CD4-Mediated Inhibition of IL-2 Production in Activated T Cells

Madeleine Bonnard,* Loralee Haughn,† and Michael Julius2*

The role of CD4 in T cell activation has been attributed to its capacity to increase the avidity of interaction with APC and to shuttle associated Lck to the TCR/CD3 activation complex. The results presented in this study demonstrate that ligation of CD4 inhibits ongoing responses of preactivated T cells. Specifically, delayed addition of CD4-specific mAb is shown to inhibit Ag- or mAb-induced responses of both primary T cells and T cell clonal variants. The Ag responses of the latter are independent of the adhesion provided by CD4; thus the observed inhibition is not due to blocking CD4-MHC interactions. Further, analysis of the clonal variants demonstrates that CD4-associated Lck is not essential for the inhibition observed, as anti-CD4 inhibits responses of clonal variants, expressing a form of CD4 unable to associate with Lck (double cysteine-mutated CD4). The inhibition is counteracted by the addition of exogenous IL-2, demonstrating that the block is not due to a lesion in IL-2 utilization, rather its production. It is demonstrated that the delayed addition of anti-CD4 results in a rapid reduction in steady-state levels of IL-2 mRNA in both primary T cells and clonal variants. The Journal of Immunology, 1999, 162: 1252–1260.

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tigen-specific activation of mature CD4+ T cells is initiated upon TCR recognition of antigenic peptides presented in the context of MHC class II molecules. Elements of the CD3 complex and ζ-chains, associated with TCR, couple Ag recognition to cellular-signaling elements that direct a cascade of biochemical events (1, 2). These ultimately lead to the de novo production of the major T cell growth factor, IL-2, and to cellular growth (3, 4). A number of accessory activation molecules, although not affecting the specificity of T cells, modify the signals emanating from the TCR/CD3 complex, and thus the cellular outcome of Ag recognition. The consequences of these modifications can be extreme, resulting in cell growth, nonresponsiveness, or death. The coreceptor molecule, CD4, plays a critical role in this regard, as anti-CD4 has been demonstrated to abrogate both Ag- and anti-TCR-induced T cell activation (5, 6). The former results may reflect the role of CD4 in enhancing the avidity of the T cell-APC interaction, thus abrogating the activation process. However, the use of anti-CD4 to block anti-TCR/CD3-mediated T cell activation in the absence of APC-presenting MHC class II led to the suggestion that CD4 may deliver inhibitory signals to T cells (5).

Upon T cell Ag recognition, the extracellular domain of CD4 interacts with the same MHC ligand as TCR (7, 8), resulting in the coaggregation of TCR/CD3 with CD4 (9). Thus, CD4 can function to increase the avidity of the T cell-APC interaction (10). Further, Ab-mediated coaggregation of TCR/CD3 with CD4 greatly enhances cell growth compared with that induced by aggregating TCR/CD3 alone (11–13). It was this latter finding that first suggested a role for CD4 in generating signals that contribute to T cell activation. The capacity of CD4 to enhance or alter signals emanating from TCR/CD3 has been attributed to its association with the Src family protein tyrosine kinase Lck (14–18). Approximately 75–95% of cellular Lck in MHC class II-restricted T cells associates with the cytoplasmic portion of CD4, involving ~85–95% of CD4 (19). This noncovalent association is mediated by two sets of cysteine residues, one present on the membrane-proximal portion of the cytoplasmic domain of CD4, and the other on Lck itself (20). Importantly, Ab-mediated aggregation of CD4 alters the phosphotyrosyl content of associated Lck on specific tyrosine residues, which in turn is associated with increased kinase activity (15, 21). Therefore, the enhanced responsiveness observed upon coaggregating CD4 with TCR/CD3 could be a consequence of juxtaposing CD4-associated Lck, presumably activated, and signaling molecules present at the site of the Ag receptor complex. Therefore, anti-CD4-mediated inhibition of T cell activation could involve both the reduction of the avidity of T cell-APC interaction as well as blocking the delivery of critical Lck-mediated function.

We have developed a number of T cell clonal variants that provide further insight into the contribution of CD4/Lck complexes to the outcome of Ag-mediated T cell activation. The Ag receptor signaling phenotypes of CD4+ and CD4− variants of an IL-2-dependent, OVA-specific T cell clone have been previously described (18). Briefly, both variants respond comparably to Ag, indicating that the Ag response is not dependent on the increased avidity supported by the expression of CD4. However, only the CD4− variant responds to mAbs specific for TCRβ. Further, the forced expression of wild-type (wt)3 CD4, but not double cysteine mutated (DC) CD4, unable to bind cellular Lck in CD4− variants, rendered cells unresponsive to mAbs specific for TCRβ. Thus, it is not the expression of CD4 per se that disables mAb-mediated responses in these variants, rather the capacity of CD4 to bind cellular Lck. This in turn suggests that CD4-mediated inhibition of

3 Abbreviations used in this paper: wt, wild type; DC, double cysteine mutated; NEO, neomycin resistance gene; IMDM, Iscove's modified Dulbecco's medium.

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responses to TCRβ-specific mAbs is due to its capacity to sequester the majority of cellular Lck. Thus, when CD4 is not coaggregated with TCR/CD3 as it is in the presence of Ag, the generation of prerequisite activation signals is prevented (18). Furthermore, the original characterization of these CD4⁺ and CD4⁻ clonal variants demonstrated that the Ag response of the CD4⁺ variant is susceptible to anti-CD4-mediated inhibition (18). Because the Ag response of this variant is not dependent on the increased avidity supported by CD4 expression, it was thought that the observed inhibition is likely due to the interference of the juxtaposition of CD4-associated Lck with the TCR/CD3 complex.

We have addressed this issue in the present study. Specifically, if the capacity of anti-CD4 to inhibit the Ag response of the CD4⁺ clonal variant is due to disrupting the delivery of Lck-derived signals, the prediction follows that anti-CD4 should not inhibit the Ag response of clonal variants expressing DC CD4, because it is unable to interact with cellular Lck. The unexpected result is that this is not the case. Rather, Ag responses of cells expressing either wt or DC CD4 are inhibited over a broad range of anti-CD4 concentrations. Moreover, the efficacy of anti-CD4-mediated inhibition is not reduced when it is added to culture after the initiation of the response. This finding suggests that CD4 ligation subsequent to cellular activation, and indeed the onset of growth, transmits a negative signal(s). Because the same phenotype is observed, albeit at lower intensity, in clonal variants expressing DC CD4, transduction of this signal(s) is at least in part independent of associated Lck. Toward characterizing the molecular basis of the anti-CD4-mediated inhibition observed, it is demonstrated that the addition of exogenous IL-2 rescues responses. Thus, CD4 ligation subsequent to the induction of cellular growth impairs processes regulating IL-2 production, rather than its utilization. Within 1 h of addition, anti-CD4 is shown to reduce steady-state levels of IL-2 mRNA more than 10-fold in Ag-activated wt CD4 clonal variants. Importantly, this novel characteristic of CD4 function is shown to apply to both Ag- and anti-TCR-mediated activation of primary T cells. Thus, the results extend the current paradigm for the function of CD4. In addition to supporting initial activation events emanating from the Ag receptor complex, CD4 may play a central role in mechanisms regulating T cell homeostasis.

Materials and Methods

Animals

At 6–8 wk of age, BALB/c and C57Bl/6 male mice were purchased from The Jackson Laboratories (Bar Harbor, ME). OVA-specific TCR transgenic mice (22), kindly provided by Dr. D. Loh (Washington University, St. Louis, MO), were housed and bred in our animal facility.

Abs and reagents

mAbs used for T cell stimulation were affinity purified on Protein A-Sepharose (Pharmacia, Baie d’Urfé, Québec, Canada), and include mAbs specific for TCRβ (H57.597; Ref. 23) and CD3ε (145.2C11; Ref. 24). mAbs used in complement-mediated lysis were used as culture supernatants and include mAbs specific for CD4 (RL.172.4H; Ref. 25), CD8 (3.168; Ref. 26), and Thy-1.2 (H013.4.9–2; Ref. 27). mAbs used in proliferation assays were either affinity purified on mouse anti-rat-IgG (MARK-1; Ref. 28) conjugated Sepharose 4B (Pharmacia), including mAbs specific for CD4 (H129; Ref. 29) and MHC class I (M1.42; Ref. 30), or affinity purified on protein A-Sepharose, including mAb specific for CD28 (37.51; Ref. 31). Affinity-purified normal syrian hamster IgG was purchased from Bio/Can Scientific (Mississauga, Ontario, Canada). Phycoerythrin-conjugated anti-CD4 (GK1.5) was purchased from Becton Dickinson (Mountain View, CA). Rabbit anti-Lck was generated by immunizing with an Lck peptide composed of the N-terminal residues 39–64, coupled to keyhole limpet hemocyanin. Rabbit anti-Lck used in precipitations was purified from immune serum using Protein A-Sepharose (Pharmacia) and immune serum was used for immunoblotting analysis.

Cell preparation and in vitro culture

Primary T cells were isolated from lymph nodes of mice as previously described (32). Briefly, lymph node cell suspensions were incubated with CD8-specific antiisera (Cytovax, Edmonton, Alberta, Canada). B cells and CD8⁺ T cells were depleted by negative selection by passing labeled cells over anti-Ig columns (Cytovax). The resulting populations were >95% TCRβ⁺ and CD4⁺, and <1% mIg⁺.

T cell-depleted APC were obtained from syngeneic splenocytes. Splenocytes were treated with mAbs described above, specific for CD4, CD8, and Thy-1.2, and guinea pig complement (Cedarlane, Hornby, Ontario, Canada). Cells were subsequently fractionated on discontinuous Percoll gradients comprised of ρ = 1.109, ρ = 1.066, and ρ = 1.00. Cells banding at the p = 1.109/ρ = 1.066 interface were harvested and irradiated (2000 rad).

The OVA-specific, IL-2-dependent CD4⁺ clone 2.10 and infections have been previously described (18). Briefly, the CD4⁺ clone 2.10 was infected with a retrovirus containing the neomycin resistance gene (NEO) alone, or in addition to either the murine cDNA encoding for wt CD4 or DC CD4. Clonal variants were maintained in serum-free Iscove’s modified Dulbecco’s medium (IMDM) containing 3 U/ml rIL-2 in the form of supernatant, and 1% soy bean lecithin (18). This medium was supplemented with 600 μg/ml active G418 (Life Technologies, Burlington, Ontario, Canada) for the propagation of the various infectants.

In proliferation assays involving T cell clonal variants, cells were harvested and washed twice in unsupplemented serum-free IMDM. T cells (5 × 10⁵) and irradiated (2000 rads) splenocytes (5 × 10⁵) or T cell-depleted splenocytes (2.5 × 10⁵), as indicated in the figure legends, were cocultured in the absence of IL-2 in a final volume of 200 μl of serum-free IMDM. Cultures were stimulated with either 100 μg/ml OVA, or 1 μg/ml OVA-receptor peptide, residues 143–157 (OVA143-157), in the presence or absence of the indicated mAbs added at either initiation of the cultures, or 12 or 18 h later, as indicated in the figure legends. Alternatively, cultures were stimulated with anti-TCRβ or anti-CD3e. At 40 or 48 h, as indicated in the figure legends, cultures received 1 μCi [³H]thymidine; 6 h later, they were collected onto filter mats and thymidine uptake was assessed by liquid scintillation spectroscopy.

Two protocols were employed to induce the proliferation of primary T cells. Primary CD4⁺ lymph node T cells were isolated from OVA-specific TCR-transgenic mice, as described above. A total of 5 × 10⁵ T cells and 10⁵ irradiated (2000 rads) syngeneic T cell-depleted splenocytes were cocultured in 200 μl of unsupplemented serum-free medium. Cultures were stimulated with 0.001 μM of specific OVA-derived peptide, residues 323–339 (OVA323-339), a kind gift of Dr. Patrice Hugo (Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada) and 12 h later received either anti-CD4 mAb H129 or anti-MHC class I mAb M1.42. Alternatively, wells of 96-well flat-bottom tissue culture plate (Nunc, Burlington, Ontario, Canada) were coated directly with 50 μl of an HBSS solution containing 1 μg/ml anti-TCRβ mAb for 1 h at 37°C. After two washes with 100 μl HBSS, wells were blocked with 50 μl of an HBSS solution containing 10 mg/ml BSA (Boehringer Mannheim). Cultures used for TCRβ and CD4 were blocked with 50 μl of an HBSS solution containing 10 mg/ml BSA (Boehringer Mannheim, Laval, Québec, Canada). After two washes with 100 μl HBSS, 5 × 10⁵ primary lymph node T cells from C57BL/6 mice were added in 200 μl of unsupplemented serum-free medium. At 40 h, cultures were pulsed with 1 μCi [³H]thymidine and proliferation was assessed as described above.

Culture of cells for Northern blot analyses involved a variety of protocols. T cell clonal variants (5 × 10⁵) and 1.5 × 10⁵ irradiated (2000 rads) syngeneic T cell-depleted splenocytes were cocultured in 1 ml serum-free medium. Cultures were stimulated with 1 μg/ml OVA143-157 peptide. Cells were harvested from 12 replicate cultures at the indicated time points, and total RNA was extracted. Alternatively, 12 h after initiation of cultures, either anti-CD4 mAb H129 (1 μg/ml) or anti-MHC class I mAb M1.42 (1 μg/ml) were added to cultures. Cells were harvested from 18 replicate cultures at the indicated times, and total RNA was extracted. Alternatively, wells of a 24-well cluster flat-bottom tissue culture plate (Nunc) were coated directly with 300 μl of an HBSS solution containing 1 μg/ml anti-TCRβ mAb for 1 h at 37°C. After two washes with 500 μl HBSS, wells were blocked with an HBSS solution containing 10 mg/ml BSA (Boehringer Mannheim). After two washes with 500 μl HBSS, 3 × 10⁵ primary lymph node T cells from C57BL/6 mice were added in 1 ml of unsupplemented serum-free medium. Eighteen hours later, either anti-CD4 mAb H129 (1 μg/ml) or anti-MHC class I mAb M1.42 (1 μg/ml) were added to cultures. Four hours later, cells were harvested from 60 replicate cultures, and total RNA was extracted.
Immunofluorescence and flow cytometry analysis

Cells (10^5) were labeled with the indicated Abs for 10 min in 100 μl PBS containing 5% FCS, followed by three washes with the same buffer. Flow cytometric analysis was performed on a Becton Dickinson FACScan.

Immunoblotting

T cells were lysed at 5 × 10^7 cells/ml in lysis buffer containing 50 mM Tris (pH 8), 20 mM EDTA, 10 μg/ml each aprotinin and leupeptin, 1 mM PMSF, 50 mM NaF, 200 μM Na orthovanadate, and 1% Nonidet P-40. After a 15-min incubation on ice, postnuclear fractions were prepared by spinning lysates at 13,000 g for 10 min. CD4 and Lck were precipitated from lysates containing 10^6 cell equivalents using Abs covalently coupled to Sepharose 4B (Pharmacia) at 4°C for 30 min. After washing in lysis buffer (without aprotinin and leupeptin), Sepharose beads were resuspended in sample buffer containing 2.3% SDS and 5% 2-ME, and boiled for 5 min before 8% SDS-PAGE. Proteins were transferred to nitrocellulose, and Lck was revealed in immunoblots by incubating membranes with rabbit anti-Lck, followed by horseradish peroxidase-conjugated protein A (ICN, Montréal, Québec, Canada). Immunoblots were developed using enhanced chemiluminescence (Amersham, Oakville, Ontario, Canada).

Northern blot analysis

Total RNA was extracted using TRIzol (Life Technologies) as per manufacturer’s instructions. Briefly, cells were lysed in TRIZol and RNA was extracted by phenol-chloroform, precipitated in 50% isopropanol, and washed in 75% ethanol. The ratio of optical densities of the RNA samples was consistently determined at 260 nm and 280 nm. Nine micrograms of each RNA sample was electrophoresed on a 1.2% agarose gel containing 3% formaldehyde, 0.02 M MOPS, 8 mM sodium acetate, and 1 mM EDTA. The RNA sample was cross-linked with UV light. The blots were prehybridized overnight at 42°C in 25 ml of 6× SSC, 50% formamide, 0.5% SDS, 0.1% dextran sulfate, 5% Denhardt’s solution, and 100 μg/ml salmon sperm DNA, and then hybridized overnight with 25 × 10^6 cpm of the indicated probe. Probes were prepared by radiolabeling the 600 bp PstI insert of pGEM-IL-2, using a commercial kit (Pharmacia). Labeled probes were separated from excess [32P]PdTCTP (DuPont) by chromatography on Sephadex G-50 columns (Pharmacia). After hybridization, membranes were washed twice with 2× SSC for 2 min at room temperature, and then with 5× SSC and 1% SDS at 65°C. Results were visualized by autoradiography, and quantitative analysis was performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Blots were stripped with 5× SSC and 1% SDS for 1 h, and hybridization was conducted as above with [32P]-labeled cDNA specific for L32 ribosomal protein that provided a loading control to which signals for IL-2 were normalized.

**Table I.** Anti-CD4 inhibits Ag induced DNA synthesis in T cell clones expressing either wt CD4 or DC CD4

<table>
<thead>
<tr>
<th>Clonal variant</th>
<th>Anti-TCRβ</th>
<th>Anti-CD3ε</th>
<th>OVA1A</th>
<th>% Control Anti-CD4/OVA</th>
</tr>
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<tbody>
<tr>
<td>NEO</td>
<td>25227.3 ± 58.1</td>
<td>43704.7 ± 1388.0</td>
<td>30654.1 ± 419.0</td>
<td>99.9</td>
</tr>
<tr>
<td>wt CD4</td>
<td>3774.3 ± 226.0</td>
<td>30779.7 ± 1601.0</td>
<td>45758.7 ± 2381.3</td>
<td>4.6</td>
</tr>
<tr>
<td>DC CD4</td>
<td>33571.3 ± 1235.2</td>
<td>70642.7 ± 2659.7</td>
<td>25042.0 ± 901.2</td>
<td>29.7</td>
</tr>
</tbody>
</table>

* Representative T cell clones from each category, NEO, wt CD4, and DC CD4, were cocultured with irradiated-H-2^b splenocytes and either TCRβ-specific mAb (1 μg/ml), CD3ε-specific mAb (0.1 μg/ml) or 100 μg/ml OVA in 200 μl serum-free IMDM. At 40 h, each culture was pulsed with 1 μCi [3H]Tdr. Six hours later, cultures were harvested onto filters and thymidine uptake assessed by liquid scintillation spectrometry. Results shown are the mean c.p.m. from three experiments, with one SE indicated.

* Alternately, cells were cultured with 100 μg/ml OVA in the presence of CD4-specific mAb. Values indicate mean percentage of the Ag response of three experiments, in the absence of anti-CD4, with one SE indicated.

Results and Discussion

Associated Lck is not obligatory for CD4-mediated inhibition of Ag responses

The previous characterization of CD4^+ and CD4^− variants of an IL-2-dependent T cell clone, specific for OVA1A, demonstrated that the expression of CD4 did not limit the response to optimal concentrations of Ag (18). This indicates that the response in these circumstances is not dependent on adhesive properties of CD4. However, as previously reported, the Ag response of the CD4^+ variant was nonetheless inhibited by mAb specific for CD4 (18). This result suggested that the observed inhibition is not likely due to disruption of the CD4-MHC class II interactions that increase the avidity of T cell-APC interaction. Rather, anti-CD4 was likely interfering with the juxtaposition of CD4-associated Lck with TCR/CD3, and thus blocking the delivery of Lck function. If so, the prediction follows that anti-CD4 should not inhibit Ag responses in clonal variants expressing DC CD4, unable to associate with cellular Lck.

To address this possibility, we assessed the capacity of anti-CD4 to inhibit Ag responses of CD4^− clonal variants in which the expression of either wt CD4 or DC CD4 was forced. Cells infected with retrovirus encoding the NEO were used as a CD4^− control. As illustrated in Table I, representative clones from each of the three categories of infectant respond to Ag and CD3ε-mediated stimulation. However, as previously described (18), the response of wt CD4 infectants to TCRβ-specific mAb was roughly 10% of that observed with DC CD4 or NEO infectants (Table I). The Ag response of the CD4^− NEO variant was not inhibited in the presence of anti-CD4, whereas that of the wt CD4 variant was inhibited as profoundly as that of the variant expressing endogenous CD4 (Table I: Ref. 18).

In contrast to the result predicted if anti-CD4 was blocking the delivery of Lck function, the Ag responses of the DC CD4 variant were also affected by anti-CD4. Therefore, when anti-CD4 was added at the initiation of culture, the ensuing Ag responses were 20–30% of that observed in control cultures (Table I). As illustrated in Fig. 1A, the less efficient inhibition of the DC CD4 variant was not due to lower levels of CD4 expression, as the distribution of CD4 in the latter variant overlapped with that of the wt CD4 variant. Further, analysis of the presence and distribution of Lck in these clonal variants demonstrated the presence of comparable levels of cellular Lck in variants of the three categories of infectants, and its association with CD4 in the wt CD4-expressing variant, exclusively (Fig. 1B). These results demonstrate that CD4 can mediate the inhibition of Ag responses in the absence of associated Lck in circumstances in which the avidity of the CD4-MHC class II interaction is not limiting the response.

The specificity of CD4-mediated inhibition of Ag responses was further characterized using an extended panel of clones from each category. As illustrated in Fig. 1C, Ag responses of both wt CD4 and DC CD4 variants were inhibited by a broad range of anti-CD4 concentrations. However, CD4-specific mAb did not alter Ag responses of CD4^− variants. This inhibitory effect is not restricted to anti-CD4 mAb H129; anti-CD4 mAb GK1.5 functions in a similar
manner (not shown). To ensure that anti-CD4 inhibition was not simply due to the generation of Ag-Ab complexes on the membranes of these clonal variants, the inhibition mediated by MHC class I-specific mAb M1.42 was assessed. The use of this marker as a negative control is appropriate in this regard as it is both isotype matched with the CD4-specific mAb, and MHC class I is expressed more abundantly than CD4 on these clonal variants. As illustrated in Fig. 1C, addition of MHC class I-specific mAb at the initiation of culture did not inhibit Ag responses of any of the three representative clones from each of the categories of infectants over the dose range tested. In addition to the MHC class I control, further evidence supports the conclusion that the observed inhibition is specific to CD4 ligation. Specifically, anti-CD8 did not inhibit the Ag response of CD8α-expressing variants of the CD42 line (not shown).

The less profound inhibition of DC CD4 variants is observed over the titration of anti-CD4 tested. Thus, although up to 50% of the response of wt CD4 infectants is inhibited at 10 ng/ml of anti-CD4, and >95% inhibition of these variants is observed at 100 ng/ml, the latter concentration was required to observe significant inhibition of DC CD4 infectants. Furthermore, although levels of inhibition reached a plateau at 100 ng/ml of anti-CD4 for both CD4-expressing variants, the amplitude of the inhibition did not exceed 80% for DC CD4 variants (Fig. 1C). Nonetheless, this inhibition was specific, and therefore the results demonstrate that the capacity of anti-CD4 to inhibit Ag-mediated activation cannot be due solely to blocking the delivery of critical activation signals mediated by CD4-associated Lck.

These results are consistent with previous reports demonstrating a role for CD4 signaling independent of its association with Lck. Corresponding to the results presented thus far, it has been reported that anti-CD4 inhibited IL-2 production in response to superantigen by hybridoma variants expressing either wt CD4 or DC CD4 to comparable degrees (33). However, in the latter study, the dependence of the responses assessed on the adhesive properties mediated by CD4 expression was not controlled. Further, and consistent with CD4 mediating positive effects that are independent of associated Lck, it was reported that enhancement of IL-2 production can be observed by coaggregating TCR to CD4 in cells expressing either wt CD4 or a truncated form of CD4, unable to associate with Lck (33). However, for the most part, studies assessing the regulatory effects of CD4 ligation in modifying signals emanating from TCR/CD3, and the role of associated Lck in this regard have focussed on effects induced at the initiation of T cell activation. Thus, ligation or aggregation of CD4 was achieved at the time of TCR/CD3 engagement.

FIGURE 1. A, Expression of exogenous CD4 by representative clones of each subset of 2.10 infectants. CD4 expression by 2.10 NEO infectant, N7, 2.10 wt CD4 infectant, NC 4.10, and 2.10 DC CD4 infectant, NDC 32, was determined by immunofluorescence staining and FACS analysis using phycoerythrin-conjugated anti-CD4 (GK 1.5). B, Differential coprecipitation of Lck with CD4 variants. CD4 and Lck were precipitated from lysates (1 × 10⁶ cell equivalents) of representative clones N7, NC 4.10, and NDC 32 using specific mAbs conjugated to cyanogen bromide-activated Sepharose beads. Abs used in precipitations include mAbs specific for CD4 and IgG isolated from rabbit anti-Lck serum. Precipitates were revealed by specific immunoblotting for Lck, as described in Materials and Methods. C, Specificity of inhibition of Ag responses. Three representative T cell clones of each subset, NEO, wt CD4, and DC CD4 were cocultured with irradiated splenocytes and 100 μg/ml OVA, as described in Table I, in the presence or absence of indicated concentrations of either CD4 specific mAb, shown in the full symbols, or MHC class I-specific mAb, shown in the empty symbols. Proliferation was assessed by thymidine uptake as described in Table I. Values shown are the mean percentage of Ag responses of three experiments with one SE indicated.
Anti-CD4 inhibits ongoing Ag responses that are rescued by exogenous IL-2

To establish whether anti-CD4 perturbs TCR/CD3-derived signals at the initiation of T cell activation, exclusively, the effects of delaying the addition of anti-CD4 were assessed. Thus, a representative clone from each category of infectant was stimulated with Ag, and the inhibition of the response, mediated by anti-CD4 added at the time of initiation of culture or 12 or 18 h later, was assessed. As illustrated in Fig. 2, the extent of inhibition observed was not significantly altered when the addition of anti-CD4 was delayed. Delayed addition of the control mAb, specific for MHC class I, had no effect (not shown). As cultures containing each of the infectants analyzed in this assay were responding to Ag at the 12 and 18 h time points as assessed by thymidine incorporation (not shown), the results suggest that ligation of CD4 may inhibit the ongoing response of activated T cells.

This result is consistent with a recent report. Specifically, it was demonstrated that anti-CD3-induced proliferation of human CD4+ PBL could be inhibited by anti-CD4 and that the addition of mAb could be delayed for several hours without altering the observed inhibition (34). A potential caveat in the latter study, also pertinent to results presented in Fig. 2 in the present study, is the absence of a formal demonstration that anti-CD4 inhibits ongoing responses of activated T cells, rather than the initiation of T cell responses recruited late within the culture period. Thus, when DNA synthesis is assessed by thymidine uptake at 40 h, it is unclear when the response of the cells incorporating thymidine was initiated. This issue will be addressed in the present study. Further, because plate-bound anti-CD3 was used as the stimulus in the previous study (34), it remains unclear if anti-CD4 treatment could override the central role of CD28 in providing costimulatory signals in T cell activation. In this context, it has been reported that gp120- or anti-CD4-mediated ligation of CD4 before anti-CD3-mediated T cell stimulation inhibited the up-regulation of CD40 ligand on T cells and of B7–1 on APC, thus reducing the available costimulatory ligand for CD28. Addition of exogenous anti-CD28 overcame the inhibitory effect of gp120 or anti-CD4 on anti-CD3-induced T cell proliferation (35). Two points merit comment in regards to this latter study. Because gp-120/anti-CD4 was used to precoat cells before stimulation in these studies, their effect on the initiation of the T cell response is being assessed, and thus the capacity of anti-CD28 to rescue the initial signals emanating from the TCR/CD3 complex. The capacity of either gp120 or anti-CD4 to inhibit ongoing T cell responses induced with anti-CD3 was not assessed in this latter study, and thus the effects of anti-CD28 in these circumstances is difficult to predict. Further, recent reports demonstrate that anti-CD28 is able to stimulate IL-2 production and ensuing T cell growth in the absence of TCR/CD3 ligation (36–38). Thus, the interpretation of the observed anti-CD28-mediated rescue of gp120/anti-CD4-mediated inhibition is complicated. Indeed it could be overcoming the negative signals induced through CD4, alternatively, anti-CD28 could be directly stimulating growth that is insensitive to gp120 and anti-CD4 pre-treatment, as for those responses induced by PMA in combination with ionomycin (35).

The results presented in Table II suggest that the effects of anti-CD4-mediated inhibition of ongoing T cell responses supercedes the costimulatory signals supported by CD28 in the protocols used in the present study. Thus, if the APC in the cultures are providing ligand for CD28, and consequently costimulation is functioning in the Ag responses assessed, addition of exogenous anti-CD28 may inhibit the process by preventing the interaction of CD28 with its natural ligand. Such effects have been reported using Fabs of CD28-specific mAb that inhibited alloresponses of T cells receiving costimulatory signals from APC in culture (39). As illustrated in Table II, the addition of CD28-specific mAb, but not control hamster IgG, at the time of initiation of culture resulted in roughly a 10-fold inhibition of the Ag response of a wt CD4 variant. Thus, CD28 is likely functioning in support of the Ag responses observed, and notwithstanding, anti-CD4 is able to override costimulatory signals provided by CD28. Because the latter contribute both to de novo transcription and to stabilization of IL-2 mRNA (40, 41) and are thus critical to the net production of IL-2, the effects of late addition of exogenous IL-2 on inhibition mediated by anti-CD4, added 12 h after the initiation of cultures, was assessed. As illustrated in Fig. 3, addition of IL-2, delayed up to 36 h after the initiation of culture, rescued Ag responses. Thus, anti-CD4-mediated inhibition of both wt CD4 and DC CD4 clonal variants was counteracted by exogenous IL-2. This demonstrates that anti-CD4 is neither inducing cell death, nor limiting the capacity to utilize IL-2. Rather, the results suggest that anti-CD4 perturbs the production of endogenous IL-2 and thus limits cellular growth. The likely involvement of CD28-mediated costimulation in this system and its characterized effects on IL-2 mRNA production and stability (40, 41), coupled with this result (Fig. 3), prompted the analysis of anti-CD4-mediated effects on IL-2 mRNA levels.

### Table II. Anti-CD28 inhibits the Ag response of wt CD4 clonal variants

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Antibody</th>
<th>% TdR-uptake</th>
<th>% Control</th>
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<tr>
<td>OVA/IA</td>
<td>–</td>
<td>20,921 ± 1,548</td>
<td>100</td>
</tr>
<tr>
<td>OVA/IA</td>
<td>Hamster IgG</td>
<td>19,592 ± 4,267</td>
<td>94</td>
</tr>
<tr>
<td>OVA/IA</td>
<td>Anti-CD28</td>
<td>1,864 ± 400</td>
<td>9</td>
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</table>

* A total of 5 × 10⁶ wt CD4-expressing cells were cocultured with 2.5 × 10⁵ irradiated H-2b T-depleted splenocytes with 1 μg/ml OVA 143–157 in the presence and absence of either CD28-specific mAb (1 μg/ml) or control hamster IgG (1 μg/ml) in 200 μl serum-free IMDM. Proliferation was assessed by thymidine uptake as described in Table I.

Anti-CD4 down-regulates IL-2 mRNA in activated wt CD4 and DC CD4 clonal variants

In the following series of experiments, the effect of delayed addition of anti-CD4 on steady-state levels of IL-2-specific mRNA was assessed by Northern blot analysis. Because the first time point...
assayed at which thymidine incorporation induced by Ag was significantly above background was 12 h, it was chosen as the time point at which the presence of IL-2 mRNA was first assessed, as well as the one at which anti-CD4 was added to cultures. Cultures were subsequently harvested 1, 2, and 4 h after the addition of anti-CD4, and steady-state levels of IL-2 mRNA were assessed. As illustrated in Fig. 4, representative clones of variants from the three categories of infectants did not express detectable levels of IL-2 mRNA at the initiation of culture; however, by 12 h after Ag stimulation, IL-2 mRNA was readily detectable in NEO, wt CD4, and DC CD4 infectants. One hour after the addition of anti-CD4, levels of IL-2 mRNA were reduced to 15 and 42% of control in wt CD4 and DC CD4 infectants, respectively (Fig. 4). This reduction was maintained and increased over the next 3 h, resulting in levels of mRNA that were 2.7 and 38% of control in wt CD4 and DC CD4 infectants, respectively. As expected, addition of anti-CD4 did not affect the levels of IL-2 mRNA observed in Ag-stimulated NEO infectants, nor did the addition of mAb specific for MHC class I down-regulate levels of IL-2 mRNA in any of the infectants (Fig. 4). Loading was controlled by stripping the blots and reprobing with cDNA specific for the ribosomal protein, L32 (Fig. 4). The numbers under each series of blots represent those derived from the densitometric analysis of the signals obtained with the IL-2 probe, after normalizing to the density of the corresponding signal obtained with the L32-specific probe.

The extent to which anti-CD4 induces the down-regulation of IL-2 mRNA parallels the extent to which it inhibits thymidine incorporation in variants from each of the three categories (compare Figs. 2 and 4). Thus in this regard, the effects of anti-CD4 can be extended to the 40-h time point (Figs. 2 and 3). However, it should be noted that due to the strict dependence on IL-2 for the maintenance of viability of these clonal variants, too few viable cells were rescued at 40 h for Northern blot analysis.

More importantly, the assessment of the kinetics of appearance of IL-2 mRNA in response to Ag enabled discrimination between the possibilities that anti-CD4 was inhibiting either the late recruitment of T cells or ongoing T cell responses. Thus, it is critical to demonstrate that the late addition of anti-CD4 is indeed altering the expression of IL-2 mRNA in cells that contain it rather than disabling the induction of mRNA in late recruits to the proliferative response. The former conclusion would be supported with the demonstration that Ag-mediated induction of IL-2 mRNA in these cell lines takes significantly longer than the time required for anti-CD4 to affect levels of IL-2 mRNA observed after treatment. The results in Fig. 5 demonstrate that this is the case. IL-2 mRNA was first detected in this assay 4–8 h after Ag stimulation in variants from each of the three categories of infectants (Fig. 5). Anti-CD4-mediated down-regulation of IL-2 mRNA reaches a plateau within 2 h (Fig. 4), thus precluding the possibility that the observed inhibition is due to blocking the late recruitment of resting T cells. Therefore, the observed inhibition is a reflection of the capacity of anti-CD4 to inhibit ongoing T cell responses through the down-regulation of endogenous IL-2.
A previous report (42) demonstrated that CD4 clustering produces immune defects in CD4+ T lymphocytes by inhibiting the induction of transcription at the IL-2 locus. Thus, pretreatment of CD4+ T cells with HIV-1 envelope glycoprotein gp120 inhibited subsequent Ag- or anti-CD3-mediated accumulation of IL-2. Thus, the capacity of CD4 to directly impede T cell activation through ablating IL-2 production is not without precedent (38). However, in these circumstances (38), the role of CD4 in perturbing the initiation of de novo transcription and translation of IL-2 was assessed. The present study extends these observations in two ways. The capacity of CD4 to interfere with IL-2 production in T cells actively transcribing IL-2 mRNA is demonstrated, and the role of CD4-associated Lck in this process is shown not to predicate this CD4-mediated effect.

Anti-CD4 inhibits activated primary T cells through inhibition of IL-2 mRNA

Toward generalizing this novel characteristic of CD4 function, it is essential to determine whether the phenotype and underlying mechanism established using T cell clonal variants can be extended to primary CD4+ T cells. This was addressed in the present study using two assay systems. The first utilized lymph node T cells derived from animals transgenic for an OVA323-339/IAα-specific TCRαβ (22). The second used primary lymph node T cells derived from conventional C57BL/6 mice. As illustrated in the left panel of Fig. 6A, the Ag response of transgenic T cells was significantly inhibited upon the addition of anti-CD4, but not anti-MHC class I Abs, at 12 h after initiation of culture. The lack of available transgenic mice precluded the assessment of steady-state levels of IL-2 mRNA in this system. However, the capacity of delayed addition of anti-CD4 to inhibit the response of primary lymph node T cells derived from conventional C57BL/6 mice to plate-bound anti-TCRβ (Fig. 6A, right panel) was amenable to...
further characterization. As illustrated in Fig. 6R, the mechanism underlying this anti-CD4-mediated inhibition is identical to that observed using the T cell clonal variants. Specifically, the delayed addition of anti-CD4, but not anti-MHC class I, added 18 h after initiation of cultures, down-regulates the steady-state level of IL-2 mRNA in these primary lymph node T cells. Importantly, and in addition to generalizing this observation to include primary T cells, this result demonstrates the capacity of anti-CD4 to affect responses initiated independently of CD4. Thus, the observed signaling through CD4 can be uncoupled from signals emanating from TCR/CD3.

The mechanism through which anti-CD4 down-regulates IL-2 mRNA remains to be characterized. It could be mediated at the level of transcription, translation, or both. The assessment of the kinetics of both IL-2 mRNA production and its down-regulation mediated by anti-CD4 reported here, coupled with the reported τ₁/₂ of IL-2 mRNA of ~15–60 min (40, 43), is consistent with a transcriptional block for wt CD4-expressing clonal variants. Thus, if transcription is blocked, and the stability of existing IL-2 mRNA is not affected by anti-CD4, one would expect roughly 6–50% of the IL-2 mRNA to remain after 60 min (1–4 half-lives). This is within the range of levels observed in the present study after 1 h of anti-CD4 treatment in wt CD4-expressing variants (Fig. 4). However, this is not the case for DC CD4-expressing clonal variants in which levels of steady-state mRNA, remaining after 1 and 4 h, respectively, are inconsistent with a complete transcriptional block, even if one assumes that the τ₁/₂ of IL-2 mRNA is 60 min (Fig. 4). Thus, after 4 h in the presence of anti-CD4, levels of IL-2 mRNA in DC CD4-expressing variants remained at roughly 40% of control. Hence, the induction of RNases affecting the stability of IL-2 mRNA (44, 45), as well as alterations in the rate of translation may be playing nonexclusive roles in the phenotype observed. Further, although recent reports highlight a number of mechanisms through which CD4-mediated transcriptional blocks could occur, including those mediated by the activation of the negative regulator of TCR-mediated IL-2 gene transcription, Rap1 (46), or perhaps through the nuclear zinc finger protein, Nil-2a (47–49), they all relate to perturbations of signals emanating from the TCR/CD3.

It remains to be determined whether these mechanisms are involved in CD4-mediated inhibition of ongoing responses. Further, the requirement for CD4-associated Lck in this regard awaits characterization. The results presented demonstrate that although its role is not essential, the differential inhibition of activated T cells expressing DC CD4 and wt CD4 indicate that the full effect of anti-CD4-mediated inhibition are revealed only if Lck is associated with CD4.

The results presented characterize a novel biological role for CD4 that may be implicated in T cell homeostasis in circumstances of normal physiology and pathology. Thus, alterations in the levels of MHC class II on APCs may play as yet uncharacterized roles in limiting T cell clonal expansion through ligation of CD4. Furthermore, there are circumstances in which chronic ligation of CD4 may directly result in the depletion of the peripheral pool of CD4+ T cells. Specifically, in HIV-infected individuals in whom circulating gp120 has been detected (50), ligation of CD4 on T cells activated in response to environmental Ag may result in blocking clonal expansion by the mechanism described, or indeed their deletion (51–53). This would progressively exacerbate the immunocompromised state and susceptibility to infection observed in these patients. The recent demonstration that the interaction of HIV gp120 with CD4 on human T cells alters the binding activity of NF-AT, NF-κB, and activator protein 1 (54) is consistent with this suggestion.

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References


