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CD4 Ligand IL-16 Inhibits the Mixed Lymphocyte Reaction[1]

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CD4 participation in TCR/CD3-associated activation through interaction with the MHC class II Ags results in formation of a CD4-TCR/CD3 complex capable of maximal signal transduction. When CD4 binds to alternative ligands such as HIV-1 gp120 or anti-CD4 Abs, Ag stimulation of TCR/CD3 is markedly inhibited, and an unresponsive state develops. To determine if the natural CD4 ligand interleukin-16 also induces unresponsiveness, we tested the effects of rIL-16 on T cell proliferation in mixed lymphocyte reactions. rIL-16 suppressed T cell proliferation in a dose-dependent manner at concentrations of 10^{-11} to 10^{-7} M. Inhibition of proliferation was present on days 5 to 9 of the mixed lymphocyte reaction. rIL-16 did not modulate membrane CD4, significantly change basal [^{3}H]thymidine incorporation in resting T lymphocytes, or alter viability. The suppressive effect was specifically blocked by preincubation with neutralizing anti-rIL-16 mAb or with recombinant soluble CD4. While the expression of IL-2R on responder cells was unaffected by rIL-16, the addition of exogenous rIL-2 did not restore T cell responsiveness. The unresponsiveness induced by rIL-16 is distinct from that of other CD4 ligands in that CD4 and IL-2R expression are unaffected. The failure of rIL-2 to restore proliferation suggests that the decrease in T cell responsiveness induced by rIL-16 may result from an interruption in the IL-2R-signaling mechanism. These results may help explain how CD4 delivers both activating and inhibitory signals and provides a rationale for the role of IL-16 in the regulation of immune responses. ***The Journal of Immunology,*** **1996, 157: 1958–1964.**

The discovery of CD4 as a receptor of the HIV-1 virus has led to increased scrutiny of the mechanisms of T cell unresponsiveness. Normally, maximal T cell activation and proliferation requires signaling through the TCR following the proper presentation of Ag to MHC class II Ags by APC (1–3). This process involves the formation of a CD4-TCR/CD3 complex, increased expression of IL-2R, and production of IL-2. Improper presentation by altered APC (4–6), inadequate costimulation (7–9), chronic exposure to excess Ag (10), or exposure to anti-CD3 mAbs (11–13) can result in T cell anergy. Anergic conditions share the feature of defective IL-2-related proliferation, which can be categorized into those in which proliferation recovers with exogenous IL-2 and those in which it does not. In the former group, exemplified by models of neonatal xenograft transplantation, anergy is difficult to induce (14, 15), while the latter group is characterized by a more stable and easily induced anergy such as that which occurs when allografting is performed across major and minor histocompatibility Ags (16–18).

Certain ligands that bind CD4 independent of CD3/TCR, such as HIV-1 envelope glycoprotein (gp120) and anti-CD4 Abs, can also induce anergy (19–24). Of interest, each of these ligands directly induces some early responses in resting T cells characteristic of activation, including chemotaxis (25, 26), activation of $p56^{lck}$ (27) and nuclear factor-κB (28), and a rise in intracellular inositol trisphosphate (IP_3)[3] and $[Ca^{2+}]_i$ although not all the functions or signals are common to all of the ligands. Mechanistically it has been demonstrated that anti-CD4 Abs as well as HIV-1 gp120 could down-modulate CD4 (17, 18, 29–35) and thereby inhibit both class II MHC-CD4 interactions (17, 29, 36, 37) and CD4-TCR/CD3 clustering (38). Moreover, these Abs appear to prime for apoptosis to a CD3 signal (39–42). In vivo, some of these Abs induce tolerance by opsonization-dependent clonal depletion of $CD4^+$ lymphocytes by reticuloendothelial cells. However, HIV gp120 and nondepleting anti-CD4 Abs exert their in vivo effects by the functional inactivation of T cells. Anti-CD4 Abs that produce clonal anergy rather than clonal deletion appear to be more effective in producing graft tolerance in animal models of transplantation and immunosuppression in patients with rheumatoid arthritis (36, 37, 43–45).

Our laboratory has described and cloned the natural human CD4 ligand, IL-16. IL-16 is secreted from $CD8^+$ lymphocytes following stimulation with either mitogen, histamine, or specific Ag. Initially described as a chemoattractant factor capable of inducing a migratory response in human $CD4^+$ lymphocytes (25, 46–48), monocytes (25), and eosinophils (49), IL-16 stimulation of resting $CD4^+$ lymphocytes also results in a transient increase in $[Ca^{2+}]_i$ and generation of IP_3 followed by an increase in cell surface expression of IL-2R (25). The activity of IL-16 is absolutely dependent upon the presence of membrane-expressed functional CD4 based on studies demonstrating that only $CD4^+$ cells are responsive to IL-16 stimulation (25, 48–51); that IL-16-induced signals are all inhibited by anti-CD4 Fab fragments (25, 46–51); that IL-16 can be immunoprecipitated by recombinant soluble CD4 (rsCD4) (51); that the association of CD4 with $p56^{lck}$ is essential for the IL-16-induced motile response (27), and finally that CD4 cDNA infection, with subsequent membrane expression of human

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[3] Abbreviations used in this paper: IP_3, inositol trisphosphate; rsCD4, recombinant soluble CD4; β-gal, β-galactosidase; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration.

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CD4 into L3T4-negative, IL-16-unresponsive, murine hybridoma cells imparts cellular responsiveness as evidenced by rises in $[Ca^{2+}]_i$ and IP_3 (50).

Since IL-16 shares agonistic effects on $CD4^+$ lymphocytes with anti-CD4 Abs and HIV-1 gp120, we investigated whether rIL-16 was also capable of inducing unresponsiveness to stimulation with allogeneic cells in mixed lymphocyte reactions. Unlike HIV-1 gp120, rIL-16 suppresses cell proliferation without modulating CD4 from the cell surface. The immunosuppressive effects are maximal when rIL-16 is added to the cells up to 24 h prior to cell activation and can still be observed when added after responder and stimulator cells are mixed. Although the induction of high affinity IL-2R on cells stimulated with allogeneic Ag is not affected by rIL-16, the inhibitory effect on the mixed lymphocyte reaction induced by rIL-16 is not rescued by the addition of exogenous IL-2.

Materials and Methods

rIL-16 and anti-rIL-16

rIL-16 and anti-rIL-16 Ab were generated as previously described (51). Briefly, a cDNA fragment containing the IL-16 open reading frame was generated by PCR and ligated into the *Escherichia coli* expression vector pET-16b. The IL-16-polyhistidine fusion protein was purified by metal-chelation chromatography and passed over a polymyxin B column to remove endotoxin (52). A control recombinant protein was generated in the same manner employing cDNA encoding for β-galactosidase (rβ-gal) and purified identically. Recombinant β-gal and rIL-16 were assayed for the presence of endotoxin using a BioWhittaker QCL 1000 LAL testing kit and stored in 7.5% glycerol with 0.0001 M HCl at −80°C. Quantification of protein was performed using a Bradford assay. All rIL-16 and rβ-gal contained less than 1.0 Endotoxin Us/ml of endotoxin and were used in experiments within 4 wk. Anti-rIL-16 Ab was generated by B cell hybridomas after immunization with rIL-16 using standard techniques (AGMED, Bedford, MA). Positive clones were identified by their ability to inhibit rIL-16-induced lymphocyte migration. Ab was purified by affinity chromatography using a rIL-16-protein A-Sepharose affinity column (Pharmacia Biotech., Uppsala, Sweden). After washing, the column was eluted with 50 mM citric acid, pH 2.5, and the pH was neutralized with the addition of 1/10 volume of 1 M Tris, pH 8.8. Total protein was quantitated by OD.

Cells and cultures

Human PBMCs were obtained from the venous blood of healthy normal human volunteers by density centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (30). T lymphocytes were selected by washing the PBMCs three times with medium 199 (M.A. Bioproducts, Walkersville, MD) supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubating on a nylon wool column for 45 min at 37°C in 5% CO_2 (53). The nylon wool nonadherent T lymphocytes eluted were >97% T lymphocytes as determined by fluorescent Ab staining with anti-CD3 mAb (Becton Dickinson, Mountain View, CA). Mixed lymphocyte reactions were performed using PBMCs (54). Stimulator cells were prepared by incubating cells at 10^6/ml with 25 μg/ml mitomycin C for 20 to 30 min. The cells were then washed four times with RPMI 1640 supplemented with 25 mM HEPES buffer, 100 U/ml penicillin, and 100 μg/ml streptomycin (RPMI 1640-HPS) and resuspended in RPMI 1640-HPS supplemented with 10% fetal bovine serum (complete medium) at 10^6 cells/ml. Responder PBMCs were prepared from an unrelated donor, suspended in complete medium at 10^6 cells/ml, and preincubated with 10^{-12} to 10^{-7} M rIL-16, identical dilutions of a rβ-gal or medium alone (control) at 37°C in 5% CO_2 for 1 h before the addition of stimulator cells. In some experiments, 10 to 100 μg/ml of anti-IL-16 Ab or 5 to 50 μg/ml rsCD4 (American Biotechnologies, Cambridge, MA) were incubated with rIL-16 or rβ-gal for 1 h at 4°C prior to the preincubation period. In other experiments, 10 to 100 U/ml of rIL-2 were added to experimental and control samples after 24-h incubation. Responder and stimulator cells were mixed in a 1:1 ratio and aliquoted into quadruplicate wells of 96-well round-bottom plates. Unless otherwise stated, the cells were pulsed with [^{3}H]thymidine on day 5, harvested with a Titretek cell harvester, and counted in a Becton Dickinson scintillation counter on day 6.

Table I. *Effects of rIL-16 on proliferation in resting T lymphocytes*

	Expt. 1[a]	Expt. 2	Expt. 3
Medium	4836 ± 1212[b]	3099 ± 636	8852 ± 1108
rβ-gal[c]	5604 ± 1598	3697 ± 285	8315 ± 1084
rIL-16[d]	5282 ± 1420	2902 ± 778	7980 ± 1198

[a] Pretreated T lymphocytes were cultured for 5 days, pulsed with [^{3}H]thymidine for 18 h, and harvested.
[b] Mean ± SD cpm from four quadruplicate wells for each sample.
[c] Equal concentration to rIL-16 based on protein quantity.
[d] 10^{-7} M.

Detection of surface Ags and receptors

CD4 expression was analyzed using fluorescein-conjugated OKT4, and phycoerythrin-conjugated OKT4A mAbs (Ortho Diagnostics). For detection of IL-2R, cells were stained with fluorescein-conjugated anti-CD25 Ab (Becton Dickinson) and phycoerythrin-labeled OKT4 Ab. Cells from appropriate cultures were washed, resuspended in PBS, pH 7.4, with 1% azide and incubated with 0.25 μg labeled Ab for 30 min at 4°C. Cells were then washed three times in cold PBS, pH 7.4, resuspended at 1×10^6 cells/ml, fixed with 10% formalin, and stored in the dark at 4°C until analysis with a Becton Dickinson FACScan as previously described (30).

Results

rIL-16 does not stimulate DNA synthesis in resting PBMCs

To determine the effects of rIL-16 on unstimulated cells, PBMCs were cultured either with rIL-16 (10^{-7} M), a similar dilution of rβ-gal (used as a negative control for the *E. coli* expression and purification steps), or complete medium alone (control) for 5 days, then pulsed with [^{3}H]thymidine for 18 h and harvested. No significant difference in [^{3}H]thymidine uptake was seen between medium alone, rβ-gal, or rIL-16 (Table I). From days 3 to 7, aliquots of cells from each group were assessed for viability as determined by trypan blue exclusion. Over the observation period, cell viability gradually decreased in all groups, but there was no significant difference between the groups (range 78 to 86%).

rIL-16 inhibits the mixed lymphocyte reaction

We next examined the effect of rIL-16 on the mixed lymphocyte reaction. rIL-16 was used in a concentration dose range (10^{-12} to 10^{-7} M) which was reported previously to induce lymphocyte migration (51). As shown in Figure 1, rIL-16 added 1 h before the cells were mixed inhibited lymphocyte proliferation in a concentration-dependent fashion compared with control medium (defined as 100%). No significant effect was observed with concentrations below 10^{-11} M.

To determine the specificity of the inhibitory effect, neutralizing anti-rIL-16 mAb (5×10^{-9} M, sufficient to neutralize 10^{-8} M rIL-16 chemotactic bioactivity) was preincubated with 10^{-12} to 10^{-8} M rIL-16 prior to the addition of responder cells. This concentration of anti-rIL-16 Ab completely inhibited the effects of rIL-16 ($p < 0.01$). As expected from the stoichiometry, a 10-fold higher concentration of anti-rIL-16 Ab was required to inhibit the effects of 10^{-7} M rIL-16 (53 ± 3% vs 84 ± 8%, $p = 0.0045$). Similar concentrations of isotype control Abs specific for human CD8 Ag did not affect rIL-16-induced inhibition (data not shown). In addition, we preincubated rIL-16 with rsCD4 (5 to 50 μg/ml). The inhibition of lymphocyte proliferation observed with 10^{-7} M rIL-16 was blocked (114 ± 11%, $p = 0.0002$) with a 10-fold excess of rsCD4 (50 μg/ml; 10^{-6} M). A total of 5 μg/ml (10^{-7} M) of rsCD4 was required to block the inhibition induced by all lower concentrations of rIL-16 ($p < 0.01$).

Kinetics of rIL-16 inhibition of mixed lymphocyte reaction

Since we harvested cells after 6 days' incubation, it was possible that the effect exerted by rIL-16 represented a delay in responder

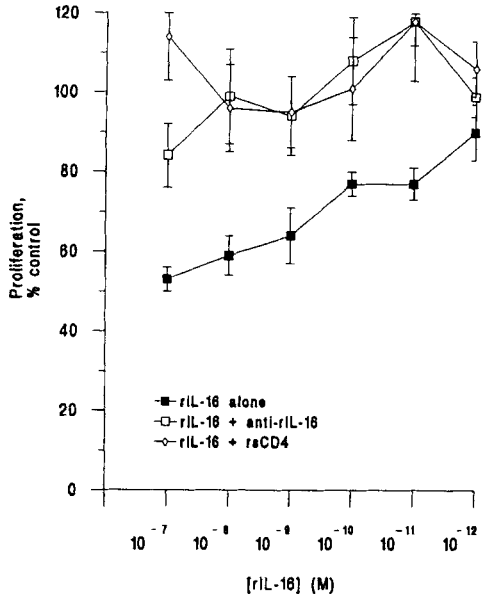

FIGURE 1. Inhibition of the mixed lymphocyte reaction by rIL-16 and reversal of effects by anti-rIL-16 Ab and rsCD4. Responder cells were incubated with 10^{-12} to 10^{-7} M rIL-16 for 1 h before the addition of stimulator cells. Mixed lymphocyte reactions (MLR) were incubated for a total of 6 days with an 18-h [^{3}H]thymidine pulse. The data, expressed as a percent of proliferation compared with medium control (defined as 100%) was determined by the formula:

$$\% \text{ Proliferation} = \frac{\text{cpm of experimental MLR} - \text{cpm of responder}}{\text{cpm of control MLR} - \text{cpm of responder}} \times 100.$$

Parallel dilutions of rβ-gal did not produce significant variations from medium control (±6%). Blocking studies were performed by preincubating rIL-16 with 100 μg of anti-rIL-16 and 50 μg rsCD4 for the 10^{-7} M concentration, and 10 μg of anti-rIL-16 and 5 μg rsCD4 for all other concentrations prior to incubation with responder cells. The concentration-dependent inhibition of the mixed lymphocyte reaction produced by all concentrations of rIL-16 ($p < 0.001$) except 10^{-12} M (90 ± 7%, $p = 0.2$) was significantly reversed by anti-rIL-16 Ab ($p < 0.01$) and rsCD4 ($p < 0.01$). The means ± SE from six experiments are shown. Statistical significance was determined by a paired Student *t* test.

cell proliferation such that the suppression of proliferation observed at day 6 would be absent later on. Conversely, rIL-16 could have augmented the proliferative response so that the suppression of proliferation represented "burn out" of the responder cells. To determine whether the effect of rIL-16 was delaying or accelerating the normal lymphocyte response, we performed mixed lymphocyte reactions in the presence of rIL-16, rβ-gal, or medium alone and harvested them serially from 3 to 9 days. There was no significant difference in cell proliferation observed between rβ-gal or medium (data not shown). However, rIL-16 treatment resulted in diminished [^{3}H]thymidine incorporation on days 5 to 9 (Fig. 2).

Time dependence of rIL-16 inhibition of mixed lymphocyte reaction

The CD4 ligands HIV-1 gp120 and anti-CD4 Abs induce reversible effects on CD4-mediated functions, which recover if the stimulus is removed. To determine if the effect caused by rIL-16 is reversible, 10^{-7} to 10^{-9} M rIL-16 was cultured with responder PBMCs for 24 h (−24 h) and 1 h (−1 h) before the addition of stimulator cells, and 1 h (+1 h) and 24 h (+24 h) after responder and stimulator cells were mixed. Figure 3 shows that the inhibition of proliferation seen at −1 h and +1 h are not significantly different for any concentration of rIL-16 added. At −24 h, there was no significant effect on proliferation with 10^{-9} M (108 ± 5%); there was minimal, but significant, inhibition with 10^{-8} M rIL-16 (85 ± 3%, $p < 0.01$); and 10^{-7} M rIL-16 inhibited proliferation to an amount comparable with that observed when added at −1 h. After initiation of the mixed lymphocyte reaction (+24), rIL-16 did not significantly inhibit proliferation at any concentration, although the reduction seen with 10^{-7} M rIL-16 approached significance (78 ± 11%, $p = 0.12$).

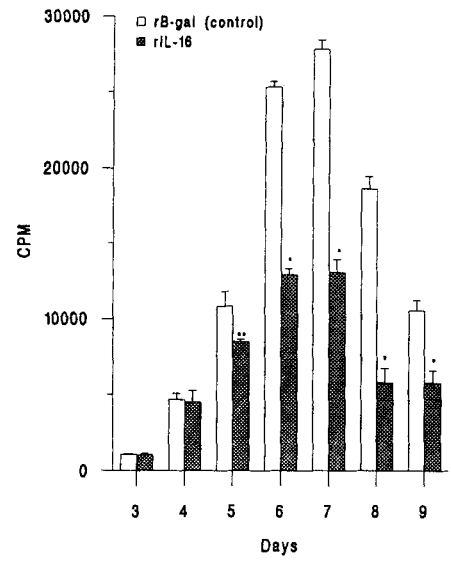

FIGURE 2. Kinetics of rIL-16 inhibition. Responder cells were incubated with 10^{-7} M rIL-16 or rβ-gal (control) 1 h before the addition of stimulator cells. Aliquots of cells were pulsed with [^{3}H]thymidine on days 2 through 8 and harvested on the following day (days 3 through 9, respectively). The ordinate represents cpm (mean ± SD) from quadruplicate wells. Statistical significance between control and rIL-16-treated samples was determined by a paired Student *t* test. (* = $p < 0.01$, ** = $p < 0.05$). One representative experiment of three is shown.

Exogenous IL-2 does not reverse rIL-16 inhibition of the MLR

We next examined whether we could restore proliferation with exogenous rIL-2. In Figure 4, we compare the proliferation of 10^{-9} to 10^{-7} M rIL-16 with and without the addition of rIL-2. No significant recovery of proliferation was observed using 10 U/ml rIL-2. Higher concentrations of rIL-2 further reduced proliferation (data not shown). The absence of IL-2 recovery was not due to a decrease in the expression of high affinity IL-2 R, since preincubation with rIL-16 did not change the cell surface expression on responder cells (Table II).

rIL-16 does not modulate CD4 expression

Incubation of CD4$^+$ lymphocytes with HIV-1 gp120 or certain immunosuppressive anti-CD4 Abs results in a rapid loss of membrane-expressed CD4 (22, 23, 30–35). It is theorized that this modulation of CD4 contributes to the observed immunosuppressive effect and priming for apoptosis observed with intact HIV-1. We investigated whether rIL-16 stimulation had a similar effect on membrane-expressed CD4. Since OKT4 Fab fragments block the effects of both natural and rIL-16, suggesting close proximity of the OKT4 and rIL-16 binding sites, we evaluated the effect of IL-16 stimulation on CD4 expression using an Ab that binds to a different domain on CD4, OKT4A. The relative amount of OKT4A labeling indicates the relative amount of membrane-expressed

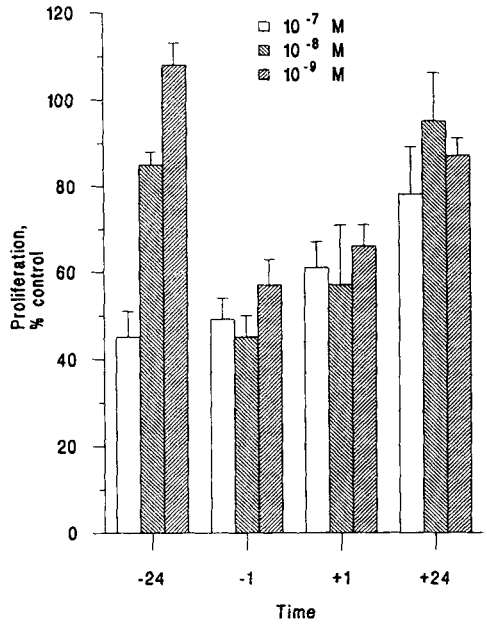

FIGURE 3. Temporal relationship of rIL-16 administration to suppression of the mixed lymphocyte reaction. Responder cells were incubated with 10^{-9} to 10^{-7} M rIL-16 for 24 h (−24 h) or 1 h (−1 h) before the addition of stimulator cells and 1 h (+1 h) or 24 h (+24 h) after responder and stimulator cells were mixed. Cultures were incubated for a total of 6 days from the time stimulator cells were added with an 18-h [^{3}H]thymidine pulse. The data, expressed as a percent of proliferation compared with control medium (defined as 100%), was determined as in Figure 1. A concentration of 10^{-7} M significantly inhibited proliferation at −24, −1, and +1 h ($p < 0.001$) but not at +24 h. Significant inhibition was also seen at −24 h with 10^{-8} M rIL-16 ($p < 0.01$) but not with 10^{-9} M. No concentration of rIL-16 significantly inhibited proliferation at +24 h. The means ± SE from three experiments are shown. Statistical significance was determined by a paired Students *t* test.

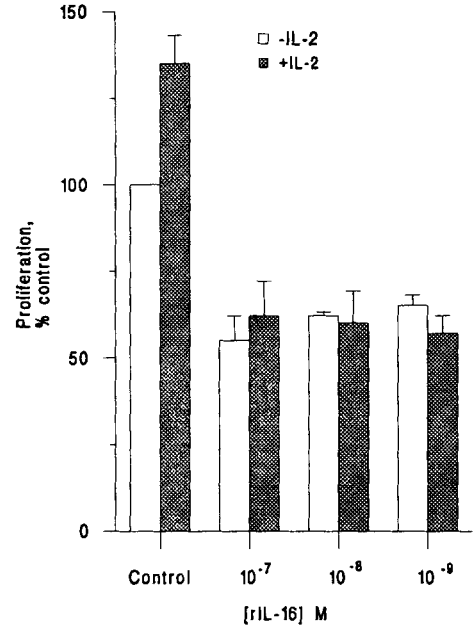

FIGURE 4. IL-2 does not induce recovery from rIL-16 inhibition. Responder cells were incubated with 10^{-9} to 10^{-7} M rIL-16 for 1 h before the addition of stimulator cells. After 24 h, 10 U/ml of rIL-2 were added and the reaction was allowed to proceed for another 5 days with an 18-h [^{3}H]thymidine pulse. The data, expressed as a percent of proliferation compared with control medium (defined as 100%), were determined as in Figure 1. rIL-2 increased the proliferative response of control cells ($p < 0.01$) but failed to reverse the inhibition in cells treated with rIL-16. The means ± SE from four experiments are shown. Statistical significance was determined by a paired Student *t* test.

CD4, while the relative amount of decrease in OKT4 labeling indicates the presence of rIL-16 bound to CD4. Figure 5 shows the effect of 10^{-7} M rIL-16 on the expression of membrane-expressed CD4. The relative amount of OKT4A labeling during the 5 days remained constant indicating that rIL-16 had no effect on surface expression of CD4. The decrease in OKT4 labeling at 2 to 6 h following addition of rIL-16 suggests some binding competition with OKT4 Ab consistent with our previous reports that OKT4 Fab fragments inhibit IL-16-induced chemotaxis and IL-2R expression (25, 48). The decrease in OKT4 binding diminishes by 24 h.

Discussion

The regulation of $CD4^+$ lymphocyte responsiveness to Ag is markedly altered by accessory molecular interactions involving CD4 and its association with the TCR/CD3 complex. A dual role for CD4 has been well established. When T cells are stimulated by Ag presented by MHC class II molecules, cell activation and proliferation are amplified. However, when CD4 is bound independent of Ag by alternate ligands (e.g., HIV-1 gp120 or certain anti-CD4 Abs) subsequent TCR/CD3-mediated proliferation is suppressed. In this study we demonstrate reversible inhibition of allogenic-induced proliferation using a recombinant form of IL-16, a naturally occurring CD4 ligand with activating properties unrelated to MHC class II Ags. The decrease in Ag responsiveness occurs without loss of cell surface CD4 and is not a result of altered kinetics of response. There is no effect on IL-2R expression or reversal by the addition of exogenous IL-2.

The expression of high-affinity IL-2R unresponsive to exogenous IL-2 in allogenic stimulated cells pretreated with rIL-16 in these studies suggests the IL-2R signaling pathway may be suppressed. Although the absence of rIL-2 recovery with normal IL-2R has been reported when the mixed lymphocyte reaction is blocked by splenocytes following total lymphoid irradiation (55), this is not the case when anti-TCR/CD3 polyclonal Ab-induced proliferation is inhibited by HIV-1 gp120 (19, 56, 57). This disparity may relate to the down-modulation of CD4 from the cell surface by HIV-1 gp120 (30–33) or may result from differences in binding sites on CD4. The latter hypothesis may explain the differences in T cell responses following binding of anti-CD4 Abs directed at different epitopes of CD4 (58). HIV-1 gp120 interacts at the D1 and D2 loci and may competitively inhibit MHC class II Ags (59, 60) while rIL-16 appears to interact with CD4 near the epitope that binds OKT4 Ab.

Since the full spectrum of rIL-16-induced, CD4-dependent responsiveness requires tetramerization (51, 61, 62), we suspect that some of the differences between the rIL-16 suppression of the mixed lymphocyte reaction and that observed following anti-CD4 Abs and HIV-1 gp120 may also be related to its ability to cross-link CD4 into tetrameric aggregates. Some anti-CD4 Abs, when cross-linked, prime cells for apoptosis when the TCR/CD3 complex is stimulated (39, 40, 63), a phenomenon that has also been reported with HIV-1 gp120 under some conditions (39, 64–68) but not in others (19, 41). We have no evidence that IL-16 primes cells for apoptosis. In fact, this appears unlikely considering the normal expression of IL-2R on stimulated $CD4^+$ cells and the

Table II. *Effects of rIL-16 on IL-2R expression in resting and stimulated T cells*

	Resting[a]	Stimulated[b]	Stimulated + rIL-16[c]	% Proliferation
Experiment 1[d]				
$CD4^+$	10.1[e]	32.7 (94)[f]	30.3 (96)	68[g]
$CD4^-$	1.9	5.5	4.3	
Experiment 2				
$CD4^+$	5.4	27.4 (129)	29.7 (128)	62
$CD4^-$	0.9	7.4	4.5	
Experiment 3				
$CD4^+$	6.1	33.6 (114)	30.3 (116)	70
$CD4^-$	1.9	4.3	4.9	

[a] Responder PBMC were cultured alone for 5 days, harvested and stained for CD4 and IL-2R.

[b] Responder and stimulator PBMC, cultured and stained under identical conditions.

[c] 10^{-9} M.

[d] Gates were set around the lymphocyte population of the control MLR based on forward and side scatter characteristics from FACScan analysis. Identical gating was used to compare resting T cells and MLR cultures containing rIL-16. Gating eliminated >99% of stimulator cells from analysis.

[e] Percent of the total gated population staining positively for IL-2R. The average of the percentage of IL-2R expression for stimulated cells cultured alone (31.2 ± 2.7), or with rIL-16 (30.1 ± 0.3) is not statistically different ($p = 0.59$).

[f] Mean fluorescence intensity of IL-2R+ cells.

[g] Expressed as percentage of proliferation of rIL-16 cells compared with control cells in a parallel MLR where:

$$\% \text{ Proliferation} = \frac{\text{cpm of experimental MLR} - \text{cpm of responder}}{\text{cpm of control MLR} - \text{cpm of responder}} \times 100$$

decreased expression of CD95 noted in response to IL-16.[4] Rather, we suspect the aggregation of CD4 into tetramers by IL-16 uncouples CD4 from the TCR and makes tetramerization with MHC II molecules impossible, resulting in the loss of mixed lymphocyte reaction-dependent proliferative activity (69–72). Our experiments do not eliminate the potential immunosuppressive effect of rIL-16-induced cytokines on the MLR, nor did we address the effects of rIL-16 on IL-2 synthesis during the mixed lymphocyte reaction. As regards the latter, prior studies have shown that IL-16 does not directly affect IL-2 synthesis (50), and the lack of recovery following addition of exogenous IL-2 makes this an unlikely complete explanation for our data.

We found that cells pretreated 24 h before initiation of the mixed lymphocyte reaction could still be inhibited from proliferating, but that the dose-response curves had shifted; lesser concentrations of rIL-16, which suppressed proliferation at −1 h, did not do so at −24 h. Interestingly, adding rIL-16 1 h after the mixed lymphocyte reaction was initiated suppressed in a fashion that was indistinguishable from instances when cells were pretreated for 1 h. However, we were unable to significantly suppress the mixed lymphocyte reaction once the reaction had been in progress for 24 h. These data suggest that the immunosuppressive effects of rIL-16 are dependent upon its presence near the time of initial Ag presentation and TCR/CD3R costimulation before complete commitment of the downstream events following the immediate second messenger cascade has occurred (73). The suppression of proliferation is reversible in that the response to previously unstimulated TCR/CD3 returns to normal once CD4 is no longer bound with rIL-16. On the other hand, suppression is continuously observed throughout the time course of the mixed lymphocyte re-

[4] W. W. Cruikshank, A. C. Theodore, K. Lim, J. Cook, G. Fine, P. F. Weller, and D. M. Center. CD3-dependent activation and proliferation is suppressed by interleukin-16. *Submitted for publication.*

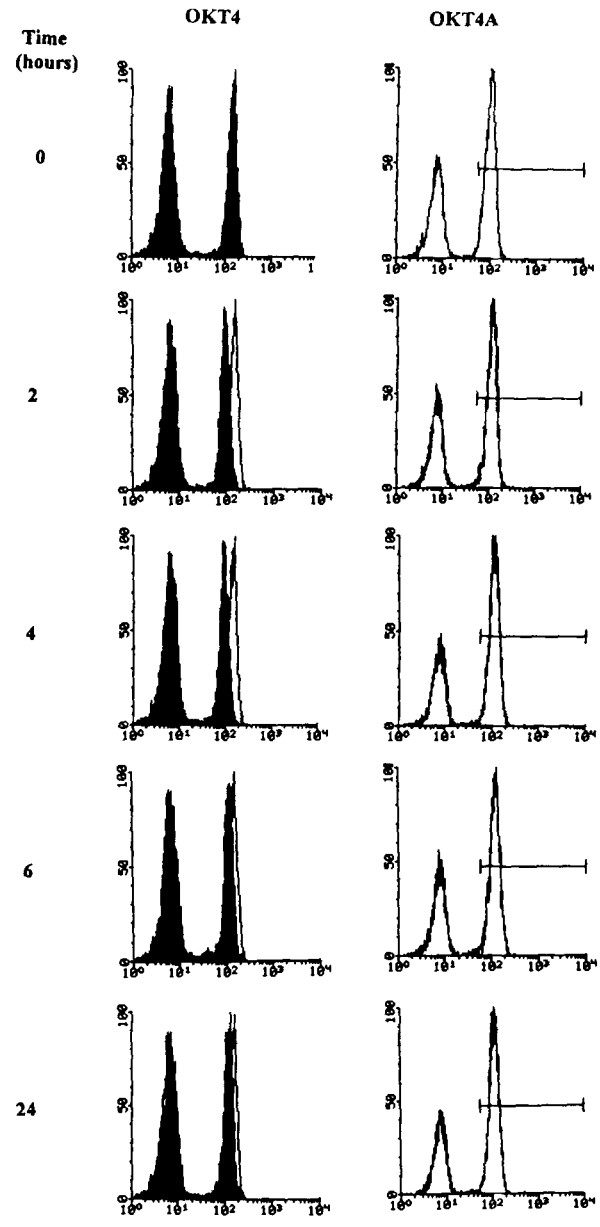

FIGURE 5. Detection of epitope-specific binding of rIL-16 to CD4. Histograms were obtained by FACScan analysis of T cells stained with FITC-conjugated OKT4 or OKT4A Ab following incubation with 10^{-7} M rIL-16 for 2, 4, 6, and 24 h. Gates were set around the lymphocyte population, and a total of 5000 cells were analyzed. Linear green fluorescence (*x*-axis) is plotted against relative cell number (*y*-axis) for each panel of the composite. A decrease in fluorescence indicates a decrease in detectable surface Ag and is reflected as a shift to the left on a histogram. The 0 h time point depicts CD4 expression as determined by OKT4 and OKT4A staining before the addition of rIL-16. At each subsequent time point for OKT4, the solid histograms represent CD4 expression of cells incubated with rIL-16 compared with CD4 expression of untreated cells harvested at the same time points (overlays). For OKT4A, the overlays of control cells are superimposed on rIL-16-treated cells shown by open histograms, but no shift is seen compared with the reference line (bar). The difference in OKT4 staining compared with OKT4A indicates displacement by a competing molecule (rIL-16) proportional to the amount of competitor bound (30, 56). The percent of positively stained cells for both OKT4 and OKT4A ranged from 50 to 60% in all experiments. One representative experiment of three is shown.

action if TCR/CD3 stimulation occurs when CD4 is bound to rIL-16.

The essential role of CD4 as a sentinel receptor is demonstrated by the effects observed following interaction with rIL-16, as compared with Ag presentation by MHC-bearing APC. Thus, when CD4 is engaged by MHC with proper Ag presentation, amplification of the TCR/CD3 response occurs; CD4 associates with TCR/CD3, IL-2R are expressed, and a proliferative signal is propagated after interaction with IL-2. However, when CD4 is engaged by rIL-16, the proliferative response to a MHC interaction via the TCR/CD3 complex is reversibly suppressed. It is likely that this effect is mediated at multiple levels, including extracellular occupancy of CD4 and receptor second messenger signal cross-desensitization. Since the interaction of rIL-16 with CD4 in the absence of TCR/CD3 stimulation results in a proinflammatory motile state, CD4 (and IL-16) may play a role in commitment to the essential T cell functions of clonal expansion, tolerance, or inflammation.

Although the regulatory role of CD4 on TCR/CD3-mediated proliferation has been well documented (69–71, 74, 75), previous studies have been able to employ only anti-CD4 Abs or preparations of HIV-1 gp120 as ligands for CD4. Our investigation is the first to be performed with a recombinant form of a natural CD4 ligand and corroborates prior observations utilizing the alternative CD4 ligands. The induction of functional unresponsiveness demonstrated with rIL-16 may elucidate the roles of natural IL-16 and provide a mechanism by which CD4 regulates CD3 in vivo.

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