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The Roles of IL-12 in Providing a Third Signal for Clonal Expansion of Naive CD8 T Cells[1]

Javier Valenzuela, Clint Schmidt,[2] and Matthew Mescher[3]

Stimulation of an effective in vitro or in vivo response by naive CD8 T cells requires three signals: TCR engagement, costimulation/IL-2, and a third signal that can be provided by IL-12. In addition to being required for acquisition of cytolytic function, IL-12 is required for optimal IL-2-dependent proliferation and clonal expansion. In experiments examining in vitro stimulation of naive CD8 T cells, IL-12 is shown to stimulate expression of the IL-2R α-chain (CD25) to much higher levels than are reached in response to just TCR and costimulation and/or IL-2. In addition, high CD25 expression is substantially prolonged in the presence of IL-12. As a consequence, the cells proliferate more effectively in response to low levels of IL-2. Examination of adoptively transferred TCR transgenic CD8 T cells responding to peptide Ag confirmed that IL-12 up-regulates CD25 in vivo, even when B7-mediated costimulation is largely blocked. TCR- and IL-2-dependent proliferation of CD8 T cells from mice deficient in CD25 was also found to increase in the presence of IL-12, indicating that CD25 up-regulation is not the only mechanism by which IL-12 increases clonal expansion of the cells. IL-2 and IL-12 both act to increase expression of both CD25 and the IL-12R, thus providing positive cross-regulation of receptor expression. These results suggest that when cross-priming dendritic cells present class I/Ag and costimulatory ligands, and produce IL-12, naive CD8 T cells will begin to produce IL-2 and both receptors will be optimally up-regulated to insure that an effective response is generated. ***The Journal of Immunology,*** **2002, 169: 6842–6849.**

Activation of naive CD8 T cells to proliferate and generate effector CTL requires MHC/peptide recognition by the TCR, B7-1/2 recognition by CD28 to result in IL-2 production, and the binding of IL-2 to its receptor IL-2R$\alpha\beta\gamma$ on the T cell. This is consistent with the two-signal model for T cell activation (1) in which the TCR provides signal 1, and costimulation by CD28 provides signal 2 to generate an effective immune response (2–5). However, recent studies have demonstrated that these two signals are not sufficient for complete activation of naive T cells; a third signal is also required (6, 7). In the case of naive CD8 T cells, IL-12 can provide this required third signal in vitro (6) and in vivo (7, 8). Although numerous studies have demonstrated augmentation of CTL responses by IL-12, the critical requirement for this third signal only became apparent when responses by highly purified naive T cells were examined in the absence of APC that might produce cytokines (6). Furthermore, memory cells require only two signals (6, 9), and the presence of even small numbers of these cells can obscure the requirement of naive cells for the third signal.

The critical importance of the third signal became even more apparent when responses of adoptively transferred naive CD8 T cells to peptide Ags were examined (7, 8). Immunization with peptide Ag in CFA resulted in massive clonal expansion of the Ag-specific CD8 T cells, development of effector function, and establishment of a long-lived memory population. In contrast, immunization with peptide Ag alone resulted in weak clonal expansion, effector function did not develop, and the cells were rendered tolerant long-term. However, when IL-12 was injected along with peptide Ag, the response was comparable to that obtained using CFA; the cells proliferated extensively and killed targets specifically, and a responsive memory population was generated (7). This was the case even when the adoptive transfer recipients were deficient in IL-12R expression, demonstrating that the IL-12 was acting directly on the CD8 T cells as a third signal (8). Thus, the presence or absence of signal 3 in the form of IL-12 can determine whether effective activation or tolerance induction occurs. IL-12 is not the only factor that can provide the necessary third signal, as evidenced by the fact that peptide Ag and CFA stimulated strong clonal expansion and generation of effector and memory cells when IL-12-deficient mice were used as the adoptive transfer recipients (7). Consistent with this, an allogeneic CTL response can be generated in IL-12-deficient mice (10). Although it is not the only third signal, IL-12 is likely to be important in many instances, because the CD8α^+ subset of dendritic cells (DC)[4] is the most effective activator of CD8 T cells, and contains the DC that produce the highest levels of IL-12 in response to adjuvants and CD40 ligation (11, 12). In vitro experiments have suggested that IL-1, but not IL-12, may provide a third signal for CD4$^+$ T cells (6). Consistent with this, IL-1, but not IL-12, could replace adjuvant in supporting an in vivo response of CD4 T cells to peptide or protein Ag (13). Thus, while CD4 T cells can respond to IL-12 by differentiating down the Th1 pathway, it is not sufficient to support their initial activation. Conversely, IL-1 cannot provide a third signal for CD8 T cells (6).

The receptors for IL-12 and IL-2 are highly regulated on T cells (for reviews, see Refs. 14–16). The IL-12R is a heterodimer of β1 and β2 chains, with IL-12 binding mediated predominantly by β1 and intracellular signaling by β2 (17, 18). Most reports agree that resting T cells do not express a high affinity IL-12Rβ1β2 (16),

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[4] Abbreviation used in this paper: DC, dendritic cell.

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although some argue that naive cells can be responsive to IL-12 even in the absence of detectable receptors (19). Both β-chains can be up-regulated by TCR stimulation, and expression is increased by B7-1, IL-2, and IFN-γ, and decreased in CD4 T cells by IL-4 and IL-10 (16).

The IL-2R complex is a trimer of α-, β-, and γ-chains; the α-chain (CD25) increases the affinity of the dimeric IL-2R$\beta\gamma$ by 10^3- to 10^4-fold, and is required for signaling at physiological levels of IL-2 in mice (20, 21). Mature resting T cells do not express CD25, but it can be rapidly and transiently up-regulated by TCR and CD28 ligation and by several cytokines including IL-2 (15). Cross-regulation of CD25 and IL-12Rβ1/β2 expression by IL-2 and IL-12 has been studied in CD4 T cells, with the results indicating that IL-2 and/or B7 costimulation can increase the expression of IL-12R (22–25), and that IL-12 can up-regulate CD25 over levels achieved by IL-2 or B7-1 (26–29).

Most of our understanding of CD25 and IL-12R regulation has come from studies examining CD4 T cells or clones, or mixed populations of T cells, or from studies using APC to provide stimulation. Given the dramatic effect of IL-12 on the proliferation and clonal expansion of naive CD8 T cells in vitro and in vivo, and the inability of IL-12 to provide signal 3 to support initial CD4 T cell activation, it was important to better understand how these receptors are regulated on the resting, naive CD8 T cells. The results described in this study demonstrate that one of the important roles of IL-12 as a third activation signal is that of increasing and sustaining expression of CD25 on the cells to allow prolonged response to the low levels of IL-2 that CD8 T cells produce. In addition, IL-12 can enhance proliferation independently of its effect on CD25 expression.

Materials and Methods

Mice and cell lines

OT-I mice (30), a gift from F. Carbone (University of Melbourne, Melbourne, Australia), express a transgenic TCR specific for an OVA-derived peptide (SIINFEKL) bound to H-2K^b. The 2C mice (31), a gift from D. Loh (Washington University, St. Louis, MO), express a transgenic TCR specific for the synthetic peptide SIYRYYGL bound to H-2K^b (32). OT-I and 2C mice were bred and housed in a specific pathogen-free environment at all times. C57BL/6 mice were purchased from National Cancer Institute (Frederick, MD), and CD25$^{-/-}$ mice (33) were purchased from The Jackson Laboratory (Bar Harbor, ME). Cells used as targets in cytotoxicity assays included EL-4 thymoma and E.G7, the EL-4 thymoma transfected with OVA (34). The cell lines were maintained in vitro in complete RPMI medium (RPMI 1640, 10% FCS, 0.2% L-glutamine, 0.1% penicillin/streptomycin, 0.1% HEPES, 0.1% nonessential amino acids, 0.01% sodium pyruvate, 0.05% 2-ME, with 400 μg/ml G418 added in the case of E.G7).

Artificial APC

Five-micron-diameter sulfated polystyrene latex microspheres (Interfacial Dynamics, Portland, OR) were coated with 2C11 anti-CD3ϵ mAb (BD PharMingen, San Diego, CA), or a biotin-H-2K^b/OVA$_{257–264}$ fusion protein (35) at 1.0 μg/10^7 beads or 0.2 μg/10^7 streptavidin beads, respectively. When used, murine B7-1-Fc (R&D Systems, Minneapolis, MN) was coimmobilized on the microspheres at 0.2 μg/10^7 beads. Ag and B7-1 densities were in the range previously shown to be effective for T cell stimulation, and were confirmed for each preparation by flow cytometry, using goat anti-hamster Ig FITC (Jackson ImmunoResearch Laboratories, West Grove, PA) to detect 2C11-coated microspheres, anti-K^b (Y3) mAb followed by goat anti-mouse Ig FITC (Jackson ImmunoResearch Laboratories) to detect K^b/OVA-coated microspheres, and anti-mouse B7-1 FITC mAb (R&D Systems) to detect B7-1 Fc. Preparation and characterization of the artificial APC have been previously described in detail (36).

Purification of naive CD8 T cells

For in vitro experiments, lymph node cells were harvested, depleted of adherent cells by incubation on plastic petri dishes for 1 h, and incubated with anti-CD4 FITC (0.5 μg/10^6 cells), goat anti-mouse IgG FITC (0.5 μg/10^6 cells), and anti-CD44 FITC (0.06 μg/10^6 cells) mAbs (BD PharMingen). After incubating for 30 min at 4°C, cells were washed and incubated with anti-FITC magnetic microbeads (l μl/10^6 cells), and passed over Midi-MACS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany), and the flow-through cells were collected. The purified cells were typically >95% CD8$^+$ T cells (<0.3% CD4$^+$ T cells) and >99% CD44^{low}. For in vivo experiments, lymph node cells from 2C mice were harvested and adherence was depleted in the same way, and the CD8 cells were purified using Cellect-plus enrichment columns (Biotex Laboratories, Edmonton, Alberta, Canada). The flow-through population was stained with anti-CD44 FITC and anti-CD8 PE, and then sorted using a FACSVantage flow cytometer (BD Biosciences, Mansfield, MA) to obtain a final population of naive CD44^{low} (>98%) CD8$^+$ (>99%) T cells.

Proliferation and cytotoxicity assays

Proliferation was determined using 5 × 10^4 responder T cells and 10^5 Ag-coated microspheres in flat-bottom microtiter wells (Falcon, Franklin Lakes, NJ) in a total volume of 200 μl of supplemented RPMI 1640 medium (6). Where indicated, human rIL-2 (2.5 U/ml), mouse rIL-12 (2.0 U/ml; Genetics Institute, Cambridge, MA), or 20 μl of hybridoma supernatant containing sheep anti-mouse IL-2Rα mAb (PC61.5.3 hybridoma) was also added. [^{3}H]TdR was added during the final 8 h of culture, and incorporation of radioactivity was determined. All conditions were tested in triplicate, and the average and SD of incorporated radioactivity are shown. Cytotoxic activity was determined in a standard 4-h ^{51}Cr release assay using E.G7 target cells, with EL-4 targets as a specificity control.

Detection of receptors by flow cytometry and RT-PCR

Surface expression of CD25 was determined using a rat anti-mouse CD25 FITC or PE mAb (BD PharMingen) and detected using the FACSVantage flow cytometer and CellQuest software (BD Biosciences). Expression of mRNA for CD25 and IL-12Rβ2 was determined using a semiquantitative two-step RT-PCR assay. Total RNA was isolated using the RNAqueous kit (Ambion, Austin, TX). cDNA was synthesized from 1 μg of starting RNA, using SuperScript First-Strand's specifications (Life Technologies, Rockville, MD). PCR amplification was done using primers for CD25 (37), IL-12Rβ2 (24), and β-actin (38). The temperature program included a 95°C hot start (10 min), followed by cycles of denaturation-annealing-polymerization (95°C (1.5 min) + 63°C (2 min) + 72°C (3 min)). Experiments showed that 27 cycles allowed for the detection of cytokine receptors and their normalization to β-actin, within the linear range of amplification.

Adoptive transfer and immunization

The in vivo response of TCR transgenic 2C (31) CD8 T cells was measured, as previously described (7, 8). Briefly, lymph node cells from 2C transgenic mice were harvested, and the naive CD8 T cells were purified as described above. A total of 3–5 × 10^6 CD8$^+$ T cells in 0.5 ml PBS were adoptively transferred by i.v. injection (tail vein) into sex-matched C57BL/6 recipient mice. Mice were immunized 1 day later, and the number and phenotype of 2C CD8 T cells in the lymph nodes were determined 3 days later using the anti-clonotypic 1B2 mAb (39) to identify the 2C cells and rat anti-mouse CD25 FITC mAb to determine CD25 expression levels. Immunizations were done using the synthetic peptide SIYRYYGL (32) (Chiron Mimotopes, Clayton, Victoria, Australia) prepared in PBS and injected on day 0 using 50 μg in 300 μl PBS (distributed between two sites on the back). Some groups also received murine rIL-12 (Genetics Institute) at the same time as peptide administration, by i.p. of 1 μg IL-12 (2.7 × 10^3 ± 1.2 × 10^3 U/μg) in 100 μl PBS containing 0.1% sterile mouse serum. In addition, some groups received CTLA-4-Ig administered i.p. at 200 μg in 100 μl PBS, 6 h before peptide/IL-12 injection and again on days 0, 1, and 2 (total of 0.8 mg/mouse). As controls, transferred animals were immunized with PBS alone (transfer only). Results shown are representative of two independent experiments.

Results

Three signals are necessary for optimal T cell proliferation and differentiation

Earlier studies showed that IL-12 was required as a third signal for the in vitro activation of naive CD8 T cells when the cells were stimulated with physiological densities of class I Ag coated on microspheres and either coimmobilized B7-1 or added IL-2 (6). However, some proliferation can occur in the absence of IL-12 when very high, nonphysiological levels of TCR engagement are

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achieved using microspheres having high surface densities of either anti-TCR mAb or refolded class I MHC protein with a homogeneous peptide Ag bound to it (Curtsinger et al., manuscript in preparation). Thus, in the experiment shown in Fig. 1, purified naive CD8 T cells were stimulated with different combinations of anti-CD3ϵ mAb coated on microspheres (2C11), IL-2, IL-12, and a blocking polyclonal anti-IL-2Rα Ab (a2R), and their proliferation was measured on day 2. None of the three stimuli alone was sufficient to induce proliferation (Fig. 1*A*). The combination of TCR engagement and IL-12 was also unable to stimulate significant proliferation, provided that anti-IL-2Rα Ab was added to block the effect of the small amount of IL-2 that is produced by the cells under these conditions. In contrast, TCR engagement and IL-2 in combination were sufficient to induce significant proliferation, and there was a further substantial increase when IL-12 was also present. The extent of synergy of IL-2 and IL-12 on proliferation varied among experiments, from minimal enhancement (Fig. 3), to almost a 2-fold increase (Fig. 1*A*). For 22 experiments, the average fold increase in the presence of IL-12 was 1.6 +/− 0.3. However, in all experiments, IL-12 was strictly required for acquisition of cytolytic function (measured on day 3), despite the substantial proliferation induced by 2C11 and IL-2 (Fig. 1*B*, and additional data not shown). The ability to drive some proliferation without IL-12 when TCR engagement levels were high allowed us to explore the role of each signal during T cell activation, under conditions in which sufficient numbers of cells could be recovered in the absence IL-12 to allow characterization.

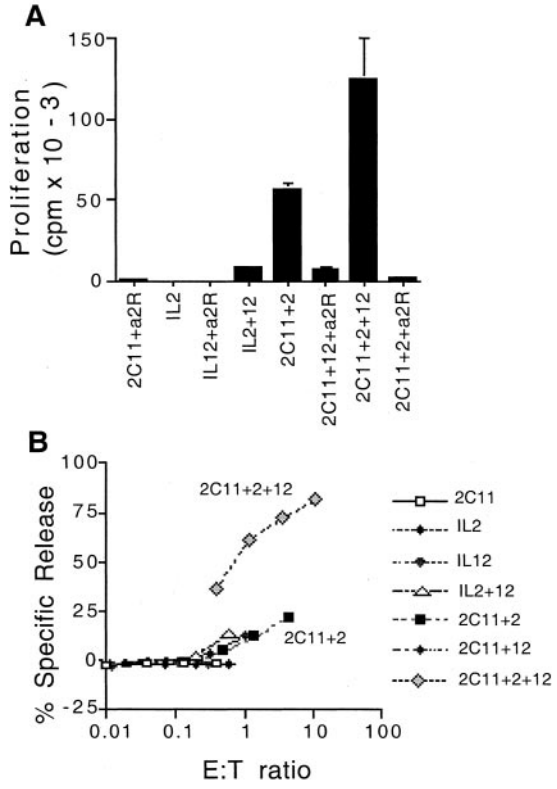

FIGURE 1. IL-12 allows optimal proliferation, and is required for CTL differentiation of purified naive CD8 T cells. *A*, $CD44^{low}$ (>99%) $CD8^+$ (>95%, <0.3% $CD4^+$) OT-1 T cells were stimulated with 5 μm-sized microspheres coated with 2C11 anti-CD3ϵ mAb (1 μg/ml/10^7 microspheres), in the absence of cytokines or with IL-2 (2.5 U/ml), IL-12 (2.0 U/ml), and/or a blocking anti-IL-2Rα mAb (20 μl of hybridoma supernatant), as indicated. Proliferation was determined on day 2 by measuring incorporation of [^{3}H]TdR. *B*, Cells stimulated as in *A* were harvested on day 3, and a 4-h ^{51}Cr release assay was done using OVA-transfected EL-4 cells (E.G.7 cell line) as targets. SDs of triplicate samples in the ^{51}Cr release assay were less than 10% of the average values shown. The requirement for IL-12 to stimulate cytolytic activity under these conditions has been consistently observed in numerous experiments.

IL-12 synergizes with IL-2 to enhance CD25 and IL-12R mRNA expression

Purified naive CD8 T cells from OT-I mice were stimulated for 48 h with 2C11 microspheres and either IL-2 alone or IL-2 plus IL-12 and compared with unstimulated cells for levels of CD25 and IL-12Rβ2 mRNA by semiquantitative RT-PCR using specific primers. Initial experiments were done to determine the number of cycles that resulted in linear amplification for the receptors and the β-actin controls (data not shown). CD25 mRNA expression was undetectable in resting cells and was weakly up-regulated in response to TCR engagement when endogenous IL-2 was blocked with anti-IL-2Rα mAb (becoming detectable in six of seven experiments). Expression increased substantially when both anti-TCR mAb and IL-2 were added, or when anti-TCR mAb and IL-12 were added in the presence of anti-IL-2Rα mAb. Thus, either signal 2 or signal 3 increases the weak expression of CD25 mRNA stimulated by TCR engagement. However, expression was greatest when all three signals, anti-TCR mAb, IL-2, and IL-12, were present. Thus, optimal CD25 mRNA expression requires all three signals. This was also seen at the level of surface protein expression of CD25 when cells were stimulated for 48 h and examined by flow cytometry following staining with an anti-mouse CD25 FITC mAb (Fig. 2*B*).

Similar results were obtained when IL-12Rβ2 mRNA expression was examined. Again, receptor message was undetectable in unstimulated cells, increased weakly in response to TCR engagement alone (becoming detectable in two of seven experiments), increased more with TCR engagment and either IL-2 or IL-12, and increased substantially when all three signals were provided (Fig.

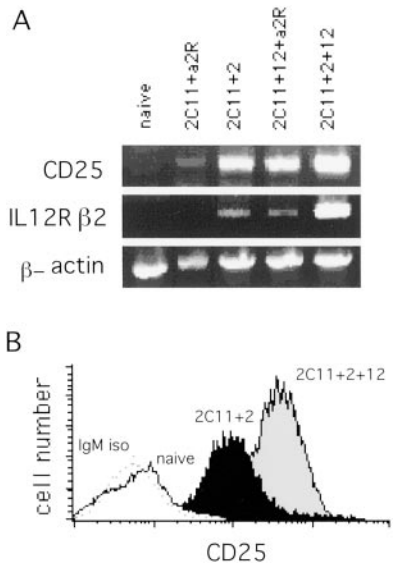

FIGURE 2. IL-12 enhances, but is not required for, expression of CD25 mRNA and protein, and IL-12Rβ2 mRNA. $CD44^{low}$ $CD8^+$ OT-I T cells were stimulated with 2C11-coated microspheres, plus the indicated cytokines, along with blocking anti-IL-2Rα mAb, where indicated. Cells were harvested after 48 h and analyzed. *A*, Expression levels of CD25 and IL-12Rβ2 mRNA were determined by RT-PCR. *B*, Cells stimulated as indicated were stained with anti-CD25 mAb or isotype control mAb (dotted line) and analyzed by flow cytometry.

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2*A*). Essentially the same results were obtained when IL-12Rβ1 expression was examined (data not shown).

IL-12 prolongs high level expression of CD25

IL-12 not only enhanced, but also prolonged, the expression of CD25. CD8 T cells from OT-I mice stimulated with 2C11 microspheres and IL-2 had peak surface expression of CD25 at 24 h, and the level subsequently declined (Fig. 3*A*). In contrast, when IL-12 was also present, CD25 expression levels were similar at 24 h, but continued to increase over the next 2 days. Similar results were obtained when OT-I CD8 T cells were stimulated with microspheres coated with Kb/OVA$_{257–264}$ complex (Fig. 3*B*). In this experiment, high CD25 expression was maintained through day 2 in the presence of IL-12, and then declined. The extent to which CD25 was increased and prolonged by IL-12 varied between experiments, ranging from 6- to 10-fold increased expression and extension by 2 or 3 days. For eight experiments, the average fold increase in CD25 expression in the presence of IL-12 was 8.4 $\pm$ 2.6. This variability was independent of the TCR stimulus being used. In contrast to CD25 expression, IL-12 did not change the kinetics of IL-12Rβ2 mRNA expression, which was maximal at 48 h (Fig. 3, *C* and *D*). It did, however, consistently increase the level of expression by 1.5- to 3-fold. Proliferation, as measured by [^{3}H]TdR incorporation, correlated with CD25 expression levels, with increased and extended responses in the presence of IL-12 (Fig. 3, *E* and *F*, and see below). These results suggest that the synergistic effect of IL-12 on growth of naive CD8 T cells is due, at least in part, to its ability to increase and sustain IL-2 signaling by increasing and sustaining expression of CD25 on the cell surface.

IL-12 effects on CD25 expression cannot be replaced with signal 1 or 2

TCR engagement and IL-2 can up-regulate CD25 in the absence of IL-12 (Fig. 2). To determine whether IL-12 is uniquely able to stimulate very high levels of CD25 expression, or if similar levels might be reached with high signal 1 or signal 2, we examined expression at varying levels of anti-TCR mAb on microspheres and varying levels of IL-2. In the presence of IL-2, CD25 expression was near maximal when microspheres were made at 0.25 μg mAb/10^7 beads, and did not increase significantly when a 4-fold higher level of mAb was used (Fig. 4*A*). A similar dose response was seen in the presence of IL-12, but maximal expression was substantially increased. Similar results were obtained when the concentration of IL-2 was varied (Fig. 4*B*). In the absence of IL-12, 2 U/ml of IL-2 stimulated maximal CD25 expression, and a 5-fold higher concentration did not increase the level. Again, a similar dose response was seen in the presence of IL-12, but the maximal level achieved was 2- to 3-fold higher than in its absence. Thus, it appears that IL-12 can stimulate CD25 expression to levels that cannot be achieved with high levels of TCR or IL-2 signaling.

Proliferation as a function of anti-TCR mAb levels on microspheres and concentrations of added IL-2 correlated well with CD25 expression levels (Fig. 4, *C* and *D*). IL-12 caused significant increases in responsiveness at all levels of TCR engagement, and supported substantial proliferation at low levels of anti-TCR mAb that caused little proliferation in its absence (Fig. 4*C*). When IL-2 levels were varied, IL-12 had the most dramatic effects at the lower

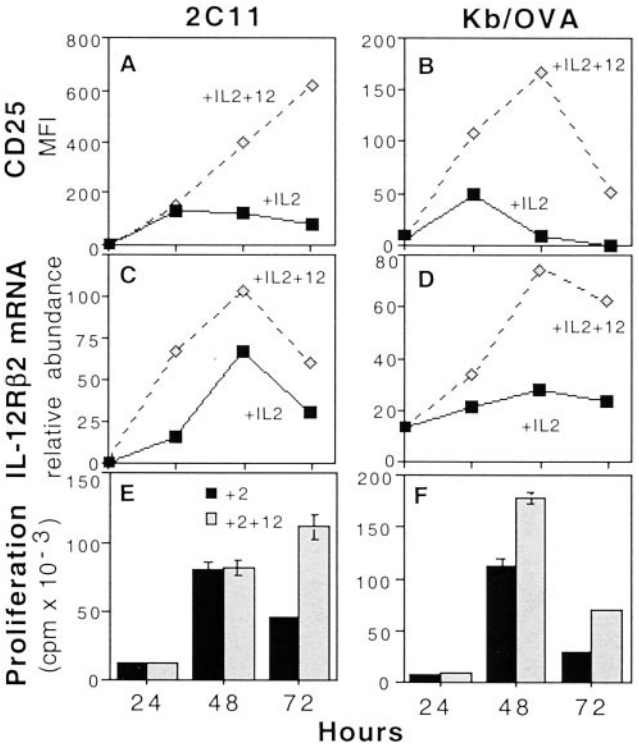

FIGURE 3. IL-12 increases and sustains CD25 expression, IL-12Rβ2 mRNA expression, and proliferation. CD44^{low} CD8$^+$ OT-1 T cells were stimulated with 2C11 (*A*, *C*, and *E*)- or Kb/OVA (*B*, *D*, and *F*)-coated microspheres, plus the indicated cytokines. CD25 surface expression (*A* and *B*), IL-12Rβ2 mRNA expression (*C* and *D*), and proliferation (*E* and *F*) were measured at the indicated times by flow cytometry, RT-PCR, and [^{3}H]TdR incorporation, respectively. Mean fluorescence intensity (MFI) values were normalized to IgM isotype controls (*A* and *B*). mRNA abundance values were normalized to their respective β-actin controls (*C* and *D*).

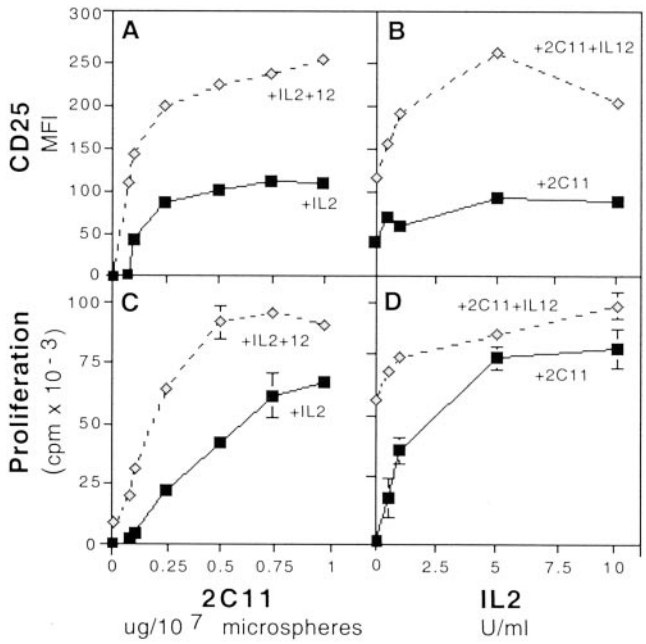

FIGURE 4. The effects of IL-12 on CD25 expression and proliferation cannot be replaced by high levels of TCR engagement or IL-2. CD44^{low} CD8$^+$ OT-1 T cells were stimulated with IL-2 (2.5 U/ml), and microspheres were coated with varied amounts of 2C11 (*A* and *C*), or with 2C11-coated microspheres (1 μg/ml/10^7 microspheres) and varied amounts of IL-2 (*B* and *D*), in the presence or absence of IL-12 (2.0 U/ml). CD25 surface expression (*A* and *B*) and cell proliferation (*C* and *D*) were measured at day 2 by flow cytometry and [^{3}H]TdR incorporation, respectively. Mean fluorescence intensity (MFI) values were normalized to IgM isotype controls (*A* and *B*).

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concentrations (Fig. 4*D*). In fact, proliferation was high in the absence of added IL-2. Under these conditions, this response depends upon the small amount of IL-2 that is made by the cells upon stimulation with anti-TCR mAb on the microspheres, because it is blocked when anti-IL-2Rα mAb is added to the cultures (Fig. 1*A*, and data not shown). Thus, IL-12 can support a vigorous response at concentrations of IL-2 too low to otherwise stimulate significant proliferation. This is most likely due, at least in part, to the cells being made more sensitive to low IL-2 concentrations as a result of higher CD25 expression.

Previous studies have demonstrated the role of B7-1 in the production of IL-2 and expression of CD25 during T cell activation (3, 40, 41). Therefore, we explored whether costimulation by B7-1 would stimulate CD25 expression to the levels achieved in the presence of IL-12. Anti-TCR mAb was immobilized on microspheres either alone (2C11) or along with B7-1 (2C11/B7), and these were used to stimulate CD8 T cells from OT-I mice in the presence or absence of IL-12. CD25 was expressed at a somewhat higher level on cells stimulated with 2C11/B7 in comparison with cells stimulated with 2C11 and IL-2, and addition of IL-2 along with 2C11/B7 did not further increase the level of expression (Fig. 5*A*). Thus, B7 costimulation increased CD25 expression more effectively than did IL-2 alone. In all cases, however, addition of IL-12 caused a further large increase in CD25 expression levels (Fig. 5*B*). The 2C11 with both IL-2 and IL-12 added resulted in higher expression than did B7 costimulation, and the highest CD25 expression was obtained when both B7 costimulation and IL-12 were present (with or without added IL-2). Proliferation in response to the various stimuli correlated with CD25 expression levels (Fig. 5*C*). The 2C11 stimulated weak proliferation in the absence of added cytokines, and this was increased by addition of IL-2, and further increased when IL-12 was also added. The 2C11/B7 stimulated a strong response, as expected when costimulation is present, and addition of IL-2 did not increase this. However, a significant increase occurred when IL-12 was also added. Thus, IL-12 acts to increase CD25 expression level, with a concomitant effect on proliferation, even when effective B7-1-dependent costimulation is provided to the cells.

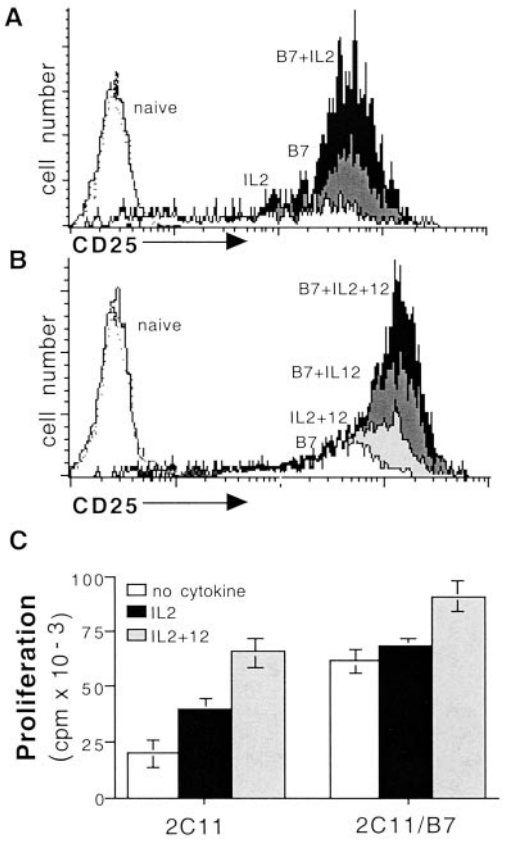

FIGURE 5. The effects of IL-12 on CD25 expression and proliferation cannot be replaced by B7-dependent costimulation. $CD44^{low}$ $CD8^{+}$ OT-1 T cells were stimulated with 2C11- or 2C11/B7-coated microspheres, plus the indicated cytokines. Surface CD25 expression (*A* and *B*) and cell proliferation (*C*) were measured at day 2 by flow cytometry and [^{3}H]TdR incorporation, respectively. The dotted line in *A* and *B* is the IgG isotype control Ab.

IL-12 increases CD25 expression in vivo

IL-12 can replace the need for adjuvant in stimulating a strong Ag-specific CD8 T cell response in vivo when it is coadministered with peptide Ag (7), and acts directly on the CD8 T cells to have this effect (8). To determine whether IL-12 up-regulates CD25 expression in vivo, as shown above in in vitro experiments, CD8 T cells from 2C TCR transgenic mice (31) having a TCR specific for SIYRYYGL peptide bound to H-$2K^b$ (32) were adoptively transferred into congenic C57BL/6 mice. Mice were immunized with PBS (transfer only) or with peptide alone, or peptide and IL-12, and clonal expansion and phenotype of lymph node cells were analyzed 3 days later. To assess the contribution of B7-dependent costimulation, one group of mice received CTLA4-Ig fusion protein 6 h before immunization with peptide and IL-12, and again on days 0, 1, and 2 (total of 0.8 mg/mouse). CTLA4-Ig binds B7-1 and B7-2 with high affinity and prevents interaction with CD28 on T cells (42). Immunization with peptide alone resulted in only a 5.8-fold clonal expansion of 2C cells in the lymph nodes, while immunization with peptide and IL-12 caused a 142-fold clonal expansion (Fig. 6*A*). The response to peptide and IL-12 was largely dependent on B7-mediated costimulation, as CTLA4-Ig blocked the clonal expansion by greater than 80%.

The 2C cells from control mice immunized with PBS (transfer only) had a very low level of CD25 expression (Fig. 6*B*). Immunization with peptide alone did not cause a detectable increase in expression levels, but expression was greatly increased on cells from mice immunized with peptide and IL-12. CTLA4-Ig treatment did not reduce the level of CD25 expression (Fig. 6*B*), despite substantial blocking of clonal expansion (Fig. 6*A*). Thus, these results confirm the in vitro experiments, demonstrating a role for IL-12 in increasing CD25 expression. Furthermore, it appears that under these in vivo conditions, B7-dependent costimulation contributes relatively little to CD25 up-regulation in comparison with that stimulated by IL-12, despite its importance for clonal expansion.

IL-12 increases proliferation of CD8 T cells in the absence of CD25 expression

Although the above results strongly suggested that IL-12 promotes CD8 T cell proliferation by increasing CD25 expression, there was the possibility that it might have additional effects. This was examined using naive CD8 T cells from $CD25^{-/-}$ mice (33). Although the IL-2$\beta\gamma$ receptors expressed by these cells cannot bind IL-2 with high affinity, they can respond if IL-2 is provided at a high concentration (28). Cells were therefore stimulated with anti-TCR mAb on microspheres and 1000 or 2500 U/ml of IL-2 in the presence or absence of IL-12, and their proliferation was compared with that of cells from normal C57BL/6 cells using 2.5 and 250 U/ml of IL-2 (Fig. 7). IL-12 was found to significantly enhance proliferation of the $CD25^{-/-}$ cells, demonstrating that CD25 is not

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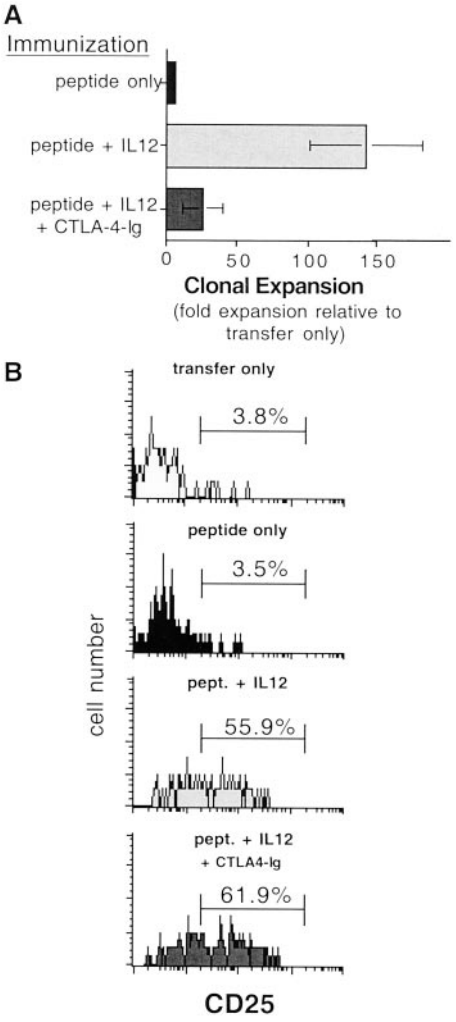

FIGURE 6. IL-12 promotes clonal expansion and CD25 expression in vivo. CD44^{low} CD25^{-} CD8^{+}2C transgenic T cells were adoptively transferred into congenic C57BL/6 mice, and the numbers and CD25 phenotype of the 2C cells were analyzed by flow cytometry 3 days after immunization. Recipient mice (two mice per group) were either not immunized (transfer only), or were immunized with SIYRYYGL peptide alone (peptide) or along with IL-12 (peptide + IL-12). One group of mice immunized with peptide and IL-12 also received CTLA-Ig by i.p. injection of 0.2 mg/mouse 6 h before immunization and again on days 0, 1, and 2 (total 0.8 mg/mouse). *A*, The number of 2C CD8 T cells in the lymph nodes was determined 3 days after immunization, as described in *Materials and Methods*. The results are expressed as the fold increase in number in comparison with the number in mice that had not been immunized, and the error bars indicate the range for duplicate mice. *B*, CD25 expression on 2C lymph node cells 3 days after immunization. Essentially the same results were obtained in an independent experiment.

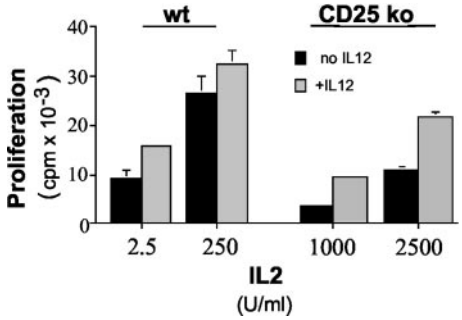

FIGURE 7. IL-12 can enhance proliferation independently of effects on CD25 expression. CD44^{low} CD8^{+} T cells from C57BL/6 wild-type or CD25$^{-/-}$ (ko) mice were stimulated with 2C11-coated microspheres, plus the indicated concentrations of IL-2, in the presence or absence of IL-12. Cell proliferation was measured at day 2 by [^{3}H]TdR incorporation.

the only mechanism by which it can contribute to proliferation and clonal expansion.

Discussion

Although the ability of IL-12 to augment CTL responses has been appreciated for some time (14, 43), its requirement as a critical third signal needed for activation of naive, but not memory, CD8 T cells has only recently become apparent (6, 8). This was first indicated by in vitro experiments examining responses to artificial APCs made by immobilizing class I Ag and B7-1 costimulatory ligands on microspheres at surface densities comparable with those on APCs (6). These artificial APC could effectively stimulate proliferation and development of cytolytic activity by memory (CD44^{high}) CD8 T cells from TCR transgenic mice, but were ineffective in stimulating naive (CD44^{low}) cells from the same mice. However, when IL-12 was added to the cultures, the naive cells mounted a vigorous response to the microspheres and developed potent cytolytic activity. Addition of IL-2 to the cultures eliminated the need for B7-1 on the microspheres, but the response remained completely dependent upon addition of IL-12.

Subsequent studies of in vivo responses supported the conclusion that naive CD8 T cells require a third signal that can be provided by IL-12 (7, 8). Using adoptive transfer of TCR transgenic T cells (44), it was shown that immunization with peptide Ag resulted in only weak clonal expansion and failure to develop lytic effector function (7). In contrast, immunization with peptide Ag in adjuvant stimulated strong clonal expansion and development of potent cytolytic activity. Administration of IL-12 along with peptide was found to be as effective as adjuvant in supporting both clonal expansion and development of function. IL-12 acts directly on the naive CD8 T cell in vivo, because it was effective when the adoptive transfer recipients were deficient in IL-12R expression, so that only the adoptively transferred CD8 T cells expressed the receptor and could respond to IL-12 (8). Furthermore, proliferative responses to IL-12 and peptide were largely dependent upon CD28 costimulation. Thus, as for in vitro responses, all three signals appear to be essential for in vivo activation of naive CD8 T cells. Interestingly, the CD8a^{+} DC are the subset of DC that are most effective in activating naive CD8 T cells, and are the DC that produce the highest levels of IL-12 in response to adjuvants or CD40 ligation (11, 12).

Although weak, some clonal expansion does occur in vivo in response to peptide in the absence of IL-12 or adjuvant, and the cells undergo several rounds of division, but do not develop cytolytic function (6, 7). Similarly, when nonphysiologically high levels of TCR engagement are achieved in vitro by using anti-TCR mAb or high levels of class I/peptide Ag complex, substantial proliferation of naive cells can occur in the absence of IL-12 without development of lytic function (Fig. 1, and data not shown). This made it possible to obtain sufficient numbers of viable cells cultured in the absence of IL-12 to allow comparison with cells grown in the presence of IL-12. Examination of CD25 was of particular interest, because the expression level of this receptor determines sensitivity to IL-2-dependent growth of T cells. Furthermore, IL-12 had previously been shown to increase CD25 expression levels on CD4 T cells and T cell clones (26–29). The significance of this is unclear, because IL-12 does not increase clonal expansion of CD4 T cells in response to Ag in vivo (13). Nevertheless, these

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observations suggested that IL-12 might have a similar effect on naive CD8 T cells, and thereby contribute to the enhanced proliferation and clonal expansion of these cells.

Some increase in CD25 expression by naive CD8 T cells occurred upon TCR engagement, and a further increase occurred in the presence of IL-2. The highest levels of CD25 protein and mRNA expression were achieved, however, when IL-12 was also present (Fig. 2). Furthermore, IL-12 makes a unique contribution, because the same CD25 expression level could not be achieved even at high levels of TCR engagement or IL-2 concentration (Fig. 4). CD28 engagement also increases CD25 expression levels on T cells (15), but again the highest levels of expression on CD8 T cells were only achieved upon addition of IL-12 (Fig. 5). This is consistent with the in vivo observation that IL-12 administration along with peptide Ag resulted in high levels of CD25 expression even when CD28 binding to B7 was prevented (Fig. 6). IL-12 also substantially extended the period of high CD25 expression, with levels declining after 24 h in the absence of IL-12, but persisting for 2–3 days in its presence (Fig. 3). IL-12-dependent increases in CD25 expression correlated well with increased proliferation of the cells in response to IL-2 (Figs. 3–5), and allowed substantial responses to occur at levels of IL-2 that stimulated little or no response in the absence of IL-12 (Fig. 4*D*). CD8 T cells make relatively little IL-2 in comparison with CD4 T cells (45), and the ability of IL-12 to enhance and prolong expression of CD25 may be critical for effective use of the IL-2 to support clonal expansion.

Although clonal expansion is weak and cytolytic function does not develop, the in vivo recognition of peptide Ag in the absence of a third signal is not without consequences. When mice are immunized with peptide and either adjuvant or IL-12, a long-lived memory population persists following the primary expansion and the cells respond rapidly to rechallenge with Ag (7). In contrast, the cells that persist long-term following immunization with just peptide are nonresponsive; they cannot be restimulated to respond to Ag in the presence or absence of adjuvant. Thus, it appears that the third signal not only determines whether an effective primary response occurs, but also dictates whether the cells that have recognized Ag become responsive memory cells or are rendered tolerant. There is evidence to suggest a model in which T cells may be rendered tolerant as a result of being weakly stimulated so that they begin proliferating, but fail to undergo a sufficient number of divisions to regain responsiveness to further stimulation (46). Thus, IL-12 might act as a third signal to prevent tolerance by up-regulating CD25 expression and thereby allowing proliferation to be sustained sufficiently for the critical number of cell divisions to occur.

The ability of IL-12 to increase and sustain CD25 expression at higher levels than can be achieved via TCR and IL-2 or CD28 indicates that IL-12R engagment activates one or more signaling pathways that contribute to CD25 induction. The highly regulated enhancer/promoter region that controls CD25 gene expression includes at least four positive regulatory regions that are the targets of several transcription factors (47). IL-2 activates the transcription factor STAT5 to bind to IFN-γ activation site motifs at positive regulatory regions III and IV in the CD25 promoter (47). IL-12 can also activate STAT5 in some T cells (48), and may contribute to CD25 up-regulation in this way. In addition, IL-12 induces STAT4 phosphorylation through activation of the mitogen-activated protein kinase kinase 6/p38 pathway (49). This pathway has recently been implicated as being important in IL-12-mediated increases in CD25 expression on CD4 T cells (28), and two STAT4-binding motifs upstream of CD25 were recently found in porcine PBMCs (50). Thus, IL-12 may contribute to up-regulation of CD25 expression by more than one signaling pathway.

It is very likely that up-regulation of CD25 contributes to the increased proliferation that occurs in the presence of IL-12. IL-12 has an additional effect, however, because it can also enhance proliferation of CD8 T cells from $CD25^{-/-}$ mice (Fig. 7). Although lacking a high affinity IL-2R, these cells can still respond to high concentrations of IL-2, and IL-12 enhances these responses. It is important to point out that IL-12 does not act independently as a growth factor for Ag-stimulated cells; very little or no proliferation occurs upon TCR engagement when IL-12 is present, but IL-2 is absent (Fig. 1*A*, and data not shown). One way in which IL-12 could enhance proliferative responses and clonal expansion beyond effects on CD25 would be through up-regulation of a survival factor(s), and this possibility is being investigated.

Although IL-12 only increases Ag-dependent proliferation of naive CD8 T cells when IL-2 is also present, it can dramatically increase homeostatic proliferation of these cells in an IL-2-independent manner (51). Homeostatic proliferation of CD8 T cells requires TCR recognition of self peptide/class I ligands, but is independent of IL-2 and costimulation (52, 53). Thus, IL-12 effects on both Ag-dependent and homeostatic proliferation of naive CD8 T cells depend upon TCR engagement, but only require IL-2 when the response is to Ag. Whether the IL-12-dependent pathway(s) involved in homeostatic proliferation is the same as that involved in CD25-independent enhancement of Ag-driven proliferation (Fig. 7) remains to be determined.

In addition to each cytokine increasing the expression of its own receptor, cross-regulation of CD25 and IL-12Rβ1/β2 expression by IL-2 and IL-12 has been demonstrated in CD4 T cells; IL-2 and/or B7 costimulation can increase the expression of IL-12R (22–25), and IL-12 can up-regulate CD25 over levels achieved by IL-2 or B7-1 (26–29). As mentioned above, the significance of CD25 regulation by IL-12 in CD4 cells is unclear, because IL-12 does not increase in vivo clonal expansion of these cells in response to Ag (44). Up-regulation of the IL-12R is important, however, for differentiation of the cells down the Th1 pathway in response to IL-12. The results described in this study demonstrate that a similar cross-regulation of CD25 and IL-12R by IL-2 and IL-12 occurs in naive CD8 T cells. In this case, however, this cross-regulation appears likely to be critical for driving clonal expansion, developing lytic effector function, and avoiding tolerance. As cross-priming DC present class I/Ag and costimulatory ligands, and produce IL-12, naive CD8 T cells will begin to produce IL-2, and both receptors will be optimally up-regulated to insure that an effective response is generated.

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