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B7 and Interleukin 12 Cooperate for Proliferation and Interferon γ Production by Mouse T Helper Clones That Are Unresponsive to B7 Costimulation

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Summary

We have previously shown that dendritic cells isolated after overnight culture, which can express B7 and are potent stimulators of naive T cell proliferation, are relatively poor at inducing the proliferation of a panel of murine T helper 1 (Th1) clones. Maximal stimulation of Th1 clones was achieved using unseparated splenic antigen presenting cells (APC). An explanation for these findings is provided in the present study where we show that FcR+ L cells transfected with B7 stimulate minimal proliferation of Th1 clones in response to anti-CD3 antibodies, in contrast to induction of significant proliferation of naive T cells. However, addition of interleukin 12 (IL-12) to cultures of Th1 cells stimulated with anti-CD3 and FcR+ B7 transfectants resulted in a very pronounced increase in proliferation and interferon γ (IFN-γ) production. Exogenous IL-12 did not affect the B7-induced proliferation of naive T cells. This showed that whereas costimulatory signals delivered via B7-CD28 interaction are sufficient to induce significant proliferation of naive T cells activated through occupancy of the T cell receptor, Th1 T cell clones require cooperative costimulation by B7 and IL-12. This costimulation was shown to be specific by inhibition of proliferation and IFN-γ production using chimeric soluble cytolytic T lymphocyte-associated antigen 4–human IgG₁Fc (CTLA4-Ig) and anti-IL-12 antibodies. Furthermore, the significant antigen specific proliferation and IFN-γ production by Th1 clones observed when splenocytes were used as APC was almost completely abrogated using CTLA4-Ig and anti-IL-12 antibodies. Thus two costimulatory signals, B7 and IL-12, account for the ability of splenic APC to induce maximal stimulation of Th1 clones. IL-10 downregulates the expression of IL-12 by IFN-γ-stimulated macrophages and this may account largely for the ability of IL-10 to inhibit APC function of splenic and macrophage APC for the induction of Th1 cell proliferation and IFN-γ production. Indeed we show that IL-12 can overcome the inhibitory effect of IL-10 for the APC-dependent induction of proliferation and IFN-γ production by Th1 clones. These results suggest that proliferation by terminally differentiated Th1 clones, in contrast to naive T cells, requires stimulation via membrane-bound B7 and a cytokine, IL-12. It is possible that these signals may result in the activation of unresponsive T cells during an inflammatory response. IL-10, by its role in regulating such innate inflammatory responses, may thus help to maintain these T cells in an unresponsive state.

Membrane-bound structures on APCs provide costimulatory signals for the activation of T cell proliferation (1). The most dominant costimulatory interaction described to date is that between B7 and CD28 (2–7). CD28 is a homodimeric cell surface protein that is found on the majority of T lymphocytes (3, 8, 9). Its ligand, B7/BB1, is a membrane glycoprotein that is induced upon activation on B cells, mac-rophages (2, 10–13), and activated T cells (14) and is also expressed on dendritic cells (15). In conjunction with TCR occupancy, the costimulatory signals that are delivered to T cells when B7 binds to CD28 result in T cell activation, lymphokine secretion and proliferation (2–7) and are resistant to cyclosporin A (16). Moreover, B7/CD28 interaction has been shown to prevent the induction of T cell anergy in-

duced when T cells are activated by occupancy of the TCR in the absence of costimulatory signals (1, 17). Cytolytic T lymphocyte–associated antigen 4 (CTLA4)[1] (18), structurally similar to CD28, is also capable of binding B7 (18, 19) and is expressed on activated T lymphocytes. CTLA4 can be made into a soluble form by fusing the extracellular domain of CTLA4 to the human IgG1 Fc region (CTLA4-Ig), and this molecule inhibits the interaction between B7 and CD28/CTLA4 (18).

In addition to membrane-bound costimulators, it is likely that APC-derived cytokines also play a role in T cell activation. A prime candidate is IL-12, a heterodimeric cytokine comprised of two distinct, but unrelated, gene products. The 40-kD (p40) and 35-kD (p35) glycosylated protein subunits must be assembled for biologically active IL-12 to be secreted (20–22). IL-12 is produced by activated macrophages and B lymphocytes (23), augments NK and T cell-mediated cytotoxicity, and stimulates IFN-γ production and proliferation of NK cells and T cells (20, 24–27), alone or in synergy with other factors such as IL-2 (24). These properties and the ability of IL-12 to promote Th1 phenotype development in both human and mouse T cell populations (28–30) may account for the ability of IL-12 to resolve infections with intracellular bacteria or parasites (31–34).

CD4$^+$ T cells can be separated both functionally (35) and based on the cytokines (36, 37) that they produce in response to antigen. Th1 cells produce IL-2, IFN-γ, and lymphotoxin, which are responsible for cell-mediated immune responses, whereas Th2 cells produce IL-4, IL-5, and IL-10, which promote humoral or allergic responses (36, 37). Our previous studies (38) suggested that Th1 clones were not maximally stimulated by dendritic cells isolated after overnight culture, which expressed B7 (15) and induced excellent proliferation of naive T cells as previously documented (38–42). In contrast, splenic APC induced maximal proliferation and IFN-γ production by Th1 clones, whereas these APC were relatively poor as compared with dendritic cells at stimulating the in vitro proliferation of naive T cells isolated from normal mice (38). In this study we show that a panel of Th1 clones require cooperative interactions between B7/CD28 and IL-12 for maximal proliferation and IFN-γ production, whereas B7/CD28 interaction is sufficient to costimulate the proliferation of naive T cells. Furthermore we present evidence that these two costimulatory signals, B7 and IL-12, account for the ability of splenic APC to induce maximal stimulation of Th1 cells.

Materials and Methods

Animals. Female BALB/cAnN, C57Bl/6N normal mice, and CB17 scid mice, between 4 and 6 wk of age, were purchased from Simonsen Laboratories, Inc. (Gilroy, CA) and used as splenic APC donors for the stimulation of antigen-specific Th1 clones or as a source of macrophages for RT-PCR.

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[1] *Abbreviations used in this paper:* CTLA4, cytolytic T lymphocyte-associated antigen 4; RT, reverse transcriptase.

Culture Medium, Reagents, and Cell Lines. Stimulation and maintenance of Th1 clones was as previously described: HDK1 (BALB/c-derived KLH-specific) (43); D1.1 (H-2^d–restricted, rabbit Ig-specific) (44); H66-61 (BALB/c-derived, autoreactive) (45); 1.31 (C57Bl/6-derived, Ovalbumin-specific) (G. Terres, unpublished observations). Clones were expanded and maintained in RPMI 1640 (JR Scientific Inc., Woodland, CA) containing 10% FCS (JR Scientific Inc.), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), hepes buffer (10 mM), sodium pyruvate (1 mM), 2-mercaptoethanol (0.05 mM; Sigma Chemical Co., St. Louis, MO), and cRPMI, supplemented with mouse recombinant (r)IL-2 (330 U/ml). In some cases clones were stored in liquid nitrogen and cells were thawed and maintained in murine rIL-2, as above, 5–7 d before assay. L cells were transfected with the FcR (CD32) plus and minus human B7 (46) and maintained in DMEM supplemented as for cRPMI but in the absence of IL-2. The untransfected L cells did not express detectable levels of B7 as determined by failure to react with CTLA4-Ig fusion protein or anti-mouse B7 mAb (data not shown). Spleen cell suspensions from BALB/c and C57Bl/6 mice were treated with 0.83% ammonium chloride to lyse red blood cells, washed, and resuspended in cRPMI.

Cytokines, mAbs, and Immunoassays. Mouse rIL-12 was obtained by transfecting COS 7 cells with the cDNAs encoding the p35 and p40 subunits of IL-12, which were obtained by PCR cloning using sequences described by Schoenhaut et al. (47). Anti-IL12 antibodies, C15.1.2 and C15.6.7 are described by Wysocka, M., and G. Trinchieri (unpublished observations). GL113 used as an isotype matched control for anti-IL12, was provided by Dr. J. Abrams (DNAX Research Institute). CTLA-4-Ig was kindly provided by Dr. Peter Lane (Basel Institute for Immunology, Basel, Switzerland) (18, 48). An isotype matched control, human IgG1 was provided by PharMingen (San Diego, CA). Biotinylated anti–mouse CD8α, B220, anti-IA^d, anti–mouse Mel-14 FITC (leukocyte cell adhesion molecule [LECAM]), anti–mouse CD4, and anti–mouse CD3-ε were obtained from PharMingen.

IFN-γ was detected in the supernatants from cell cultures using a two-site sandwich ELISA (49), and the sensitivity was 150 pg/ml.

Proliferation of Th1 clones was measured by incorporation of [^{3}H]thymidine (1 μCi/well, specific activity, 2 Ci/mMol; New England Nuclear, Boston, MA), added to cultures after supernatants were removed for cytokine determination. Plates were pulsed for 8–12 h and then processed for scintillation counting.

Stimulation of Naive T Cells and Th1 Clones. CD4$^+$ T cells were enriched by negative selection using a Magnetic Activated Cell Sorter as previously described (MACS; Biotec GMBH; Miltenyi, Germany) (50), and then further purified to homogeneity using a FACStar$^{Plus®}$ flow cytometer (Becton Dickinson & Co., Mountain View, CA) to achieve 99.8% CD4$^+$ T cells. At this stage Mel-14^{dull} cells were also removed by sorting to remove previously activated or memory T cells (51). T cell clones (2 × 10^4/well) were stimulated with the appropriate antigen and MHC matched splenic APC (4 × 10^5/well) (3,000 rads), or with soluble anti-CD3 (50–2,000 ng/ml) and L cells transfected with CD32 plus or minus human B7 (250–4,000 cells/well) (7,000 rads). Cells were incubated in 96-well flat bottomed plates at 37°C in a humidified incubator (5% CO$_2$). Cultures were in the presence or absence of any of the following either alone or in combination: IL-12 (50–75 U/ml) (mock control Cos supernatant, not shown, had no effect at 1 in 80 dilution and greater, at which concentration the Cos IL-12 was used); IL-10 (100 U/ml); anti-IL-12 mAbs (100 μg/ml) or an isotype matched control GL113 mAb (100 μg/ml); CTLA4-Ig (10 μg/ml) or a human IgG1 control (10 μg/ml). 100 μl of supernatant was removed at 60 h and assayed for IFN-γ production.

Proliferation was measured by adding [³H]thymidine to the remaining culture and incubating for an additional 8–12 h.

RNA-PCR. RNA was prepared using a modification of the guanidinium isothiocyanate (GuSCN)/cesium chloride method (52) from CB17 scid peritoneal cells (10⁶ cells/sample) stimulated overnight with IFN-γ (100 U/ml), IFN-γ (100 U/ml) and IL-10 (100 U/ml), or no stimulation. Reverse transcription (RT) and PCR were performed for p40 and p35 IL-12 mRNA expression, using the RT-Semi-quantitative PCR method previously described (53). IL-12 sequences used were: P35 (IL-12) Sense 5′-GAG GAC TTG AAG ATG TAC CAG-3′, Size (325) Antisense 5′-TTC TAT CTG TGT GAG GAG GGC-3′, Probe 5′-AAG CTC TGC ATC CTG CTT CAC GCC TTC-3′; P40 (IL-12) Sense 5′-GAC CCT GCC CAT TGA ACT GGC-3′, (Size 415) Antisense 5′-CAA CGT TGC ATC CTA GGA TCG-3′, Probe 5′-TGT CTG CGT GCA AGC TCA GGA-3′.

Results and Discussion

Cooperative Effects of B7 and IL-12 on IFN-γ Production and Proliferation of Th1 Clones.   We have previously shown that a panel of Th1 clones, in contrast to naive T cells, responded poorly to antigen presented by dendritic cell APCs isolated after overnight culture in contrast to splenic APCs (38). The relatively poor capacity of these dendritic cells isolated after overnight culture to stimulate Th1 clones was surprising since these cells expressed B7, a dominant costimulator for T cell proliferation, possibly as a result of some activation during isolation. To dissect further the requirements for optimal Th1 proliferation and IFN-γ production, as compared with naive T cells, soluble anti-CD3 antibody and stable FcR-bearing L cell transfectants expressing high levels of B7, or parental FcR⁺ L cells without B7, were used to stimulate a panel of Th1 clones or naive CD4⁺ T cells. (The subline of L cells used in these studies does not express murine B7 as determined by their failure to stain with CTLA4-Ig or anti–mouse B7.) B7 transfectants resulted in significant costimulation for anti-CD3–induced proliferation of naive CD4⁺ T cells, but minimal proliferation of Th1 clones (Fig. 1 A). FcR⁺ L cells lacking B7 gave no proliferation of Th1 clones or naive CD4⁺ T cells stimulated with soluble anti-CD3. Moreover, addition of IL-12 gave no proliferation of naive CD4⁺ T cells and minimal to no proliferation when Th1 cells were stimulated with parental FcR-bearing L cells and anti-CD3. By contrast, a very significant cooperative effect on proliferation of all the Th1 clones was observed when IL-12 was added to the B7 transfectants, whereas IL-12 did not increase the proliferation of naive CD4⁺ T cells costimulated with B7 transfectants. In contrast to the lack of effect of IL-12 on naive CD4⁺ T cell proliferation, cooperation between B7 and IL-12 for stimulation of IFN-γ production by both Th1 clones and naive CD4⁺ T cells was observed (Fig. 1 B). Intermediary levels of IFN-γ were induced in the Th1 clones with either B7 transfectants or IL-12 alone in contrast to the minimal proliferation. The cooperative effect of B7 and IL-12 for Th1 proliferation was IL-2 dependent (data not shown). This was shown by inhibition with anti-IL-2 and anti-IL-2 receptor mAbs.

To ensure that the effects of B7 or IL-12 on Th1 or naive CD4⁺ T cell proliferation and IFN-γ production were spe-

cific, CTLA4-Ig and anti-IL-12 antibodies were included in the stimulation cultures. CTLA4-Ig almost completely inhibited the proliferation of naive CD4⁺ T cells (Fig. 2 A) induced by FcR⁺ B7 transfectants and anti-CD3, and to a lesser extent the proliferation of Th1 clones (Fig. 2 A). The effect of CTLA4-Ig on IFN-γ production by Th1 clones was minimal, perhaps reflecting that IFN-γ is often inducible in the absence of B7 (Fig. 2 B). The cooperative effect of IL-12 and B7 on Th1 proliferation was partially blocked by CTLA4-Ig, more so by anti-IL-12 alone, and essentially completely with a combination of both reagents, confirming specificity of the interactions (Fig. 2 B). Anti-IL-12 had no effect on the proliferation of naive CD4⁺ T cells stimulated with anti-CD3 and B7 transfectants. The low levels of IFN-γ produced by CD4⁺ T cells, stimulated with anti-CD3 together with B7 and IL-12 were abolished by inclusion of CTLA4-Ig or anti-IL-12 mAbs (Fig. 2 B).

In summary, interaction of B7 and CD28 is sufficient to induce maximal proliferation of naive CD4⁺ T cells stimulated by anti-CD3, and in no circumstances can this stimulation be enhanced by addition of IL-12. In contrast, costimulation with B7 of Th1 clones stimulated by anti-CD3 induced minimal proliferation and this was significantly enhanced by addition of IL-12. Although IL-12 did not show any effect on the anti-CD3–induced proliferation of CD4⁺ T cells, it cooperated with B7 to induce very low levels of IFN-γ (Fig. 1 B). IL-12 or B7 alone induced minimal levels of IFN-γ production from anti-CD3 stimulated CD4⁺ T cells. It is possible that the IFN-γ is being produced by a small number of contaminating memory or preactivated T cells, although measures were taken to avoid this by FACS® sorting Mel-14^bright cells (51).

Splenic APC–induced Th1 Proliferation and IFN-γ Production Are Inhibited by CTLA4-Ig and Anti-IL-12 Antibodies.   To determine whether the Th1 cell proliferation and IFN-γ production induced when splenocytes were used as APC involved B7 or IL-12, CTLA4-Ig and anti-IL-12 antibodies were included in cultures of Th1 clones stimulated with specific antigen presented by splenic APCs. Again, proliferation of Th1 clones was partially blocked by CTLA4-Ig, to a greater extent by anti-IL-12 antibodies, and was almost completely abrogated by addition of the two reagents (Fig. 3 A). A similar trend was observed for the inhibition of IFN-γ production by Th1 clones (Fig. 3 B), although still to a slightly lesser extent than the effects of CTLA4-Ig and anti-IL-12 antibodies on proliferation (Fig. 3 A). This confirmed the physiological relevance of our findings in the anti-CD3 system demonstrating an important role for B7 together with IL-12 in inducing optimal proliferation in an antigen-specific system.

These results may have important implications for the regulation of the immune response and induction of anergy (1). Th1 clones may represent terminally differentiated T cells that produce lower amounts of IL-2 upon stimulation with antigen but high levels of effector cytokines such as IFN-γ, in contrast to naive T cells that produce high levels of IL-2 and only low levels of other cytokines such as IL-3, IFN-γ, and GM-CSF (54). Since B7 alone is not sufficient to activate a high level of proliferation in terminally differentiated Th1
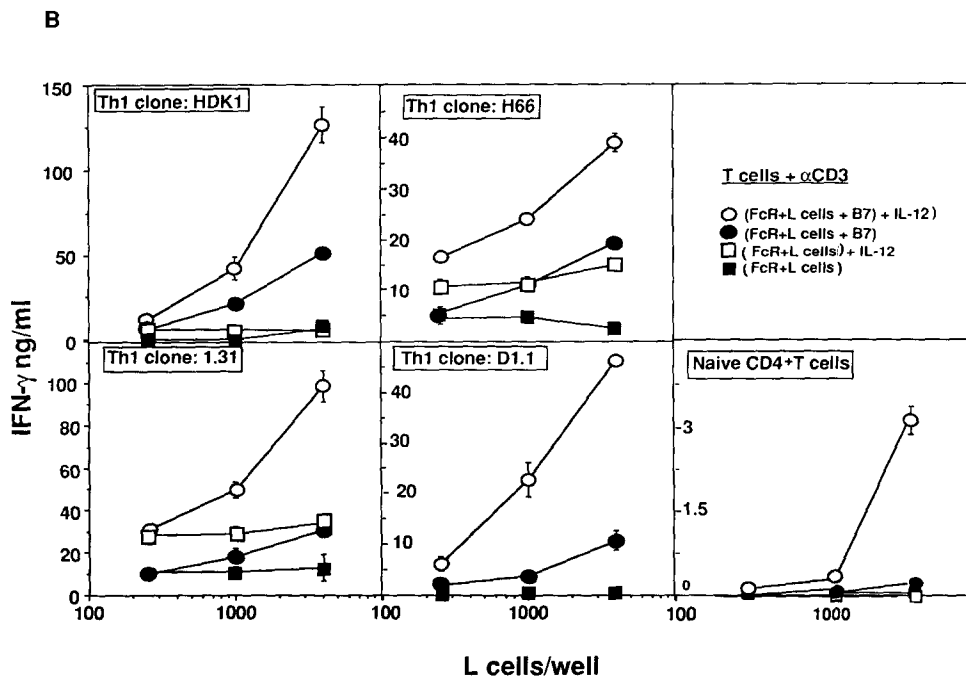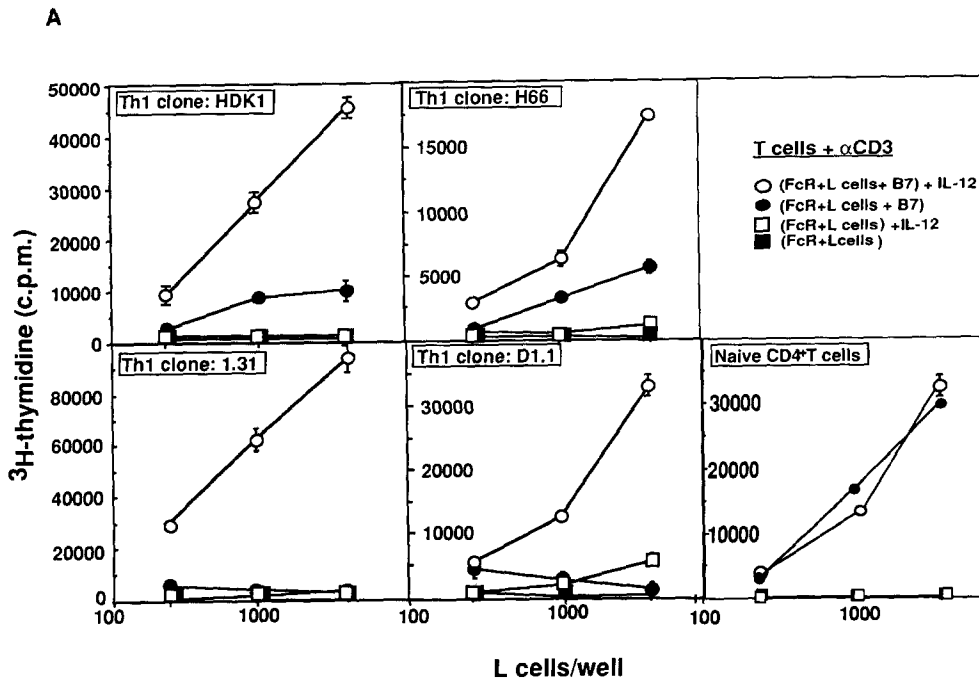
A

Th1 clone: HDK1

50000
40000
30000
20000
10000
0

^{3}H-thymidine (c.p.m.)

Th1 clone: H66

15000
10000
5000
0

T cells + αCD3

○ (FcR+L cells+ B7) + IL-12
● (FcR+L cells + B7)
□ (FcR+L cells) +IL-12
■ (FcR+Lcells)

Th1 clone: 1.31

80000
60000
40000
20000

Th1 clone: D1.1

30000
20000
10000

Naive CD4+T cells

30000
20000
10000

100   1000   100   1000   100   1000

L cells/well

B

Th1 clone: HDK1

150
100
50
0

IFN-γ ng/ml

Th1 clone: H66

40
30
20
10

T cells + αCD3

○ (FcR+L cells + B7) + IL-12)
● (FcR+L cells + B7)
□ (FcR+L cells) + IL-12
■ (FcR+L cells)

Th1 clone: 1.31

100
80
60
40
20
0

Th1 clone: D1.1

40
30
20
10

Naive CD4+T cells

3
1.5
0

100   1000   100   1000   100   1000

L cells/well

Figure 1. Cooperative effects of IL-12 and B7 on proliferation and IFNγ production by a panel of Th1 clones and naive CD4$^+$ T cells. L cells transfected with CD32 (FcR) plus or minus B7 were titrated in 96-well plates and Th1 clones (*first four panels*) or naive CD4$^+$ T cells (*last panel*) were added (2 × 10^4/ well) with soluble anti-CD3 in the presence or absence of IL-12 (50–75 U/ml), for 48–60 h. (*A*) Proliferation of Th1 clones and naive CD4$^+$ T cells was measured by [^{3}H]thymidine incorporation. (*B*) IFNγ production by Th1 clones and naive CD4$^+$ T cells was measured in supernatants removed at 60 h. The data are shown as the mean of triplicate cultures ± SD.

clones, IL-12 may play an important role in determining the responsiveness of such T cells. Since IL-12 is produced by macrophages after stimulation with bacterial or other intracellular pathogens (23, 28), it is possible that innate inflammatory immune responses may lead to stimulation of T cells, which otherwise appear unresponsive. The interaction of B7 with CD28 expressed on Th2 clones was shown to result in increased proliferation and cytokine production (data not shown), supporting the recently published studies of McAr-

thur and Raulet (55). However, in contrast to its effects on Th1 clones, IL-12 showed no costimulatory effect for proliferation and cytokine production by Th2 clones (data not shown), supporting a recently published study (56).

IL-12 Overcomes the Inhibitory Effect of IL-10 on Splenic APC Function for Induction of Proliferation and IFN-γ Production by Th1 Clones. We (57) and others (58, 59) have shown that IL-10 inhibits Th1 IFN-γ production and proliferation by inhibiting splenic and macrophage APC function. In addi-
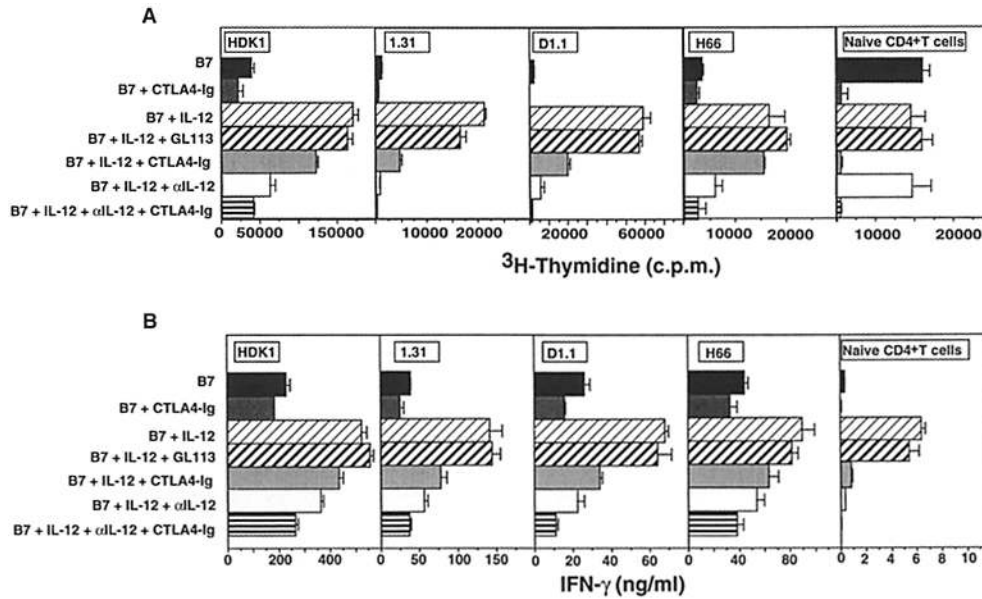
A

^{3}H-Thymidine (c.p.m.)

B

IFN-γ (ng/ml)

Figure 2. Effect of anti-IL-12 and CLTA4-Ig on B7 and IL-12 stimulation of Th1 or naive CD4$^+$ T cell proliferation and IFN-γ production. L cells transfected with CD32 (FcR) and B7 were titrated in 96-well plates (single point shown) and Th1 clones or naive CD4$^+$ T cells were added (2 × 10^4/well) with soluble anti-CD3 (50–2,000 ng/ml) in the presence or absence of IL-12 (50–75 U/ml), anti-IL-12 (100 μg/ml), GL113 (100 μg/ml), and/or CTLA4-Ig (10 μg/ml) singly or in combination, as indicated. Th1 clones plus or minus CD32$^+$ L cells plus anti-CD3 gave, alone 100 –900 c.p.m. or plus IL-12, 800-4,500 c.p.m., and 50–100 ng/ml IFN-γ, and without anti-CD3 gave <500 c.p.m. and 2–20 IFN-γ ng/ml. Naive CD4$^+$ T cells alone, with no accessory cells gave 46 c.p.m. and undetectable IFN-γ with anti-CD3, and 59 c.p.m. and undetectable IFN-γ, with anti-CD3 plus IL-12. (A) Proliferation of Th1 clones and naive CD4$^+$ T cells was measured by [^{3}H]thymidine incorporation. (B) IFN-γ production by Th1 clones and naive CD4$^+$ T cells was measured in supernatants removed at 60 h. The data are shown as the mean of triplicate cultures ± SD.

tion, IL-10 inhibits macrophage induced Th1 development which is mediated by IL-12 (50, 60). These observations and the present findings that B7 and IL-12 cooperate for Th1 proliferation and IFN-γ production induced by splenic APCs prompted us to test whether IL-10's regulatory effects resulted from downregulation of IL-12. Fig. 4, A and B demonstrate that IL-10 inhibited Th1 clone proliferation and IFN-γ production when splenocytes were used as APCs as we have previously shown (57, 61). Addition of IL-12 to these cultures completely restored proliferation by IL-10–inhibited Th1 clones. The ability of IL-12 to overcome the inhibition of IFN-γ production by IL-10 was often not complete. This

could reflect a need for other costimulators for induction of IFN-γ production that are also inhibited by IL-10. Possible candidate molecules are, B7, whose expression has been shown to be downregulated by IL-10 on murine macrophages (62) and human monocytes (62a). Moreover, Kubin et al., in a parallel study (62a) show that IL-12 synergizes with B7/CD28 interaction in inducing efficient proliferation and cytokine production by activated human T cells. However, since IL-12 can completely overcome the inhibition of Th1 cell proliferation, but not IFN-γ production, by IL-10, it is likely that other factors, whose induction by macrophages (61) and monocytes (63) is also inhibited by IL-10, may be required for op-
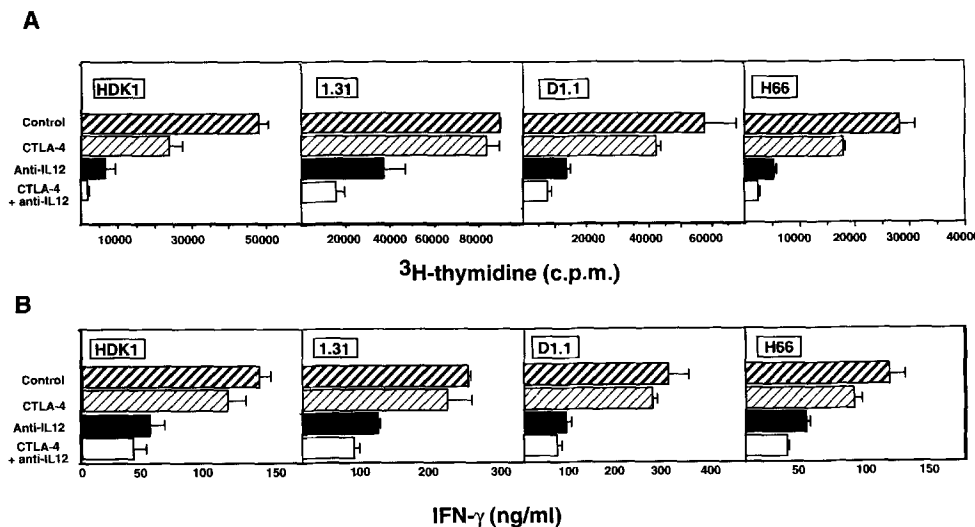
A

^{3}H-thymidine (c.p.m.)

B

IFN-γ (ng/ml)

Figure 3. Anti-IL-12 and CTLA4-Ig specifically block splenic APC function for induction of proliferation and IFN-γ production by Th1 clones. Spleen APCs were titrated (single point shown) in 96-well plates and Th1 clones were added (2 × 10^4) with specific antigen and stimulated in the presence of IL-12 (50–75 U/ml), anti-IL-12 (100 μg/ml) and/or CTLA4-Ig (10 μg/ml) singly or in combination as indicated. (A) Proliferation of Th1 clones was measured by [^{3}H]thymidine incorporation. (B) IFN-γ production by Th1 clones was measured in supernatants removed at 60 h as described in Materials and Methods. The data are shown as the mean of triplicate cultures ± SD.
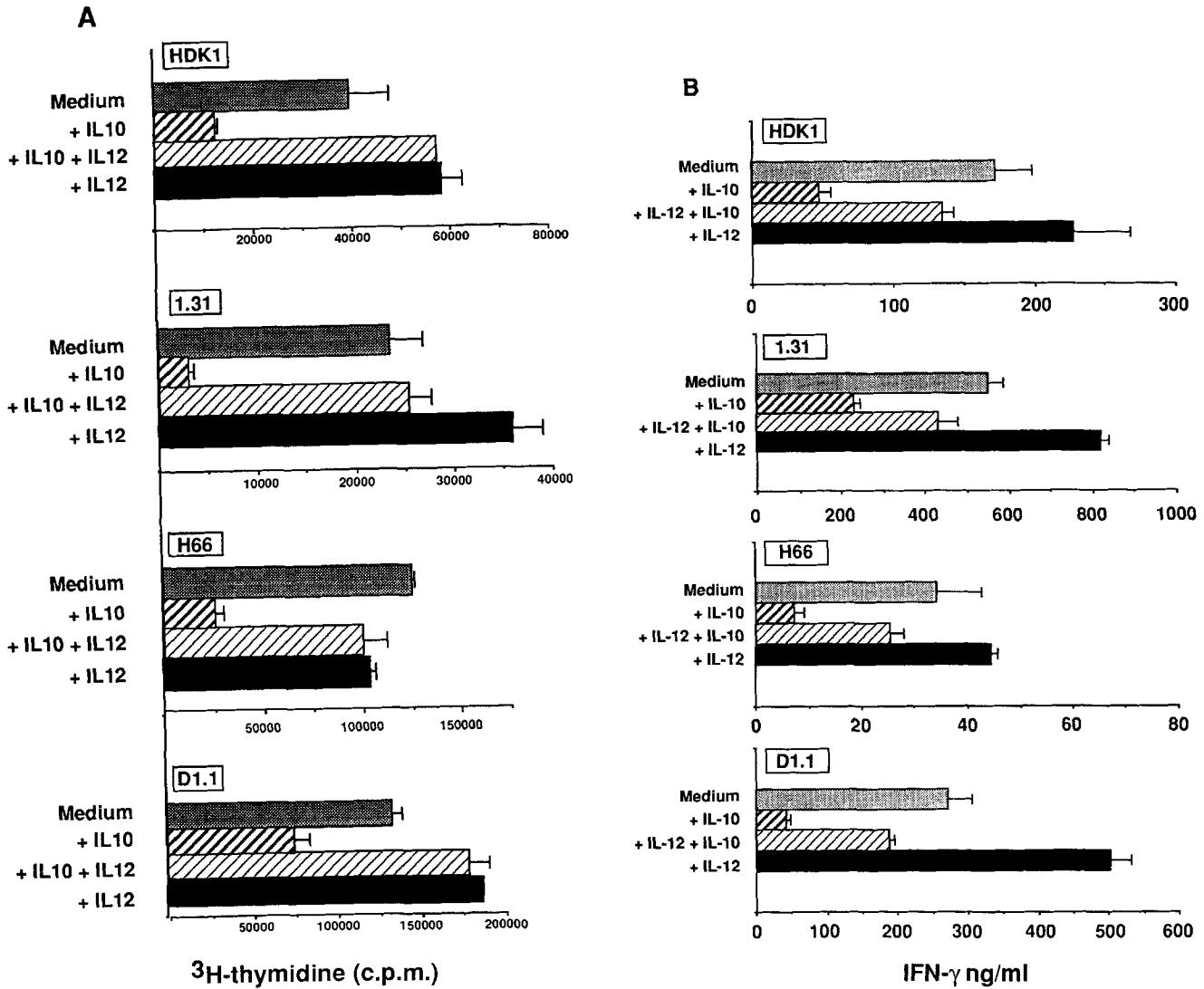
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A

HDK1

Medium
+ IL10
+ IL10 + IL12
+ IL12

20000   40000   60000   80000

1.31

Medium
+ IL10
+ IL10 + IL12
+ IL12

10000   20000   30000   40000

H66

Medium
+ IL10
+ IL10 + IL12
+ IL12

50000   100000   150000

D1.1

Medium
+ IL10
+ IL10 + IL12
+ IL12

50000   100000   150000   200000

^{3}H-thymidine (c.p.m.)

B

HDK1

Medium
+ IL-10
+ IL-12 + IL-10
+ IL-12

0   100   200   300

1.31

Medium
+ IL-10
+ IL-12 + IL-10
+ IL-12

0   200   400   600   800   1000

H66

Medium
+ IL-10
+ IL-12 + IL-10
+ IL-12

0   20   40   60   80

D1.1

Medium
+ IL-10
+ IL-12 + IL-10
+ IL-12

0   100   200   300   400   500   600

IFN-γ ng/ml

Figure 4. IL-12 overcomes the inhibitory effect of IL-10 on splenic APC function for induction of proliferation and IFN-γ production by Th1 clones. Spleen APCs were titrated (single point shown) in 96-well plates and Th1 clones were added with specific antigen in the presence or absence of IL-12 (50–75 U/ml), IL-10 (100 U/ml), or IL-12 and IL-10 together. (*A*) Proliferation of Th1 clones was measured by [^{3}H]thymidine incorporation. (*B*) IFN-γ production by Th1 clones was measured in supernatants removed at 60 h. The data are shown as the mean of triplicate cultures ± SD.
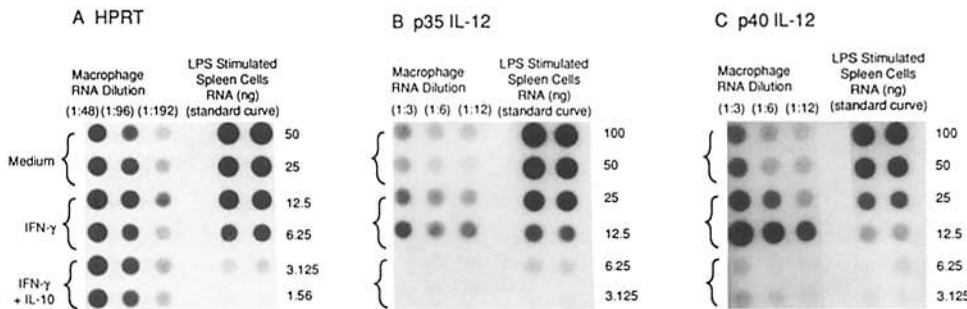
A HPRT

Macrophage RNA Dilution
(1:48)(1:96)(1:192)

LPS Stimulated Spleen Cells RNA (ng) (standard curve)

Medium
IFN-γ
IFN-γ + IL-10

50
25
12.5
6.25
3.125
1.56

B p35 IL-12

Macrophage RNA Dilution
(1:3) (1:6) (1:12)

LPS Stimulated Spleen Cells RNA (ng) (standard curve)

100
50
25
12.5
6.25
3.125

C p40 IL-12

Macrophage RNA Dilution
(1:3) (1:6) (1:12)

LPS Stimulated Spleen Cells RNA (ng) (standard curve)

100
50
25
12.5
6.25
3.125

Figure 5. IL-10 downregulates p35 and p40 IL-12 mRNA expression in IFN-γ–stimulated peritoneal macrophages. Scid peritoneal cells (10^6/well) were used as a source of enriched macrophages and incubated in RPMI as described in Materials and Methods with IFN-γ (100 U/ml) in the presence or absence IL-10 (100 U/ml), or medium alone and incubated for 12 h at 37°C in a humidified incubator (5% CO_2). RT-PCR was performed as previously described (49). (*A*) mRNA (starting from ~4,000 cell equivalents) levels were assessed for hypoxanthine ribosyl transferase expression to ensure that all samples had similar input RNA. Samples were diluted (as indicated) so that message levels fell within the linear part of the standard curve. (*B*) mRNA expression of p35 IL-12 (dilutions of cDNA were made starting from ~25,000 cell equivalents). (*C*) mRNA expression of p40 IL-12 (dilutions of cDNA were made starting from ~25,000 cell equivalents). For a uniform level of HPRT expression equivalent in all three duplicate samples the arbitrary units (a.u.) of expression for IL-12 were: Macrophages (i) + medium only, p35 = 45 a.u; p40 = 79 a.u.: (ii) + IFN-γ, p35 = 39 a.u; p40 = 158 a.u.: (iii) + IFN-γ + IL-10, p35 = 7 a.u; p40 = 27 a.u.

timal IFN-γ production by Th1 cells. Indeed, D'Andrea et al. (64), have shown that TNF-α and IL-1 are required in addition to IL-12 to overcome the inhibitory effects of IL-10 on IFN-γ production by human NK cells. In addition, we have recently shown that these costimulators are required for maximal IFN-γ production by committed Th1 TCR-transgenic CD4$^+$ T cells, although IL-12 has a dominant effect (Macatonia, S. E., C.-S. Hsieh, M. Wysocka, G. Trinchieri, K. M. Murphy, and A. O'Garra, manuscript submitted for publication). Furthermore, IL-12 could also enhance proliferation to a small extent and IFN-γ to a larger extent, by Th1 clones stimulated with splenic APCs and antigen.

IL-10 Inhibits IFN-γ-induced Macrophage Expression of IL-12. To confirm that IL-10 was inhibiting APC function by downregulating the expression of IL-12, we analyzed expression of mRNA encoding P40 and P35 IL-12 in enriched populations of macrophages from the peritoneal cavity of *scid* mice after stimulation in the presence and absence of IL-10. IFN-γ was shown to upregulate expression of both P35 and p40 IL-12 mRNA, although the effect on p40 expression was more marked (Fig. 5). IFN-γ-induced expression of p35 and p40 IL-12 mRNA by macrophages was completely abrogated

by the addition of IL-10, bringing the levels to below those observed in the absence of stimulation. These results suggest that IL-10 may not only inhibit Th1 development from naive T cells by inhibiting IL-12 production (28, 29), but in addition downregulates the costimulatory activity of IL-12 on already differentiated Th1 cells for induction of effector function and proliferation.

In summary, B7 and IL-12 cooperate for maximal induction of proliferation and IFN-γ production by terminally differentiated Th1 clones, which are poorly stimulated by either signal alone. These results suggest that IL-12 together with B7 may play a role in activating unresponsive or anergic T cells. That together these two costimulatory signals play an important role in T cell activation is supported by similar findings using human T cells (62a). B7 is expressed on dendritic cells and can be upregulated on macrophages by concomitant signals that upregulate IL-12. Thus insult with infectious agents may lead to inflammatory responses that could influence otherwise unresponsive T cells, and IL-10 by its role in regulating such innate inflammatory responses (31, 32, 61, 63, 64, 65) may thus help to maintain these T cells in an unresponsive state.

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