Interleukin 12 Synergizes with B7/CD28 Interaction in Inducing Efficient Proliferation and Cytokine Production of Human T Cells

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Summary

Several receptors and counter-receptor pairs on T cells and on antigen-presenting cells (APCs) deliver costimulatory signals to T cells during antigen presentation. The CD28 receptor on T cells with its ligand B7 represents one of the best characterized and most important examples of this costimulation. We show here that interleukin 12 (IL-12), a cytokine also produced by APCs (monocyte/macrophages and B cells) and active on T and natural killer cells, has a strong synergistic effect with the B7/CD28 interaction in inducing proliferation and cytokine production in both mitogen-activated and freshly isolated peripheral blood T cells. Together with anti-CD28 antibodies, IL-12 induces proliferation of T cells to levels higher than those obtained with IL-2 stimulation and it is effective at IL-12 concentrations 100- to 1,000-fold lower than effective concentrations of IL-2. The proliferative effect of anti-CD28 and IL-12 is resistant to moderate doses of cyclosporin A and is largely independent of endogenous IL-2. IL-12, in synergy with anti-CD28 or B7-transfected cells, is most effective in inducing interferon γ (IFN-γ) production, but production of tumor necrosis factor α and granulocyte/macrophage colony-stimulating factor is also observed. IL-12-induced IFN-γ production in peripheral blood mononuclear cells is inhibited by the chimeric molecule CTLA-4 immunoglobulin, which prevents binding of CD28 to B7, suggesting that endogenous B7 on the mononuclear cells and IL-12 cooperate in inducing IFN-γ production. IL-10 inhibits both IL-12 production and B7 expression on monocytes. These two effects are largely responsible for the ability of IL-10, acting on accessory cells, to inhibit IFN-γ production by lymphocytes, because anti-CD28 antibodies and IL-12 can reverse the inhibitory effect of IL-10 on IFN-γ production. Our results in vitro suggest that the synergy between B7 and IL-12, a surface antigen and a soluble product of APCs, respectively, plays a role in regulating T cell activation and immune response in the microenvironment of inflamed tissues.

APCs such as macrophages and dendritic cells deliver costimulatory signals that are required for optimal activation of T lymphocytes. Several receptor counter-receptor pairs mediate adhesion between APC and T cells and deliver activating signals to T cells. The interaction of B7 with its CD28 receptor on T cells represents the best characterized example of this costimulation. Unlike surface molecules expressed on APCs, the role of soluble cytokines produced by APCs in regulating the response of T cells received less attention, although a role for IL-1 and, to some extent, for IL-6 and TNF-α, has been demonstrated. IL-12 is a heterodimeric cytokine (1–3) produced by monocyte-macrophages, B cells, and other accessory cell types especially upon bacterial stimulation (4), which has immunomodulatory effects on T and NK cells (1, 5–8). IL-12 induces lymphokine production and is especially potent in inducing production of IFN-γ alone or in synergy with other inducers (5); it also induces proliferation of activated T and NK cells (6, 9), augments the cytotoxicity of NK cells (1, 10), and favors the generation of cytotoxic T cells and lymphokine-activated killer cells (11). Because IL-12 is a likely physiologic inducer of the generation and maturation of T helper type 1 cells (Th1) (7, 8, 12) and, through its ability to induce IFN-γ production, is instrumental in activating phagocytic cells in response to infection (4, 13, 14), a central role for IL-12 in inflammation and immune response as a product of accessory cells and APCs is being increasingly recognized (15). However, the proliferative effect of IL-12 on T cells has always been reported to be modest in comparison to the proliferation induced by IL-2 and restricted to a temporal window during activation of T cells (6, 9). Depending on the cell type, IL-12 may display a modest additive effect with IL-2 in inducing proliferation or, as in the case of NK cells or of T cells with TCR-γ/δ, it may inhibit IL-2–induced proliferation (6). These findings

suggest a regulated expression of what is probably a heterogeneous receptor for IL-12, able to deliver both proliferative and antiproliferative signals (16, 17). IL-12, even in association with IL-2, has no or an extremely low ability to induce proliferation in fresh resting peripheral lymphocytes (6, 9). However, especially in cultures of 5–6 d, IL-12 has an enhancing effect on T cell proliferation induced by a variety of stimuli, including mitogenic lectins, allogeneic cells, anti-CD3 antibodies, and phorbol diesters. A synergistic effect of IL-4 and IL-12 was reported for proliferation of human CD56^{+} NK cells but not of human T cells (18). Although in certain conditions, IL-12 can elicit low levels of IL-2, the neutralization of endogenous IL-2 in most cases does not diminish the proliferation induced by IL-12 or its enhancing effect on the proliferation induced by other mitogenic stimuli; only the enhancing effect of IL-12 on phorbol diester–induced T cell proliferation was inhibited by anti-IL-2 antibodies (6).

The interaction of the CD28 receptor, present on most T cells, with its counter-receptor B7, expressed on dendritic cells and activated monocytes, B cells, and repeatedly activated T cells, represents an important pathway of T cell stimulation (19, 20). Not only is a costimulus through B7/CD28 interaction essential for optimal T cell stimulation after interaction of the TCR with peptide antigen in the context of the MHC (21, 22), but activation through this pathway also prevents the induction of T cell anergy (23), observed in many conditions in which the TCR alone is challenged (24). CD28 is a homodimeric molecule of 44-kD subunits expressed on most CD4^{+} and about half of CD8^{+} human T cells but not on NK cells (25). Antibodies against CD28 augment the proliferation and production of cytokines by T cells in response to TCR or CD3 stimulation, to stimulation by anti-CD2 antibodies, and to phorbol diesters (21, 22, 26). Part of the costimulatory activity of CD28 antibodies is due to a cyclosporin A (CsA)[1]-resistant posttranscriptional mechanism that results in increased stability of the IL-2 message (27, 28). However, anti-CD28 antibodies also have a direct transcriptional, CsA-sensitive mechanism to induce IL-2 gene expression, especially when the antibodies are cross-linked on the cell surface or when IgM antibodies are used (29–31). CTLA-4 is another T cell surface receptor, with some homology to CD28, that binds to the counter-receptor B7 (32). CTLA-4Ig is a chimeric immunoglobulin Cγ fusion protein that binds with high avidity to B7 and blocks several T cell–dependent immune responses in vitro and in vivo (32, 33).

In this study, we investigated whether a cooperative effect between IL-12 and B7, a soluble and surface-bound product of APCs, respectively, may be required for optimal T cell activation. Our results show that the B7/CD28 stimulatory pathway synergizes with IL-12 in inducing both efficient proliferation and cytokine production in human T cells.

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[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; CsA, cyclosporin A; TPA, 12-O-tetradeanoylphorbol 13-acetate.

Materials and Methods

Cytokines and Reagents. Chinese hamster ovary (CHO) cell–derived rNKSF/IL-12 was provided by Dr. S. Wolf (Genetics Institute, Boston, MA); recombinant IL-2 (10^7 U/mg) was provided by the Division of Cancer Treatment (National Cancer Institute, Bethesda, MD); and CHO cell–derived hIL-10 (1.5 × 10^7 U/mg), by Dr. K. Moore (DNAX Research Institute, Palo Alto, CA). The following reagents were obtained from commercial sources: PHA (Sigma Chemical Co., St. Louis, MO); heat-fixed *Staphylococcus aureus* Cowan strain 1 (Pansorbin; Calbiochem-Behring Corp., La Jolla, CA); 12-O-tetradecanoylphorbol 13-acetate (TPA; Sigma Chemical Co.); CsA (Sandoz Forschungsinstitut GmbH, Vienna, Austria).

Antibodies. mAb OKT3- (IgG2a, anti-CD3) and OKM1-(IgG2b, anti-CD11b) producing cells were obtained from American Type Culture Collection, Rockville, MD); CK248 (IgM, anti-CD28) (22) and 93 (IgG1, anti-CD25) were kindly provided by Dr. Lorenzo Moretta (Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy); 1.12 (IgG2a, anti-HLA-DR) were provided by Dr. Roberto Accolla (University of Verona, Verona, Italy); and B52.1 (IgM, anti-CD14) (34) was produced in our laboratory. The mAb 9.3 (IgG2a, anti-CD28) (35) was donated by Dr. J. Hansen (Fred Hutchinson Cancer Center, Seattle, WA); mAb B7.4 (IgG, anti-B7) and mAb CD28.1, CD28.2, CD28.3, CD28.5, 15E8 (IgG1, anti-CD28) (31) were obtained through participation in the 5th International Conference on Human Leucocyte Differentiation Antigens, Boston, MA, Nov. 3–7, 1993. The specificity of all the anti-CD28 antibodies used was assigned at the Conference Workshop and confirmed in our laboratory by reactivity in immunofluorescence with CD28-transfected J32 cell line, but not with the CD28-negative parental cell lines. In immunofluorescence, mAb 9.3 inhibits the binding of mAb CD248 to CD28^{+} T cells. Anti-IL-2 polyclonal goat antiserum was prepared in our laboratory and, at 1:200 dilution, completely neutralizes the activity of ⩾100 U/ml of IL-2. CTLA-4Ig (32) and B7-transfected CHO cells (36) were kindly provided by Dr. Peter S. Linsley (Bristol-Meyers Squibb Pharmaceutical Research Institute, Seattle, WA); B7-transfected L cells (37) by Dr. Lewis Lanier (DNAX Research Institute).

Cytokine Assays. RIAs for human IFN-γ and human TNF-α were performed as described (38, 39) using mAbs B133.1/B133.5 and B154.9/B154.7, respectively. GM-CSF was measured by a newly developed double determinant RIA using antibody C9.1 bound to plastic as the capture antibody and antibody C16.3 as the ^{125}I-labeled detection antibody according to the same protocol previously described for IFN-γ and TNF-α RIAs (38, 39). Recombinant cytokines were used to standardize all assays.

Preparation of Human PBMC. Peripheral blood obtained from healthy donors was anticoagulated with heparin. PBMC were separated on Ficoll-Hypaque (Lymphoprep; Nyegaard and Co., Oslo, Norway) density gradient, and lymphocytes (PBL) were obtained after adherence of PBMC to plastic flasks (1 h, 37°C). In some experiments, purified preparations of small high density lymphocytes were purified on a discontinuous Percoll gradient (40). These preparations were devoid of cycling cells, as determined by propidium iodide staining and flow cytometry analysis, and did not express significant levels of activation markers CD25, CD69, CD71, and 4F2. Approximately two thirds of the CD4^{+} cells contained in these populations were CD45 RA^{+}, a phenotype attributed to naive T cells. PHA-blasts (>98% activated T cells), as determined by indirect immunofluorescence with antileukocyte subset antibodies, were obtained after a 5-d culture of PBL in the presence of 5 μg/ml of PHA (5, 41). Cells were cultured in 200 μl RPMI 1640 medium supplemented with 10% heat-inactivated FCS (Ir-

vine Scientific, Santa Ana, CA) in 96-well round-bottomed plates (Flow Laboratories Inc., McLean, VA) with 0.2 × 10⁶ cells/well for proliferation and 10⁶ cells/well for cytokine production. For detection of thymidine ([³H]TdR) uptake, 1 μCi/well of [³H]TdR was added to the wells in the last 6 h of culture; cells were collected on a glass filter and radioactivity measured. All cultures were performed in triplicate.

Immunofluorescence Assay. This was performed as described (42). Briefly, cells were sequentially incubated (30 min, 4°C) with saturating concentrations of antibodies in PBS containing 1% gelatin, 1% human plasma, and 0.1% NaN₃, washed three times, and incubated with FITC-conjugated goat F(ab')₂ anti–mouse Ig (absorbed to human IgG-Sepharose before use; Cappel Laboratories, Cochranville, PA), washed again, and analyzed for fluorescence and light scatter using an Ortho Cytofluorograf 50H connected to a 2100 Data Handling System (Ortho Instruments, Westwood, MA).

Results

IL-12 Synergizes with Anti-CD28 Antibodies in Inducing Proliferation of PHA Activated and Freshly Isolated Peripheral Blood Human T Cells. IL-12 has a minimal proliferative effect on resting PBL, whereas a dose-dependent proliferation is induced by IL-12 on PHA-activated T cell blasts or on activated NK cells (6, 9). Maximal proliferation induced by IL-12 on activated T cells is, however, always lower than that induced by IL-2 (6, 9). Furthermore, maximal responsiveness to IL-12, unlike that to IL-2, is restricted to ∼2–3 d of stimulation with PHA, perhaps reflecting a more transient expression of the IL-12 receptor compared with the IL-2

receptor (17). The PHA-activated blast cell preparations used in the preset studies were collected 5 d after PHA stimulation in order to avoid the background proliferation observed in PHA-induced cells collected at an earlier time of culture. As shown in Fig. 1, increasing concentrations of IL-12 induced modest proliferation, as measured by [³H]TdR uptake, in PHA-blasts both at days 3 and 6 of culture, whereas IL-2 alone induced efficient proliferation, with a maximum effect observed around 10 ng/ml. The anti-CD28 antibody CK248, in the absence of IL-12 or IL-2, induced proliferation at day 3 when used at a concentration of ascites fluid of 1:500 or higher. Proliferation at day 6 was lower. Addition of antibody CK248 induced a modest synergistic enhancement of IL-2–induced proliferation but a strong synergistic enhancement of IL-12–induced proliferation. IL-12 in the presence of CK248 was effective at concentrations as low as 5 pg/ml and induced equivalent [³H]TdR uptake at concentrations 2–3 orders of magnitude lower than those required with IL-2. Antibody CK248 was found to induce a proliferative effect only early in the culture, whereas the modest proliferation induced with IL-12 was observed from 1 to 6 d and the proliferation induced by IL-2, CK248 and IL-2, or CK248 and IL-12 was maximum at day 6 (Fig. 2) and declined at later times (not shown). When anti-CD28 antibodies with distinct binding properties were tested for their ability to induce PHA blast proliferation in conjunction with IL-12, it was observed that mAb 9.3, CD28.2, and 15E8, which in the presence of phorbol diesters have been reported to induce IL-2 production by the Jurkat cell line, synergize with IL-12, although less efficiently than CK248. mAb CD28.1, CD28.3, and CD28.5, which have been reported not to induce Ca²⁺ flux or IL-2 production in Jurkat cells (31), failed to synergize with IL-12 (Table 1). CK248 was the only antibody to induce consistent levels of proliferation in PHA blasts in the absence of IL-12; this result parallels the reported ability of CK248 to induce IL-2 production by Jurkat cells in the absence of phorbol diester (31). Indeed, in the presence of the phorbol diester TPA, mAb 9.3 was shown to be as efficient
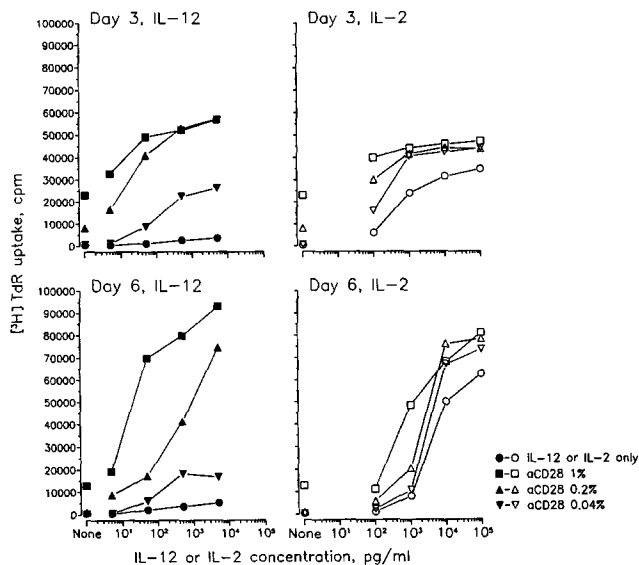

Figure 1. Anti-CD28 antibody CK248 synergizes with IL-12 and IL-2 in inducing proliferation of PHA-blasts. PHA-blasts were obtained after 5 d culture of PBL in the presence of 5 μg/ml PHA and incubated for 3 or 6 d with the indicated concentrations of IL-12 (*solid symbols*) or IL-2 (*open symbols*) and no antibodies (●, ○) or 0.04% (▼, ▽), 0.2% (▲, △) or 1% (■, □) ascites containing antibody CK248. [³H]TdR uptake was evaluated after a 6-h pulse at the end of the culture period. Results are average of values obtained with PHA-blasts from three donors.
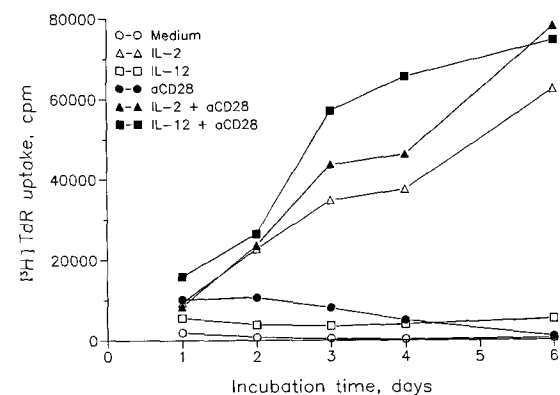

Figure 2. Kinetics of proliferation of PHA blasts in response to IL-2, IL-12, and anti-CD28 antibodies. PHA-blasts were incubated with the anti-CD28 antibody CK248 (ascites 1%), IL-2 (100 ng/ml), or IL-12 (0.5 ng/ml) and [³H]TdR uptake was evaluated at 1, 2, 3, 4, and 6 d after a 6-h pulse. Results are average of values obtained with three donors.

Table 1. *Proliferation and IFN-γ Production of PHA Blasts Stimulated by Anti-CD28 Antibodies in the Presence or Absence of IL-12*

mAb*	[³H]TdR uptake, cpm		IFN-γ, U/ml	
	no IL-12	IL-12, 1 ng/ml	no IL-12	IL-12, 1 ng/ml
Medium	4,988	24,579	5	37
CK248	43,391	71,978	85	1,042
9.3	8,753	42,423	7	184
CD28.1	5,199	25,866	7	86
CD28.2	7,740	42,276	8	405
CD28.3	4,601	25,548	6	96
CD28.5	6,177	28,563	7	155
15E8	12,660	56,518	11	717

* PHA blasts were cultured in the presence or absence of 1 ng/ml IL-12 in the presence of ascites (0.5%) containing the indicated mAb. 9.3 mAb was used at 10 μg/ml. IFN-γ was measured in the cell free supernatant fluid collected after 18-h stimulation and [³]TdR uptake was measured after a 6-h pulse at day 3 of stimulation.

as CK248 in synergizing with IL-12 in inducing PHA blasts proliferation (results not shown). A synergistic proliferative effect with IL-12 was observed by stimulating PHA blasts with L cells transfected with the CD28-ligand B7, but not with nontransfected cells (Fig. 3).

We tested the ability of anti-CD28 antibody CK248 to synergize with IL-2 and IL-12 in inducing proliferation of freshly isolated PBL (not shown). Synergistic effect with IL-12 was observed at day 6, but not at day 3, although a modest enhancement of IL-2-induced proliferation was already observed at day 3. A similar kinetics of proliferation, with maximum [³H]TdR uptake at day 6, was also induced by mAb CD248 and IL-12 in two experiments using Percoll-separated high density small lymphocytes (results not shown).

We analyzed the role of endogenous IL-2 in the synergistic effect between IL-12 and anti-CD28 antibodies by using neutralizing anti-IL-2 and anti-IL-2 receptor antibodies. Because CsA has been demonstrated to differentially affect the different pathway by which CD28 signal transduction affect IL-2 gene expression, we also analyzed the ability of CsA to inhibit this synergy. The results are shown in Fig. 4. The proliferative effect induced by anti-CD28 alone was almost completely inhibited both by blocking endogenous IL-2 (with a mixture of a goat anti-human IL-2 antibody and an anti-CD25 mAb; referred to in Fig. 4 and in this section as anti-IL-2) or by adding CsA (20 ng/ml). In both conditions, a similar but less complete inhibition of proliferation induced by plastic-bound anti-CD3 mAb was observed, whereas the
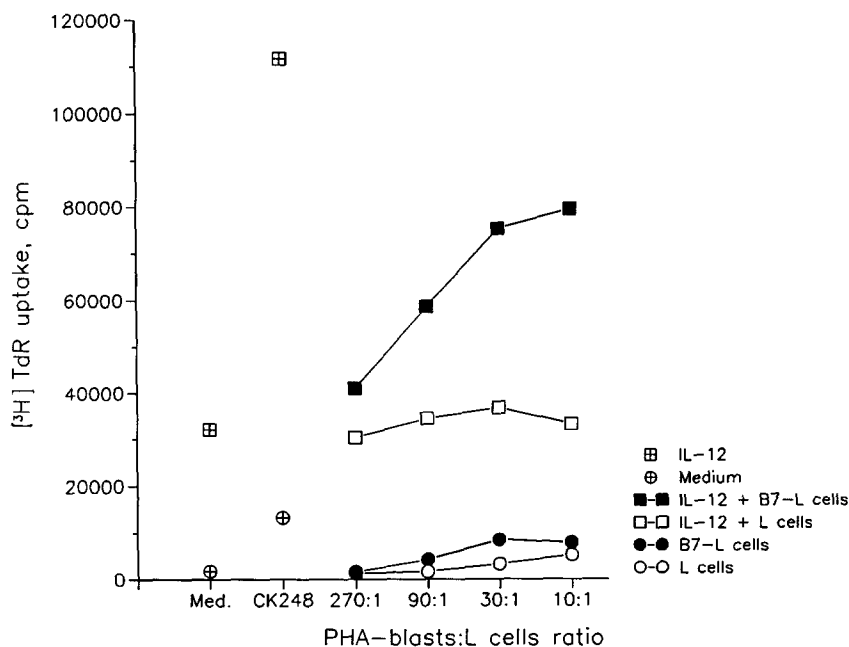

Figure 3. B7-transfected L cells synergize with IL-12 in inducing proliferation of PHA blasts. PHA blasts were incubated with anti-CD28 antibody CD248 (ascites 0.2%) or with different numbers of L cells transfected or not with the B7 antigen in the presence or not of IL-12 (1 ng/ml). [³H]TdR uptake (cpm) was evaluated at 3 d after a 6-h pulse. Results are from one experiment representative of three performed.
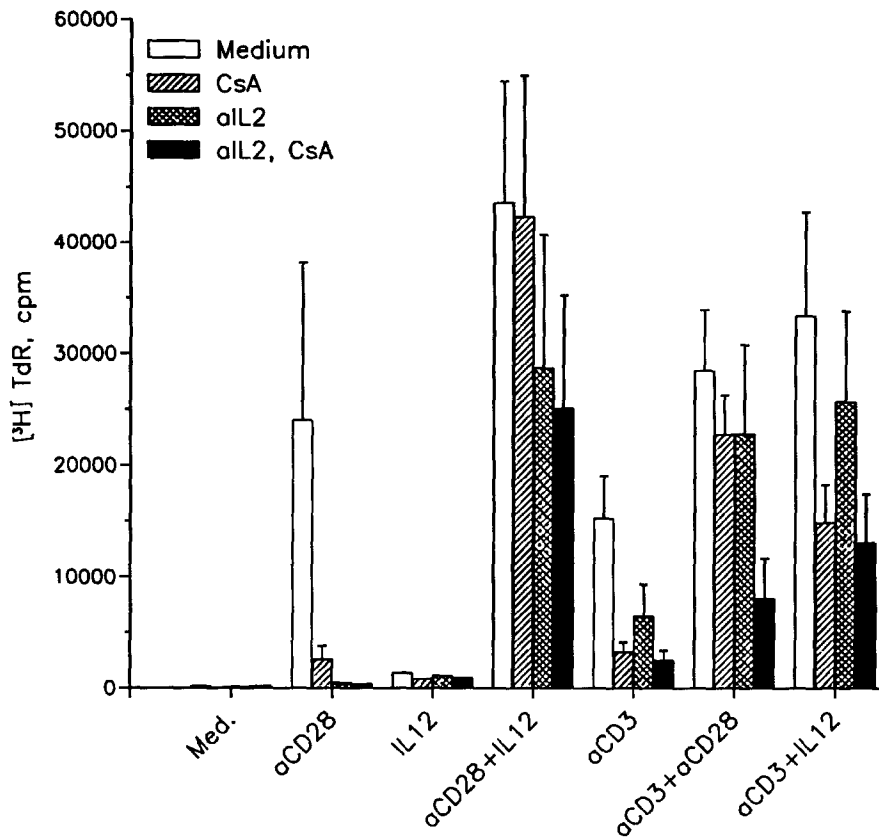
Figure 4. Effect of CsA and inhibition of endogenous IL-2 on PHA-blast proliferation. PHA-blasts were incubated for 3 d in culture medium in the presence of anti-CD28 antibodies (1% ascites fluid containing CK248 mAb), IL-12 (1 ng/ml), solid-phase bound anti-CD3 OKT3 antibody (5 μg/ml) or their combination. CsA was used at 20 ng/ml, anti-IL-2 was a mixture of goat anti–human IL-2 serum (1:200) and anti-CD25 mAb 93 (ascites 1:100). [^{3}H]TdR uptake was measured by scintillation counting after a 6-h pulse at the end of the culture time. Results are mean ± of four experiments.

low proliferation induced by IL-12 was largely unaffected by either anti-IL-2 or CsA. The proliferation induced by anti-CD3 and anti-CD28 mAb together was largely unaffected by CsA or anti-IL-2, although the effect of each antibody alone was inhibited by CsA and required endogenous IL-2 activity. Unexpectedly, CsA significantly inhibited proliferation induced by anti-CD3 and anti-CD28 antibodies when the IL-2 activity was neutralized. The proliferation induced by the synergistic effect of anti-CD28 and IL-12 was unaffected by CsA and only partially dependent on endogenous IL-2. Unlike the synergistic effect of anti-CD28 and IL-12, the enhanced proliferation in response to IL-12 induced by anti-CD3 mAb was still partially affected by CsA. When higher concentrations of CSA were used ($\geqslant$200 ng/ml), a more generalized inhibition of proliferation was observed.

IL-12 Synergizes with Stimulation of CD28 by Antibodies or by Its Ligand B7 in Inducing Cytokines in T Cells. IL-12 synergizes with the anti-CD28 antibody CK248 or with B7-transfected CHO cells in inducing production of IFN-γ from PHA-blasts (Fig. 5). Control parental CHO cells (Fig. 5) or control ascites (not shown) had no significant effect. Identical results were obtained with B7-transfected L cells (not shown). The anti-CD28 IgG2a mAb 9.3, unlike the IgM mAb CK248, did not induce IFN-γ production by itself, and enhanced IFN-γ production induced by IL-12 only twofold on average (Table 2). mAb 9.3, which competes for binding to the CD28 antigen on T cells (not shown), in-

hibited the induction of IFN-γ and the synergy with IL-12 of CK248 (Table 2). Two other anti-CD28 mAb synergized with IL-12 in inducing IFN-γ production from PHA-blasts (CD28.2 and 15E8) whereas three mAbs (CD28.1, CD28.3, and CD28.5) showed only minor effects (Table 1). The ability

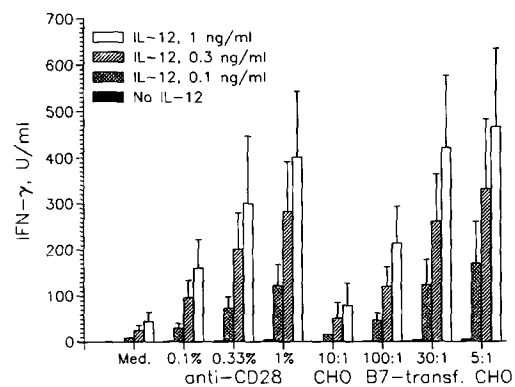**Figure 5.** Anti-CD28 antibody CK248 and B7-transfected CHO-cells synergize with IL-12 in inducing IFN-γ production from PHA-blasts. PHA blasts were cultured for 18 h in the presence of medium or increasing concentrations of IL-12, and the indicated dilutions of ascites containing the anti-CD28 mAb CK248, parental CHO cells or B7-transfected CHO cells at the PHA-blast CHO-cell ratios indicated. At the end of the culture, cell-free supernatant fluids were collected and IFN-γ assayed by RIA. Results are mean ± SE of four experiments.

Table 2. *IFN-γ Production in PHA-blasts Induced by IL-2 and/or IL-12 in the Presence of Different Anti-CD28 mAbs*

Antibodies‡	IFN-γ (Mean ± SE)			
	Medium*	IL-2	IL-12	IL-2 + IL-12
			U/ml	
PX63	1 ± 0	20 ± 9	35 ± 4	515 ± 29
9.3	1 ± 1	23 ± 10	70 ± 45	477 ± 122
CK248	69 ± 34	132 ± 57	1,075 ± 96	1,190 ± 71
CK248 + 9.3	1 ± 1	37 ± 23	77 ± 29	633 ± 68

* PHA-blasts were cultured for 18 h in the presence of the following inducers: medium; IL-2 (10 ng/ml); IL-12 (1 ng/ml); or a combination of the two cytokines. IFN-γ was assayed in the cell-free supernatant fluid by RIA. Results are mean ± SE from four experiments.
‡ Antibodies PX63 (irrelevant IgG1) and CK248 (anti-CD28) were added as a 1% dilution of ascites fluid; antibody 9.3 (anti-CD28) was added at 10 μg/ml.

of the different anti-CD28 mAb to synergize with IL-12 in inducing IFN-γ parallels their ability to enhance IL-12–induced proliferation (Table 1). As for IL-2 induction in Jurkat cells (31) or proliferation of PHA blasts (Table 1), CK248 was the only antibody to induce significant IFN-γ production in the absence of IL-12. Although mAb 9.3 alone was relatively inefficient in synergizing with IL-12, when used in combination with the phorbol diester TPA and IL-12, this mAb induced similar levels of IFN-γ production as observed with CK248 and IL-12 (results not shown).

Anti-CD28 mAb CK248 and B7-transfected CHO cells induced the production by PHA blasts not only of IFN-γ, but also of TNF-α and GM-CSF, and this effect was enhanced by costimulation with IL-12 or plastic-bound anti-CD3 mAb, and, to a lesser extent by IL-2 (Fig. 6). However, IL-12 was particularly effective in stimulating IFN-γ production as indicated by the fact that anti-CD28 mAb or B7-CHO induced maximal IFN-γ production when in combination with IL-12, whereas they induced TNF-α and GM-CSF more efficiently when combined with plastic-bound anti-CD3. A strong synergy of IL-12 with IL-2 or anti-CD3 was also observed in the induction of IFN-γ production, but not of TNF-α or GM-CSF (Fig. 6).

mAb CK248 also synergized with IL-12 in inducing IFN-γ but not TNF-α production by freshly isolated PBL (Table 3). The limited ability of IL-12 to induce TNF-α production was underscored by the fact that in the same cultures in which IL-12 failed to enhance the TNF-α production induced by CK248, the phorbol diester TPA had a dramatic synergistic effect in combination with CK248 (Table 3). Although the PBL preparations used in these experiments were depleted of adherent cells, they were not monocyte-free; however, because IL-12 and anti-CD28 are specific stimuli for T (and, in the case of IL-12, NK) cells, it is likely that most of the TNF-α measured in these cultures was produced by lymphocytes.

To analyze whether the B7-CD28 interaction plays a role in the IL-12–induced IFN-γ production by PBMC, we tested the ability of the chimeric recombinant soluble B7-ligand CTLA-4Ig to inhibit IFN-γ production (Fig. 7). CTLA-4Ig
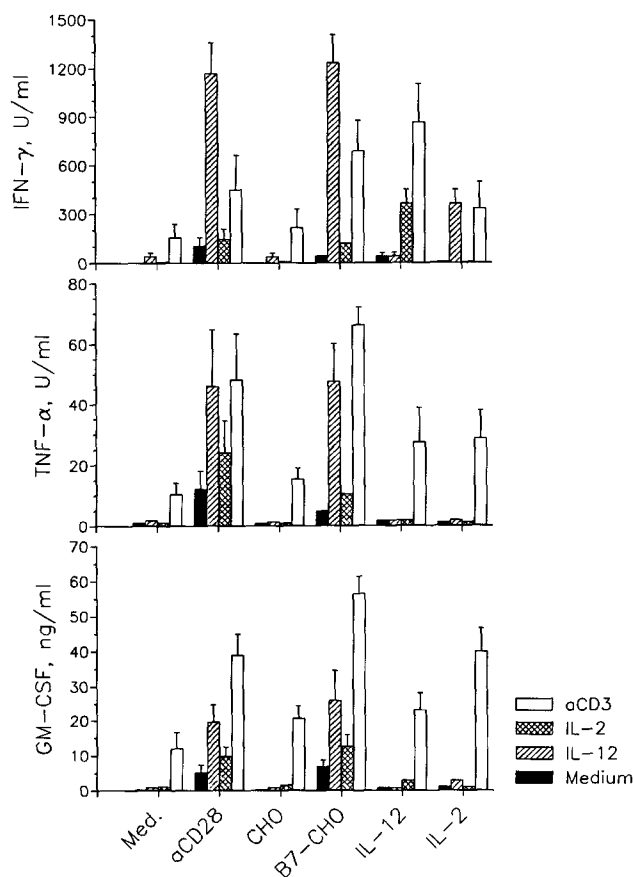

Figure 6. Anti-CD28 antibody CK248 and B7-transfected CHO cells augment cytokine production by PHA-blasts in response to various inducers. PHA-blasts were cultured for 18 h in medium or in the presence of anti-CD28 mAb CK248 (ascites 1:100), parental CHO cells, or B7-transfected CHO cells (ratio PHA-blast/CHO cells, 10:1). As indicated by the differently shaded columns, cultures were also stimulated in the presence of IL-12 (1 ng/ml), IL-2 (100 ng/ml), or plastic bound anti-CD3 OKT3 mAb (5 μg/ml). For comparison, combinations of these three stimuli with IL-12 and IL-2 (final concentrations, was 1 ng/ml and 100 ng/ml, respectively) are shown in the two rightmost groups of bars. At the end of the 18-h culture, cell-free supernatant fluids were collected and IFN-γ, TNF-α, and GM-CSF measured by RIA. Results are mean ± SE of three experiments.

Table 3. *Induction of Cytokine Production in Fresh PBL by IL-2, IL-12, or TPA in Combination with Anti-CD28 mAb CK248*

Stimuli	CK248	IFN-γ	TNF-α
		U/ml	pg/ml
Medium	–	2 ± 0	9 ± 5
	+	41 ± 13	196 ± 103
IL-2	–	9 ± 4	10 ± 5
	+	124 ± 36	197 ± 57
IL-12	–	11 ± 4	11 ± 6
	+	298 ± 77	156 ± 33
IL-2 + IL-12	–	85 ± 26	18 ± 10
	+	539 ± 96	273 ± 71
TPA	–	11 ± 6	119 ± 44
	+	263 ± 69	960 ± 182

PBL were cultured for 18 h in the presence or absence of the indicated stimuli and anti-CK248 mAb (ascites 1:100). IL-2 (10 ng/ml), IL-12 (1 ng/ml), or TPA (1.6 × 10⁻⁸ M) were used to stimulate cytokine production. IFN-γ and TNF-α were measured by RIA in the cell-free supernatant fluid at the end of the culture. Results are mean ± SE from nine experiments.

efficiently inhibited the production of IFN-γ in PBMC preparations stimulated with IL-12 at the same concentration at which it inhibited the enhancing effect of B7-CHO cells on IFN-γ production by PHA-blasts, stimulated or not with IL-12. Equivalent concentrations of normal human IgG had no effect on IFN-γ production.

Effect of IL-10 on IFN-γ Production and B7 Expression in PBMC Stimulated in the Presence of Anti-CD28 Antibodies and IL-12. We previously showed that IL-10 inhibits IL-12 pro-
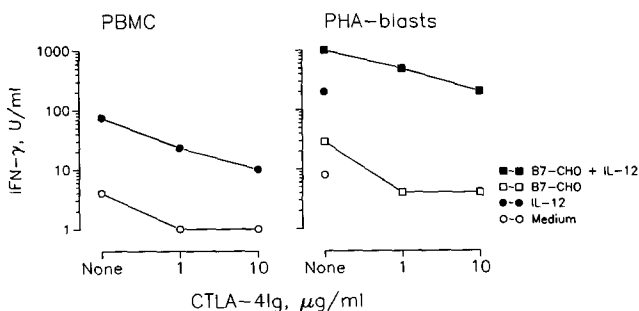
duction by monocytes stimulated by various inducers, e.g., *S. aureus*, and that IL-10 ability to suppress IFN-γ production in T and NK cells is largely due to inhibition of IL-12 production from accessory cells (43). However, because IL-10 also partially inhibits IL-12–induced IFN-γ production, other IL-10–mediated effects including inhibition of IL-1β and TNF-α production (44, 45), and also possibly inhibition of expression of surface-bound stimulatory molecules (46) were suggested to play a role in the inhibition of IFN-γ production (43). In the experiments presented here (Fig. 8), IL-10 reduced IFN-γ production induced by IL-12 by approximately one half, confirming the previously reported results (43), and had no effect on IFN-γ production induced by anti-CD28 or by the synergistic effect of anti-CD28 and IL-12 (Fig. 8). IFN-γ production induced by *S. aureus* or by the combination of anti-CD28 and *S. aureus* was almost completely inhibited by IL-10, whereas IL-10 only partially affected IFN-γ induced by anti-CD28 and *S. aureus* when IL-12 was also added (Fig. 8). Because the data obtained from the CTLA-4Ig inhibition experiments suggested a role for the B7/CD28 interaction in IL-12–mediated induction of IFN-γ in PBMC, we tested whether IL-10 affects monocyte expression of B7 antigens. As shown in Fig. 9, incubation of PBMC with IL-10 induced a severalfold decrease of both the constitutive expression and the IFN-γ-enhanced expression of B7 and HLA-DR antigens on monocytes, whereas it had no effect on the expression of CD14 or CD11b antigens. These data
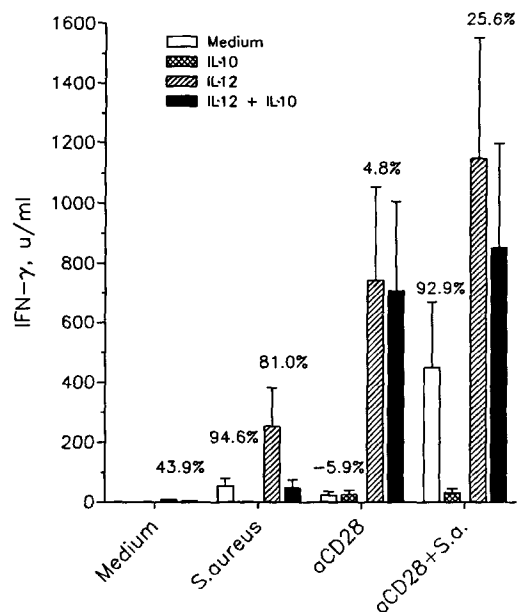

Figure 7. CTLA-4Ig inhibits IFN-γ production in PBMC or in PHA-blasts stimulated in the presence of B7-transfected CHO cells. PBL or PHA-blasts were cultured for 18 h in medium or in the presence of the indicated concentrations of the chimeric protein CTLA-4Ig, and stimulated with the indicated combination of IL-12 (1 ng/ml) or B7-transfected CHO cells (PHA-blast/CHO cell ratio, 10:1). At the end of the culture, cell-free supernatant fluid was collected and IFN-γ assayed by RIA. Representative experiment out of four performed.

Figure 8. Ability of IL-10 (100 U/ml) to inhibit IFN-γ production by PBMC in response to *S. aureus* (1:10,000 vol/vol final dilution), anti-CD28 mAb CK248 (ascites, final dilution 1:100), or a mixture of anti-CD28 mAb and *S. aureus*, in the presence or absence of IL-12 (1 ng/ml). PHA-blasts were cultured for 18 h in the presence of the indicated combination of cytokines or antibodies. At the end of the culture period, cell-free supernatant fluids were collected and IFN-γ was assayed by RIA. Results are mean ± SE of six experiments. Percent inhibition of IFN-γ production is given.

Medium   IFN-γ

Green fluor., CD14   Green fluor., CD14

Green fluor., CD11b   Green fluor., CD11b

Green fluor., HLA–DR   Green fluor., HLA–DR

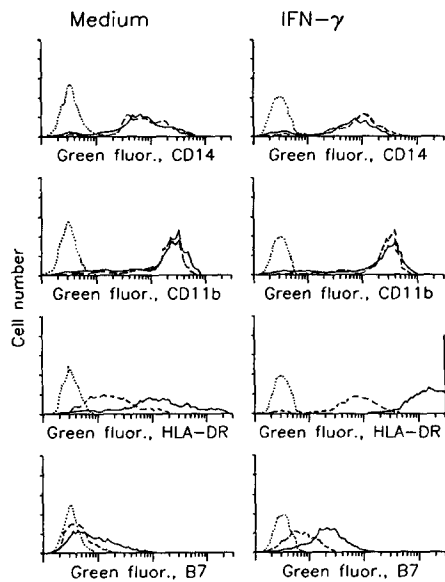Green fluor., B7   Green fluor., B7

Cell number

Figure 9. IL-10 inhibits constitutive and IFN-γ-induced expression of B7 and HLA-DR on cultured human monocytes, but not expression of CD11b and CD14 antigens. PBMC were cultured for 18 h in the presence or not of IL-10 (100 U/ml) and/or IFN-γ (1,000 U/ml). At the end of the culture, PBMC were collected, washed, and analyzed by indirect immunofluorescence (flow cytometry), gating on cells with light scatter characteristics of monocytes, for the expression of CD14 (mAb B52.1), C11b (mAb OKM1), HLA-DR (mAb 1.12), and B7 (mAb B7.4). Graphs in the left column are PBMC cultured without IFN-γ, and in the right column, in the presence of IFN-γ. Dotted lines indicate control negative staining in the absence of a primary antibody in PBMC cultured without IL-10 (PBMC cultured with IL-10 or stained in the presence of irrelevant isotype control antibodies gave similar background staining); continuous lines indicate antibody staining of cells cultured in the absence of IL-10; broken lines indicate antibody staining of cells cultured in the presence of IL-10. Representative experiment out of six performed.

are consistent with the hypothesis that inhibition of IL-12 production and inhibition of B7 expression on accessory cells both have a role in the inhibition of IFN-γ production mediated by IL-10.

Discussion

IL-12 is considered to be a major factor for the generation and activation of Th1 cells as well as for the generation of cytotoxic CD8^{+} T cells and NK cells (8, 15). Unlike the modest proliferative effects on IL-12 reported until now, our results demonstrate that in the presence of an appropriate costimulus, i.e., signaling through the CD28 receptor induced by B7-transfected cells or certain anti-CD28 antibodies, IL-12 can induce powerful and prolonged proliferation in activated T cells. No only were the maximal levels of proliferation obtained with IL-12 higher than those induced by IL-2, but IL-12 was maximally effective at concentrations 100- to 1,000-fold lower than effective concentrations of IL-2. Moreover, the synergistic proliferation induced by IL-12 and anti-CD28 antibody was also observed with freshly isolated PBL. On both freshly isolated PBL or activated PHA-blasts,

anti-CD28 antibodies or B7-transfected cells also synergized with IL-12 in inducing cytokine production.

As with previously reported data on IL-12–induced T cell proliferation, it is difficult to interpret the mechanism underlying the proliferative interactions of IL-12 with T cells without knowledge of the characteristics and control of expression of the IL-12 receptor. The reported affinity of 200–600 pM for the IL-12 receptor identified by IL-12–binding assay (16) is difficult to reconcile with the proliferative effect of IL-12 described here at concentrations <0.1 pM. To further examine the mechanism by which anti-CD28 and IL-12 exert their synergistic effect, we tested the participation of endogenous IL-2 and the inhibitory effect of CsA on the proliferative response induced by this type of stimulation. The low level of proliferation induced by IL-12 was not affected by low doses (20 ng/ml) of CsA and was IL-2 independent, whereas the proliferation induced by the IgM anti-CD28 mAb CK248 was completely abolished by either CsA or by neutralization of endogenous IL-2. These results suggest that the IgM antibody, like the cross-linked IgG anti-CD28 on Jurkat cells (29, 31, 47), induces IL-2 production by a CsA-sensitive mechanism. However, the synergistic effect of anti-CD28 plus IL-12 on proliferation was insensitive to CsA and only partially dependent on endogenous IL-2, suggesting that the biochemical signaling pathway by which anti-CD28 synergizes with IL-12 is different from that by which it directly induces IL-2–driven proliferation. As expected from published results (28), CsA did not inhibit the proliferation induced in T cells by the combination of anti-CD3 and anti-CD28, two stimuli that separately are CsA sensitive. It is also of interest that the proliferation induced by anti-CD3 and IL-12 was still sensitive to inhibition by CsA, suggesting that both the direct proliferative effect of anti-CD3 and its cooperative effect with IL-12 are mediated by a possibly common, CsA-sensitive signal transduction mechanism.

The synergistic effect of the B7/CD28 costimulatory pathway with IL-12 was observed not only for the proliferation of T cells, but also for the production of cytokines, particularly IFN-γ. The combination of anti-CD28 or B7-transfected cells with IL-12 is the most efficient condition for stimulation of IFN-γ production in freshly isolated or lectin-activated T cells yet described. Other cytokines such as TNF-α and GM-CSF were also induced by a combination of IL-12 and CD28 receptor stimulation, but in this case, other stimuli such as anti-CD3 or TPA were more effective than IL-12 in synergizing with CD28 stimulation. These data show that IL-12, although able to induce low level production of various cytokines, is particularly efficient in inducing IFN-γ production, especially when used in synergy with other stimuli. This is consistent with previous findings that many of the physiologic effects of IL-12 in vivo depend on its ability to induce IFN-γ (13, 14, 48).

Whereas the IgM antibody CK248 was very efficient in synergizing with IL-12 in inducing IFN-γ production and T cell proliferation, the IgG2a antibody 9.3 was only moderately effective. The specificity of the effect of CK248 was, however, suggested by the fact that antibody 9.3, which is able to compete with CD248 for binding to T cells, inhibits

the IFN-γ–inducing effect of CK248, alone or with IL-12. The fact that CK248 has more dramatic effects on T cells than do other IgG anti-CD28 antibodies and, unlike the IgG antibodies, induces IL-2 production and proliferation by itself, has been reported (31). Cross-linking of the IgG2a mAb 9.3 on the Jurkat T cell line induces IL-2 production by a CsA-sensitive mechanism (47). In light of our findings that the ability of CK248 to induce T cell proliferation and presumably IL-2 production is CsA-sensitive, it is possible that the IgM mAb CK248 induces a biologic response similar to that of cross-linked IgG antibodies. Using several anti-CD28 antibodies with different binding characteristics, we have shown that the synergistic effect with IL-12 for both proliferation and IFN-γ production is observed only with those antibodies that induce efficient Ca^{2+} flux and, in the presence of phorbol diester, IL-2 production in Jurkat cells (31). Three anti-CD28 antibodies that have been previously reported to be inefficient in both functions (31) also failed to show significant synergism with IL-12. mAb CK248 stands alone in this as in a previous study (31) from its ability to induce by itself IL-2 production in Jurkat cells and low but significant proliferation and IFN-γ production in human T cells. As discussed above, this unique property of CK248 may depend on its IgM isotype, which may induce cross-linking of CD28, possibly a sufficient signal for IL-2 production (47). Indeed, CK248 synergistic effects with IL-12 are very similar to those observed when the IgG2a mAb 9.3 is used together with the phorbol diester TPA. The sensitivity of the direct effect of CK248 to CsA and its dependence to IL-12 compared with the resistance to CsA of its synergistic effect with IL-12 suggests that different mechanisms are responsible for the direct proliferative effect and the synergism with IL-12. Because the synergistic effects of CD28 stimulation with IL-12 are also observed using the physiologic CD28 ligand B7, it is likely that the response observed with CD248 and other anti-CD28 mAb in the presence of IL-12 reflects the physiologic response of activation of the B7/CD28 costimulatory pathway.

Our results with human T cells suggest that optimal CD28 stimulation in combination with IL-12 drives proliferation and cytokine production by a mechanism that is largely independent of IL-2 production. Although proliferation induced by anti-CD28 and IL-12 was more efficient and was observed earlier with preactivated PHA blasts than with PBL, production of IFN-γ was similarly induced in both cell types. It should, however, be considered that NK cells, present in PBL preparations, are responsible for a proportion of the IFN-γ produced in response to IL-12 stimulation (41). Although we could not demonstrate any effect of mAb CK248 on IFN-γ production by purified NK cells (not shown), it can not be excluded that NK cells might be activated by factors produced by T cells activated by anti-CD28 and IL-12 and that might amplify the IFN-γ production observed using total PBL. The results with human PBL contrast with those obtained with mouse naive T cells in which we did not observe a responsiveness to IL-12, even when the cells were costimulated by specific antigen and B7 (37). These results may be explained by the presence among PBL of a proportion of memory T cells, which might have been rendered responsive

to IL-12 by previous exposure to antigen. The kinetics of proliferation of freshly purified PBL, with maximal proliferation observed at day 6, suggests the possibility that nonspecific stimuli related to the culture conditions and possibly, but not necessarily, acting through the TCR, activate a proportion of T cells to respond to CD28 and IL-12 stimulation. The kinetics of proliferation observed in this condition is reminiscent of that observed with stimulation with PHA and IL-12 (1) that is interpreted as due to activation by PHA of IL-12 receptors in a conformation required for induction of proliferation (17). Whether CD28 stimulation directly renders naive and memory freshly isolated peripheral blood T cells responsive to IL-12, or whether a nonspecific, possibly TCR-dependent stimulus, is required for induction of responsiveness to the proliferative response to CD28 and IL-12 stimulation, remains to be determined.

CTLA-4Ig inhibited the production of IFN-γ more than fivefold in PBMC stimulated with IL-12 alone, suggesting that interaction of T cells with B7 expressed on accessory cells in PBMC has an important role in the responsiveness of fresh peripheral blood cells to IL-12. We previously showed that accessory cells (nonmonocytic, non-B, HLA-DR-positive), possibly dendritic cell–like, were necessary for optimal IFN-γ production by resting PBL (41) but not for enhancement of the cytotoxic effect of NK cells (CD28-negative) (49). Although these accessory cells might be required for IFN-γ production because of their constitutive expression of B7 antigen, we also observed low level expression of B7, at least at the end of an 18-h incubation period, on monocytes.

IL-10 (43) inhibits IL-12 production by human PBMC, and we have shown that the ability of IL-10 to inhibit IFN-γ production induced by a variety of stimuli, e.g., S. aureus, is largely dependent on the inhibition of IL-12 production from accessory cells and not on a direct effect on T cells (43). However, because IL-10 also partially inhibited the IFN-γ production induced by IL-12 itself, it was clear that the IL-10–mediated inhibition of IFN-γ production was not due uniquely to inhibition of IL-12 production by accessory cells. We identified TNF-α and IL-1β as two other accessory factors that cooperate with IL-12 in inducing IFN-γ production and proposed that downmodulation of accessory cell surface molecules might also be involved (43). In the mouse, IL-10 was recently shown to prevent IFN-γ–induced upregulation of B7 on macrophages but not on dendritic cells (50). We observed that, IL-10 efficiently prevented not only IFN-γ–induced upregulation of B7, but also inhibited the constitutive expression of B7 observed in the 18-h culture of human peripheral blood monocytes. IL-10 did not affect CD11b and CD14 expression (51), but, unlike observations in the mouse (52), profoundly inhibited class II MHC expression on human monocytes (51). Thus, it is possible that IL-10 suppresses IFN-γ production in human PBMC by inhibiting IL-12 production as well as by decreasing B7 or other CD28-ligand expression on some accessory cells. Indeed, the ability of IL-12 and anti-CD28 antibodies combined to induce IFN-γ production in PBMC was completely resistant to IL-10. The synergistic effect of S. aureus and anti-CD28 was still inhibited by IL-10, as expected because S. aureus induces IFN-γ by trig-

gering IL-12 production in PBMC, an IL-10–inhibitable mechanism. However, when IL-12 and anti-CD28 were added together to S. aureus, an almost complete resistance to IL-10 was obtained. The partial inhibition by IL-10 in this case might be explained by its inhibition of other cofactors, e.g., TNF-α and IL-1β (43–45). Because B7 is upregulated by IFN-γ, it is likely that this represents an amplifying feedback mechanism when IFN-γ is induced by IL-12 in vitro or in vivo. Indeed, B7 expression on monocytes was upregulated after 1–3 d of culture of PBMC with IL-12 or with the IL-12–inducing S. aureus; this effect was inhibited by anti-IFN-γ antibodies (not shown).

The ability of monocyte-macrophages and B cells to present a surface counter-receptor and to secrete a soluble cytokine that cooperate with each other in a potent synergistic effect in inducing T cell activation, both in terms of proliferation and cytokine production, is likely to have physiologic significance in vivo during inflammation and immune response. Note that IFN-γ induced by IL-12 and B7 can enhance both secretion of IL-12 (53) and expression of B7 in monocytes (54), thus providing a double positive feedback loop. T cells activated by the B7/CD28 interaction and IL-12 also produce GM-CSF and TNF-α, which may also participate in macrophage activation and enhanced production of IL-12 (Kubin, M., and G. Trinchieri, unpublished observations). This effect is likely to amplify both phagocytic cell and T cell activation, e.g., in bacteria-induced inflammation, until negative feedback mechanisms mediated by IL-10 and possibly by IL-4, TGF-β, and other pharmacologic mediators interrupt this amplifying circle. Because T cell activation mediated by B7 and IL-12 is at least in part independent of IL-2 production, these mechanisms may represent an early, nonspecific response to IL-12–inducing situations, e.g., bacterial or parasitic infection. Although activation of naive T cells by B7 and IL-12 may not be, by itself, a sufficient physiological stimulus for T cell proliferation and even for IFN-γ production, a proportion of preactivated or memory T cells able to respond to these stimuli, is probably present within circulating T cells. Furthermore, stimulation of TCR by bacterial-derived superantigens (55) or other stimuli, possibly activating alternative stimulation pathways such as CD2 (26) or CD5 (56), may render naive T cells responsive to B7 and IL-12 stimulation for both early cytokine production and, eventually, proliferation. This response could represent a first line of defense against infections, and also, depending on the predominant cytokine pattern induced, affect development of antigen-specific CD4+ and CD8+ cells. An early nonspecific ability to produce IFN-γ and other phagocytic cell–activating cytokines has usually been attributed to NK cells rather than to T cells (14, 57). However, our in vitro results strongly suggest that in the presence of IL-12 and B7 expression on accessory T cells, T cells are likely to participate in these nonantigen-specific mechanisms. The participation of T cells may be dependent on and follow NK cell activation and production of IFN-γ; our data both in human and mouse have indeed shown a possible requirement for NK cells in the IL-12–induced differentiation of Th1 cells (7, 58).

In a parallel study (37), we demonstrated a similar synergistic effect of B7 and IL-12 on proliferation and cytokine production in murine Th1 clones; the ability of splenic APCs and antigen to induce proliferation and IFN-γ production in Th1 clones was shown to depend on B7 expression on the APC and on IL-12 secretion. Preliminary results (Kubin, M., F. Gerosa, and G. Trinchieri, unpublished observation) show that human CD28+ T cell clones can also be activated synergistically by anti-CD28 and IL-12, provided that responsiveness to IL-12 in the clones is induced by PHA, similar to the requirement for antigen activation in the murine clones. It is therefore likely that the synergistic effect between B7 and IL-12 is important for the activation of differentiation of quiescent T cells as well as for the maintained activation of the effector functions of established mature Th1 clones.

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