the presence of anti-CD86 mAb, indicating that IL-12 substitutes for CD28-costimulatory signaling via an IL-2-dependent mechanism.

IL-12 fails to enhance IL-2 synthesis or the expression of CD25, CD80, or CD86 in T-cell cultures activated in the presence of anti-CD86 mAb

We first considered the possibility that IL-12 might upregulate IL-2 synthesis in T-cell cultures that were activated with anti-CD3 mAb in the presence of anti-CD86 mAb. Culture supernatants were collected at 24 h of culture and assayed by IL-2-specific sandwich ELISA. Levels of IL-2 in anti-CD86 mAb plus IL-12-treated T-cell cultures were comparable to the reduced IL-2 levels found in cultures treated with anti-CD86 alone, indicating that the addition of IL-12 to anti-CD86-treated activated T-cell cultures does not result in enhanced IL-2 synthesis (**Table 3**). Similar results were obtained when IL-2 production was measured after 8 h of culture (unpublished results). As a positive control, some cultures were treated with IL-12 plus anti-CD86 and anti-CD28 mAb. Abundant IL-2 was detected in these cultures as a result of optimal costimulation of T cells through mAb cross-linked CD28. To address the possibility that any additional IL-2 induced by IL-12 might be consumed by the proliferating T cells and, therefore, not be detected by ELISA, we also examined IL-2 mRNA expression by semiquantitative RT-PCR. IL-2 mRNA levels in T cells activated in the presence of anti-CD86 mAb, with or without IL-12, were equivalent (unpublished results), thereby confirming that IL-12 does not upregulate IL-2 production by T lymphocytes activated under these culture conditions.

Next, we examined the effect of IL-12 on CD25, CD80, and CD86 expression in anti-CD3- activated T-cell cultures performed in the presence of anti-CD86 mAb, because upregulation of IL-2R expression could lead to more effective utilization of available IL-2, and increased CD80 and/or CD86 expression would be expected to enhance IL-2 synthesis. Flow cytometric analysis revealed a substantial decrease in the percentage of CD25-bearing cells in T-cell cultures activated in the presence of anti-CD86 mAb compared with control cultures, which received anti-CD3 mAb only plus an irrelevant rat IgG (**Table 4**). Mean channel fluorescence for CD25, which correlates roughly with surface-molecule density, was unaffected. T cells activated in the presence of anti-CD86 mAb plus exogenous IL-12 expressed CD25 at close to the same level as T cells activated in the presence of anti-CD86 mAb. IL-12 alone had no effect on CD25 expression by anti-CD3-activated T cells. In

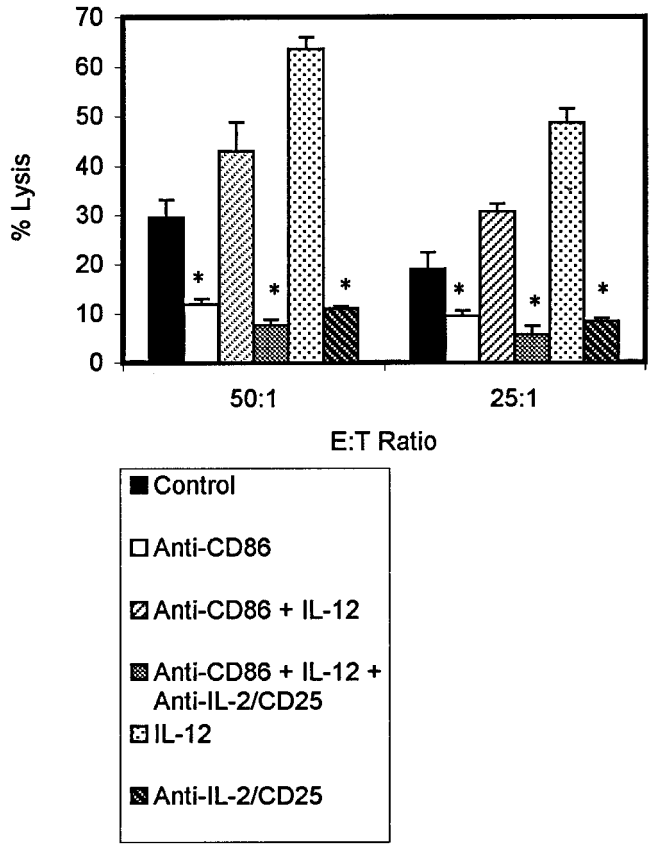

Fig. 3. IL-12 substitutes for CD86-dependent costimulation of CTL via an IL-2-dependent mechanism. T cells were stimulated with anti-CD3 mAb in the presence of rat IgG or anti-CD86 mAb (~4 μg/ml final concentration) with or without IL-12 (10 ng/ml), anti-IL-2 plus anti-CD25 mAb (both at 10 μg/ml), or IL-12 in combination with anti-IL-2 plus anti-CD25 mAb. Following 48 h of culture, cytotoxicity against P815 target cells at the indicated E:T ratios was determined by ^{51}Cr-release assay. Results are expressed as mean percent lysis (±SD) and are representative of three independent experiments. Asterisk denotes a statistically significant reduction in cytotoxic activity compared with the control, as determined by Student's *t*-test.

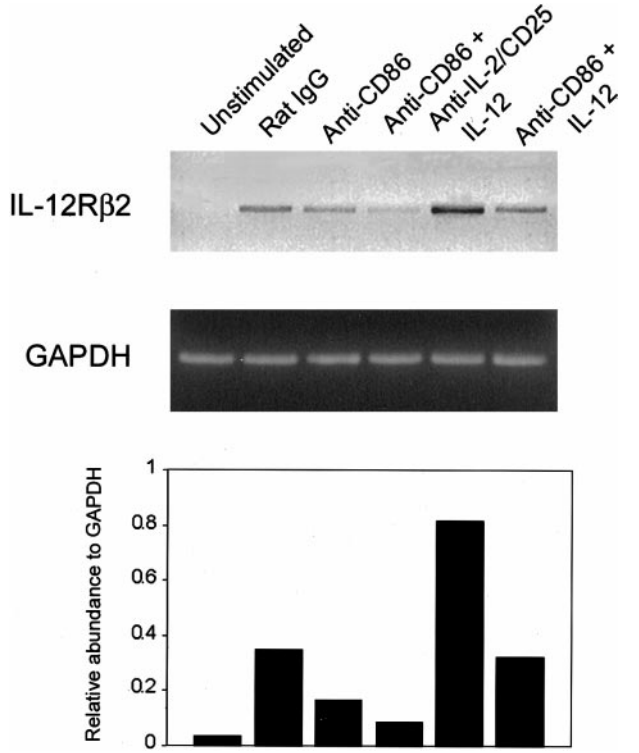

Fig. 4. T-cell expression of IL-12Rβ2 is IL-2-dependent and is upregulated by IL-12 in the presence of anti-CD86 mAb. T cells were cultured alone or stimulated with anti-CD3 mAb in the presence of rat IgG, anti-CD86 mAb (~4 μg/ml final concentration), anti-CD86 with anti-IL-2 plus anti-CD25 mAb (both at 10 μg/ml), IL-12 (10 ng/ml), or anti-CD86 mAb plus IL-12. Total RNA was isolated following 24 h of culture and reverse-transcribed, and semiquantitative PCR with exon-binding, intron-bridging primers for IL-12Rβ2 was performed. GAPDH mRNA levels were determined by RT-PCR also. Amplicons were resolved by gel electrophoresis and visualized by ethidium-bromide staining. The images were scanned and inverted, and IL-12Rβ2 expression was quantified by densitometric analysis relative to the steady-state expression of GAPDH. Data are from one experiment representative of two independent experiments.

line with our finding that IL-12 does not upregulate IL-2 synthesis in T-cell cultures activated in the presence of CD86-blocking mAb (Table 4), CD80 and CD86 expression in anti-CD3-activated T-cell cultures was unaffected by IL-12 (unpublished reuslts). These data indicate that the ability of IL-12 to substitute for CD86-dependent costimulation is not a result of increased expression of CD25, CD80, or CD86.

TABLE 3. IL-12 Fails to Induce IL-2 Synthesis by T Cells Activated in the Presence of Anti-CD86 mAb

Additions to culture[a]	IL-2 (pg/mL)[b] Expt. 1	Expt. 2
Anti-CD3 + rat IgG	160 ± 29	187 ± 3
Anti-CD3 + anti-CD86	20 ± 44*[c]	35 ± 12*
Anti-CD3 + anti-CD86 + IL-12	14 ± 30*	24 ± 25*
Anti-CD3 + anti-CD86 + anti-CD28	1604 ± 86*	2528 ± 904*

[a] T cells were stimulated with anti-CD3 mAb in the presence of rat IgG, anti-CD86 mAb (~4 μg/mL), or anti-CD86 mAb plus rIL-12 (10 ng/mL). [b] IL-2 levels in culture supernatants after 24 h of culture were evaluated by IL-2-specific sandwich ELISA (SD from triplicate samples is indicated). [c] Asterisk indicates $P < 0.001$ by Student's *t*-test.

IL-12Rβ2 expression is enhanced by IL-12 in the presence of low levels of IL-2

We observed earlier that blockade of CD86 interaction with CD28 results in ~50% inhibition of IL-2 synthesis in anti-CD3-activated T-cell cultures (Table 1). IL-2 has been shown to synergize with IL-12 during CTL activation [31] through a mechanism that most likely involves IL-2-induced expression of the IL-12Rβ2 subunit, which is critical for T-cell responsiveness to IL-12 [33]. We, therefore, employed semiquantitative RT-PCR to examine the effect of IL-2 and IL-12 on IL-12Rβ2 expression by T cells in our model system (**Fig. 4**). Unstimulated T cells failed to express detectable IL-12Rβ2 mRNA. T cells activated with anti-CD3 mAb in the presence of anti-CD86-blocking mAb exhibited decreased IL-12Rβ2 expression relative to control cells activated in the presence of rat IgG. Elimination of IL-2 bioactivity in anti-CD86-treated activated T-cell cultures by the addition of anti-IL-2 plus anti-IL2R (anti-CD25) mAb ablated IL-12Rβ2 expression virtually,

indicating that IL-2 is required for T-cell expression of IL-12Rβ2, as well as strongly suggesting that reduced IL-12Rβ2 expression in the presence of anti-CD86 mAb is the result of diminished IL-2 synthesis (Table 1). T cells stimulated with anti-CD3 mAb in the presence of IL-12 displayed dramatically increased IL-12Rβ2 mRNA expression, indicating that IL-12 upregulates expression of the IL-12Rβ2 subunit. Furthermore, T cells activated in the presence of anti-CD86-blocking mAb plus IL-12 exhibited close-to-control levels of IL-12Rβ2 expression. Taken together, these data suggest the requirement that IL-2 be present for IL-12Rβ2 expression by T cells, and subsequent IL-12 responsiveness accounts for the IL-2-dependent nature of the compensatory effect of IL-12 on anti-CD3-activated CTLs, which develop in the absence of CD28-costimulatory signaling.

IL-12 restores Gzm B expression by CTL induced in the presence of anti-CD86 mAb

We have shown previously that blockade of CD28-CD86 interaction during MHC-unrestricted CTL induction by anti-CD3 mAb leads to diminished Gzm B but not perforin or CD95L gene transcription [25]. This led us to determine whether the addition of IL-12 to anti-CD3-activated T-cell cultures that contain anti-CD86 mAb was able to reverse the inhibitory effect of CD86 blockade on Gzm B mRNA expression. T cells were activated with anti-CD3 mAb in the presence of an irrelevant rat IgG, anti-CD86 mAb, 25 ng/ml IL-12, or anti-CD86 mAb in combination with 25 ng/ml IL-12, and total RNA was isolated after 48 h of culture for one-step RT-PCR analysis with mouse Gzm B-specific primers. To show that an equal amount of RNA was used in each RT-PCR, identical aliquots of RNA were electrophoresed also, and ribosomal RNA bands are presented for comparison. As shown in **Figure 5**, Gzm B mRNA expression was abrogated in cultures containing anti-CD86 mAb, whereas cultures containing IL-12 expressed heightened levels of Gzm B mRNA relative to control cultures. T cells activated with anti-CD3 mAb in the presence of anti-CD86 mAb and exogenous IL-12 expressed Gzm B mRNA at a level comparable to that of control cultures, suggesting that IL-12 reverses the inhibitory effect of CD86 blockade on CTL induction by upregulating Gzm B gene expression.

To confirm the restorative effect of exogenous IL-12 on Gzm B gene expression in anti-CD86 mAb-treated activated T-cell cultures, we next examined Gzm B enzymatic activity in post-nuclear cell lysates obtained from T cells activated with anti-CD3 mAb in the presence of an irrelevant rat IgG, anti-CD86 mAb, 25 ng/ml IL-12, or anti-CD86 mAb in combination with 25 ng/ml IL-12. **Figure 6** shows that, compared with control cultures, Gzm B enzymatic activity was reduced substantially in anti-CD86 mAb-treated T-cell cultures and enhanced in T-cell cultures activated in the presence of IL-12. Gzm B enzymatic activity in lysates of T cells activated in the presence of anti-CD86 mAb and IL-12 was equivalent to that observed in lysates of control anti-CD3-activated T cells. Taken together, these data confirm that the enhancing effect of IL-12 on Gzm B expression compensates for the inhibitory effect of CD86 blockade on Gzm B synthesis and suggest that this accounts for the ability of IL-12 to restore cytotoxic activity to control levels when CTLs are induced in the absence of CD28-CD86 interaction.

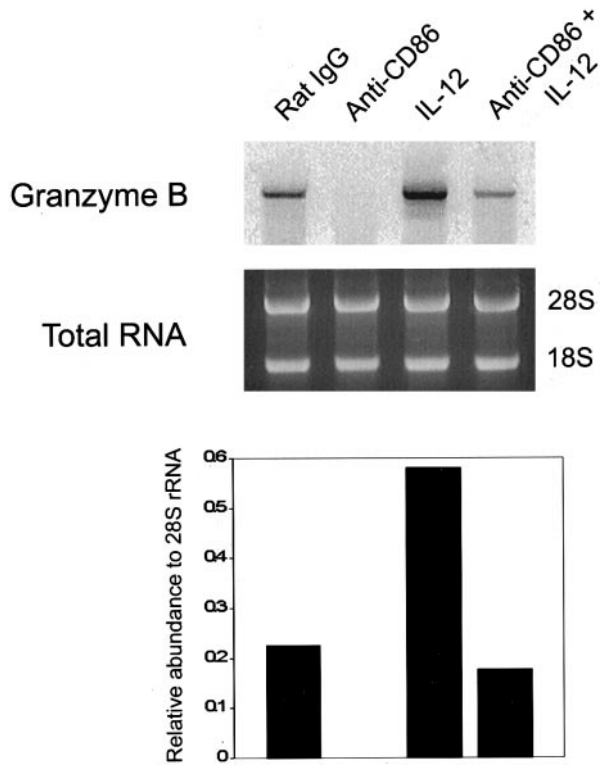

Fig. 5. IL-12 restores Gzm B mRNA expression by CTL induced in the absence of CD28-CD86 interaction. T cells were stimulated with anti-CD3 mAb in the presence of rat IgG or anti-CD86 mAb (~4 μg/ml final concentration) with or without IL-12 (10 ng/ml). Total RNA was isolated following 48 h of culture, and one-step RT-PCR beads were used to reverse-transcribe single-stranded cDNA from 0.5 μg RNA with random hexamers. Amplicons generated by PCR reaction with exon-binding, intron-bridging primers specific for GzmB were resolved by gel electrophoresis and ethidium-bromide staining. Densitometric analysis was performed to quantitate GzmB mRNA expression. Equal RNA-template loading is shown by electrophoresis of the same vol of RNA used in the one-step RT-PCR procedure. Data are from one experiment representative of two independent experiments.

DISCUSSION

Recent evidence indicates that the costimulatory CD28/CD86 receptor/ligand system regulates the development of *in vitro* and *in vivo* murine CTL responses [25, 34]. Triggering the CD28-activation pathway of T helper cells that have been stimulated through the T-cell receptor/CD3 complex enhances dramatically the production of cytokines such as IL-2 and IFN-γ [9], which are known to be important for CTL development [29, 30]. We have shown previously that ligation of CD28 by CD86 and subsequent activation of the CD28 signaling pathway of $CD8^+$ CTL precursors directly regulates the development and differentiation of nonspecific CTL in response to anti-CD3 mAb [25]. In contrast, the contribution of CD80 as a costimulator of MHC-unrestricted cytotoxicity in anti-CD3-activated mouse T-cell cultures is, at best, minimal.

We show here that blockade of CD28-CD86 interaction results in diminished IL-2, -4, -6, and -10 and IFN-γ produc-

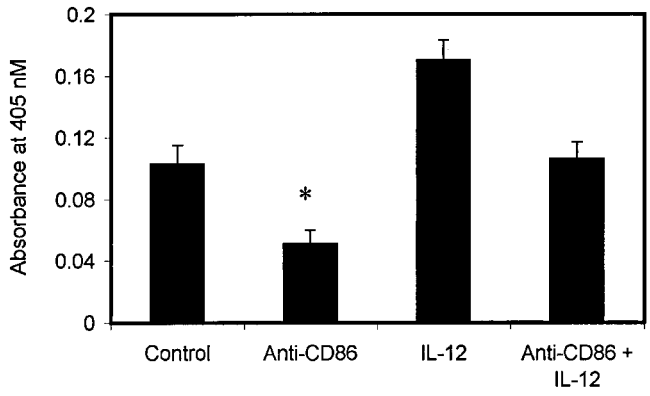

Fig. 6. IL-12 rescues Gzm B enzymatic activity of CTL induced in the absence of CD28-CD86 interaction. T cells were stimulated with anti-CD3 mAb in the presence of rat IgG or anti-CD86 mAb (~4 μg/ml final concentration) with or without IL-12 (25 ng/ml). Following 48 h of culture, postnuclear lysates were prepared from equal numbers of T cells and added to a colorimetric reaction mixture containing synthetic Gzm B substrate. Absorbance at 405 nM (mean±SD of quadruplicate samples) indicates the esterolytic activity/10^6 cells. Data are representative of two independent experiments. The asterisk denotes a statistically significant reduction in enzymatic activity compared with control, as determined by Student's *t*-test.

tion by anti-CD3-activated mouse T cells, and TNF-α synthesis is unaffected (Table 1). These results confirm that T lymphocytes activated in the absence of CD86-dependent CD28 costimulation are prevented from synthesizing normal levels of IL-2 and IFN-γ [27] as well as revealing that optimal production of IL-4, IL-6, and IL-10 by T cells is dependent on CD28 signaling also. The inhibitory effect of CD86 blockade on the production of type 1 and 2 cytokines by anti-CD3-activated T lymphocytes agrees well with the recent finding that CD86 on antigen-presenting cells, in the genetically assured absence of CD80, can prime T cells to synthesize type 1 and type 2 cytokines [35]. It is interesting that the failure of CD86 blockade to affect TNF-α synthesis suggests that TNF-α expression in anti-CD3-activated mouse T-cell cultures occurs independently of CD28 signaling.

We observed that IFN-γ synthesis was almost nonexistent in T-cell cultures activated under conditions of CD86 blockade (Table 1). Given that IL-12 is a potent inducer of IFN-γ synthesis [20] and cytotoxic effector cells [29, 31], we reasoned that the addition of exogenous IL-12 might override the inhibitory effect of anti-CD86 mAb on anti-CD3-induced MHC-unrestricted CTL development. IL-12 was, indeed, able to restore cytotoxicity to control levels in cultures of T cells activated in the presence of anti-CD86 mAb alone (Fig. 1A) or in combination with anti-CD80 mAb (Fig. 1B) to prevent any possible CD28 costimulation because of low levels of CD80 known to be expressed in these T-cell cultures [25]. Moreover, pure $CD8^+$ T cells activated in the presence of combined anti-CD80 and anti-CD86 mAb developed into competent effector cells if IL-12 was also present (Table 2), indicating that CTL precursors are responsive to IL-12 in the absence of CD28-costimulatory signaling. IL-12 does not exert this effect through an increase in the available CD28 ligands, because IL-12 treatment did not increase CD80 or CD86 expression in anti-CD3-activated T-cell cultures (unpublished results). Taken together, these data indicate that the IL-12R provides a redundant signal similar to CD28-costimulatory signaling for CTL activation and are consistent with the observation that CTLs do not necessarily require CD28 signaling to become activated [36].

IL-12 has been shown to enhance the synthesis of IFN-γ by $CD4^+$ T cells from wild type and CD28 knockout mice [37]. Because IFN-γ is known to be important for CTL generation [29, 30], and synthesis of this cytokine was inhibited strongly by CD86 blockade (Table 1), it seemed possible that IL-12-induced upregulation of IFN-γ production might account for the ability of anti-CD3-activated T lymphocytes to develop cytotoxicity in the absence of CD28 costimulation. However, T cells activated in the presence of anti-CD86 mAb plus exogenous IL-12 in combination with neutralizing anti-IFN-γ mAb and blocking anti-IFN-γR mAb still developed high levels of cytotoxicity (Fig. 2A). Moreover, the addition of exogenous IFN-γ to T cells activated in the presence of anti-CD86 mAb failed to restore cytotoxicity to control levels (Fig. 2B), confirming that IL-12-induced upregulation of IFN-γ synthesis in anti-CD3-activated T-cell cultures does not account for the ability of IL-12 to substitute for CD28-costimulatory signaling. Taken together, these data suggest that IL-12R signaling contributes to the induction of other genes, in addition to IFN-γ, which are involved in CTL development.

Experiments in which T cells were activated in the presence of anti-CD86 mAb in combination with anti-IL-2 mAb and blocking anti-CD25 mAb revealed the IL-2-dependent nature of the compensating effect of IL-12 on CTL induction in the absence of CD28-CD86 interaction (Fig. 3). We considered the possibility that IL-12 might be upregulating IL-2 production or IL-2R expression in anti-CD3-activated T-cell cultures. However, results from ELISA experiments indicated that IL-12 does not increase IL-2 production in anti-CD3-activated T-cell cultures performed in the presence of anti-CD86 mAb (Table 3). This finding, which was confirmed by semiquantitative RT-PCR analysis of IL-2 mRNA expression, is consistent with a recent study that exogenous IL-12 does not enhance IL-2 production by T cells in a mixed-tumor reaction [33]. Furthermore, IL-2R (CD25) expression in anti-CD3-activated T-cell cultures, although inhibited by CD86 blockade, was not enhanced by IL-12 (**Table 4**). Thus, although previous studies have shown that IL-12 can synergize with CD80 (present at low levels in anti-CD3-activated T-cell cultures) [25] to upregulate

TABLE 4. IL-12 Fails to Enhance IL-2R (CD25) Expression by T Cells Activated in the Presence of Anti-CD86 mAb

	% $CD25^+$ cells (MCF)[b]	
Additions to culture[a]	Expt. 1	Expt. 2
Anti-CD3 + rat IgG	79 (42)	54 (63)
Anti-CD3 + anti-CD86	60 (37)	25 (54)
Anti-CD3 + IL-12	ND[c]	56 (69)
Anti-CD3 + anti-CD86 + IL-12	63 (39)	31 (57)

[a] T cells were stimulated with anti-CD3 mAb in the presence of rat IgG, anti-CD86 mAb (~4 μg/mL final concentration), or anti-CD86 mAb plus IL-12 (10 and 25 ng/mL in expts. 1 and 2, respectively). CD25 expression was measured at 18 h of culture by flow cytometric analysis. [b] MCF, mean channel fluorescence. [c] ND, not determined.

CD25 expression by mouse Th1 cell clones [38], we failed to find evidence of this effect of IL-12 on a polyclonal mouse T-cell population.

The importance of Gzm B as a CTL-effector molecule is demonstrated convincingly by the failure of Gzm B-deficient CTL to kill target cells [39]. We found that IL-12 restored Gzm B mRNA and protein expression in anti-CD86-treated activated T-cell cultures to near control levels (Figs. 5 and 6, respectively). These data suggest that the IL-12-induced restoration of cytotoxic activity in anti-CD3-activated T-cell cultures performed in the absence of CD28-costimulatory signaling (Fig. 1) is likely a result of more Gzm B protein being produced. Because IL-12 independently and in synergy with IL-2 can upregulate Gzm B and perforin expression in IL-2-dependent human CTL lines [31], it is likely that the interaction between exogenous IL-12 and the relatively low levels of IL-2 present in mouse T-cell cultures activated through the T-cell receptor/CD3 complex in the presence of anti-CD86 mAb accounts for the ability of IL-12 to substitute for CD28-costimulatory signaling during CTL development. Indeed, we have observed that mouse T cells cultured in the presence of IL-12 in combination with IL-2 develop a higher level of cytotoxic activity than that induced by culture in IL-2 alone, and IL-12 alone fails to induce any substantial T-cell cytotoxic activity (unpublished results). We have demonstrated also that low levels of endogenous IL-2 produced by T cells activated in the absence of CD28-CD86 interaction (Table 1) are sufficient to induce IL-12Rβ2 expression, which confers IL-12 responsiveness upon T cells and allows for subsequent IL-12Rβ2 upregulation by exogenous IL-12. This finding is in good agreement with the recent study from Chang *et al.* [33] that IL-2 is necessary for induction of IL-12Rβ2 expression and supplies a mechanistic explanation for the IL-2-dependent nature of the compensating effect of IL-12 on CTL induction in the absence of CD28-costimulatory signaling.

The finding that IL-12 can substitute for CD28-costimulatory signaling during mouse CTL induction may have important implications for the immunotherapeutic treatment of human cancers. Because most human tumors do not express CD80 or CD86 [40], they are unlikely to costimulate tumor-specific CTL development effectively. Moreover, tumor-infiltrating and peripheral T lymphocytes from tumor-bearing individuals are compromised frequently in their ability to synthesize IL-2 [41]. Based on our findings, locoregional administration of IL-12 by gene therapy, for example, would be predicted to synergize with even low levels of IL-2 present in the tumor microenvironment to induce tumor-reactive CTL.

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