Intracellular Trafficking of CTLA-4 and Focal Localization Towards Sites of TCR Engagement

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Summary

T lymphocyte receptor CTLA-4 binds costimulatory molecules CD80 (B7-1) and CD86 (B7-2) with high avidity and negatively regulates T cell activation. CTLA-4 functions at the cell surface, yet is primarily localized in intracellular vesicles. Here, we demonstrate cycling of CTLA-4 between intracellular stores and the cell surface. Intracellular vesicles containing CTLA-4 overlapped with endocytic compartment(s) and with perforin-containing secretory granules. Cell surface expression of CTLA-4 was rapidly increased by raising intracellular calcium levels. During T cell activation, intracellular and cell surface CTLA-4 became focused towards sites of TCR activation. Cycling and directional control of CTLA-4 expression may regulate its functional interaction with APCs bearing peptide-MHC complexes of appropriate specificity and avidity.

Introduction

Full activation of T cells requires engagement of the T cell antigen receptor (TCR)-CD3 complex and ligation of costimulatory receptor(s) (Mueller et al., 1989). Among the key costimulatory receptors are CD28 and CTLA-4, homologous members of the immunoglobulin superfamily, which bind B7-1 (CD80) and B7-2 (CD86) molecules on antigen-presenting cells (APCs). Following engagement of their counterreceptors, CD28 and CTLA-4 transmit signals important for optimal T cell activation (Schwartz, 1992; Linsley and Ledbetter, 1993; Guinan et al., 1994; June et al., 1994; Allison, 1994). CD28 has low avidity for B7 molecules (Linsley et al., 1994) and is expressed on resting and activated T cells. In contrast, CTLA-4 has high avidity for B7 molecules (Linsley et al., 1991, 1994) and is expressed on T cells only after activation (Brunet et al., 1987). CD28 and CTLA-4 apparently have different functions during T cell activation. CD28 triggering strongly up-regulates T cell cytokine production and cellular activation (Schwartz, 1992; Linsley and Ledbetter, 1993; Guinan et al., 1994; June et al., 1994; Allison, 1994). CTLA-4 triggering with specific monoclonal antibodies (MAbs) does not produce the same effects (Linsley et al., 1992; Damle et al., 1994; Walunas et al., 1994; Krummel and Allison, 1995), and may down-regulate CD28-mediated effects (Krummel and Allison, 1995). CTLA-4-deficient mice have a severe lymphoproliferative disorder, and show early lethality (Tivol et al., 1995; Waterhouse et al., 1995). Thus, CTLA-4 is an important negative regulator of T cell activation in young animals.

Although CTLA-4 has sequence characteristics and ligand binding properties of a plasma membrane receptor (Harper et al., 1991), it is primarily an intracellular membrane protein, characterized by polarized perinuclear expression (Leung et al., 1995). In transfected cells, the intracellular localization of CTLA-4 was regulated by a tyrosine-containing 11 aa motif in its cytoplasmic tail. This motif targeted CTLA-4 to a Golgi or post-Golgi compartment (Leung et al., 1995), thereby limiting its expression at the cell surface.

The intracellular localization of CTLA-4 contrasts with its presumed function as a cell surface receptor and raises several questions about how this molecule functions during T cell activation. One question is whether the small amount of CTLA-4 found on the cell surface under steady-state conditions is responsible for the biological effects of CTLA-4. The preferential intracellular localization of CTLA-4 may reflect the need to limit the levels of this potent high avidity molecule expressed at the cell surface. Another question is whether expression of CTLA-4 at the cell surface is static, or whether it is dynamically regulated such that the molecule transits in response to environmental stimuli between intracellular stores and the cell surface.

Finally, there is the question of whether intracellular localization of CTLA-4 represents a mechanism(s) to direct the localization of its expression. Studies in both lymphoid and nonlymphoid cells have shown that following cell-cell contact, the Golgi apparatus is typically reoriented such that it faces the site of cell contact (Singer, 1992). This typically results in directional release of secretory proteins such as cytokines (Poo et al., 1988; Kupfer et al., 1991, 1994), or the contents of cytotoxic granules (Berke, 1994) towards sites of cell-cell contact. Since secretory and membrane proteins share common pathway(s) of protein export, directional release of membrane proteins such as CTLA-4 may also occur. In this study, we have investigated the relationship between intracellular and cell surface-localized CTLA-4. We show that CTLA-4 expression at the cell surface is dynamically and directionally regulated by continuous processes of export and internalization.

Results

Cycling of CTLA-4 Between Intracellular Stores and the Cell Surface

Although CTLA-4 is expressed mainly in intracellular vesicles, it might still cycle between the cell surface and intracellular stores. If this were so, then anti-CTLA-4 MAbs added to culture medium of CTLA-4-expressing cells should accumulate intracellularly, even though little CTLA-4 is expressed at the cell surface. To test this possibility, we compared accumulation of BODIPY-conjugated anti-CD28 and anti-CTLA-4 MAbs by phytohemagglutinin (PHA) blasts at 37°C or 4°C (Figure 1). When cells were incubated with BODIPY-conjugated anti-CD28 MAb, identical staining intensities were seen during incubations from 1-4 hr at 37°C or 4°C (Figures 1A
Figure 1. Uptake of Anti-CTLA-4 MAb by Activated T Cells

(A and B) PHA blasts were incubated with BODIPY-conjugated anti-CD28 MAb 9.3 at 37°C (A) or 4°C (B) for 1 hr (gray line), 2 hr (white line), or 4 hr (black line). Cells were then washed and a total of 10,000 cells was analyzed by flow cytometry. The shaded histograms represent autofluorescence of unstained samples. An additional sample incubated at 37°C for 3 hr has been omitted for clarity. (C and D) PHA blasts were incubated for various periods of time with BODIPY-conjugated anti-CTLA-4 MAb 11D4 at 37°C (C) or 4°C (D). Labeling of the curves is as in (A) and (B).

(E) The uptake of both BODIPY-conjugated whole anti-CTLA-4 11D4 MAb (closed symbols, solid line) and BODIPY-conjugated 11D4 Fab (open symbols, dashed line) was measured in an experiment identical to the one shown in (C). Mean fluorescence intensities were plotted versus time of incubation at 37°C. Similar results using intact MAb were obtained in more than five separate experiments.

(F) Cells were incubated with BODIPY-conjugated MAb 11D4 at 37°C for 3 hr, fixed, counterstained with propidium iodide (red fluorescence), and examined by confocal microscopy. BODIPY-conjugated MAb was found in intracellular vesicles (red fluorescence, indicated by arrows).

and 1B). The amount of cell-associated MAb at 4 hr was reduced by treatment at pH 3, indicating that much of it had been associated with the cell surface (data not shown). In contrast, cells incubated with anti-CTLA-4 MAb 11D4 at 37°C showed a time-dependent uptake of labeled MAb (Figure 1C); cells incubated at 4°C did not show this increase (Figure 1D). Other experiments (data not shown) showed that this uptake was blocked by addition of CTLA-4Ig, indicating that it was antigen specific; and that cell-associated MAb after 4 hr was not released by treatment of cells at pH 3, suggesting that it had been internalized. The rate of anti-CTLA-4 MAb uptake was linear over a period of more than 4 hr (Figure 1E). In more than five separate experiments, the amount of cell-associated fluorescence doubled approximately every 1–2 hr. The rate of uptake of Fab fragments of anti-CTLA-4 MAb was identical to that of intact MAb (Figure 1E), indicating that uptake did not require bivalent MAb binding. Examination by confocal microscopy of cells incubated at 37°C with BODIPY-conjugated anti-CTLA-4 MAb (Figure 1F) or BODIPY Fab fragment (data not shown) revealed numerous fluorescence-tagged vesicles or endosomes inside these cells. In contrast, when cells were incubated with BODIPY-conjugated anti-CD28 MAb, fluorescence was mainly cell surface associated, as determined by confocal microscopy (data not shown). These results showed differential endocytosis of anti-CD28 and CTLA-4 MAb. Anti-CTLA-4 MAb becomes internalized during incubation at 37°C, whereas anti-CD28 MAb primarily remains attached to the cell surface.

Overlap Between Endosomes and Intracellular CTLA-4

Data shown in Figure 1 suggested cycling of CTLA-4 between the cell surface and intracellular stores. This would imply overlap between intracellular stores of CTLA-4 and endosomal compartment(s). To test this possibility, we determined whether the distribution of intracellular CTLA-4 overlapped with that of endosomes involved in the well-characterized endocytic pathway involving transferrin and its receptor (Figure 2).
PHA blasts were incubated with Texas Red-conjugated transferrin (TR Tf) to achieve steady-state labeling of transferrin receptor-containing endosomes. TR Tf-labeled cells were then fixed and permeabilized and stained for intracellular CTLA-4. As shown in Figure 2, there was considerable overlap between TR Tf-containing endosomes and intracellular CTLA-4. Thus, intracellular stores of CTLA-4 intersect with an endocytic compartment(s).

**Focal Localization of CTLA-4 Towards Sites of TCR Engagement**

Because of the reorientation of the Golgi apparatus that typically accompanies cell-cell contact (Singer, 1992), we hypothesized that during T cell activation, CTLA-4 expression might be preferentially localized towards the source of activating stimuli. Previous studies (Leung et al., 1995) showed a polarized distribution of CTLA-4 in activated T cells. However, since these cells were examined in solution, their orientation towards the activating stimulus could not be determined.

To test for directional release of CTLA-4 during T cell activation, we devised a system for examining the distribution of lymphocyte membrane proteins relative to an immobilized activating antibody. The procedure was then used to determine the relative distributions of CD28 and CTLA-4. A typical experiment is shown in Figure 3. Peripheral blood mononuclear cells were immobilized on chambered coverslips coated with anti-CD3 MAb. Adherent cells were then stained for various markers and immunofluorescence was examined by confocal microscopy. The distribution of CD28 molecules on immobilized cells was uniform and typical of a cell surface membrane protein; this was evident in both horizontal (Figures 3A–3C) and vertical (D) optical sections. In contrast, CTLA-4 in permeabilized cells was distributed preferentially towards the sites of cell attachment to the immobilized MAb (Figures 3E–3J). Intracellular CTLA-4 expression was typically (in >90% of positively staining cells) beneath the nucleus, i.e., between the nucleus and the coverslip. In some cells (Figure 3H) CTLA-4 staining was very close to the plane of the cell surface membrane, whereas in other cells, (I–J) it was more broadly distributed beneath the nucleus. CD2 (Figure 3K) and CD5 (L) showed typical surface fluorescence, similar to CD28. In other experiments, CD4, CD25, and CD69 also showed typical surface fluorescence distribution, indicating that the focal distribution of CTLA-4 was not a general property of T cell activation antigens. Staining with CTLA-4Ig was also uniform, indicating that CD80/CD86 molecules on T cells (which bind CTLA-4Ig) do not colocalize with CTLA-4 molecules.

The experiment shown in Figure 3 thus demonstrates that intracellular CTLA-4 is directed towards the activating anti-CD3 MAb on the glass coverslip. Since CTLA-4 functions as a cell surface receptor, it was of interest to determine whether cell surface CTLA-4 also showed directional localization. In cells permeabilized prior to staining with anti-CTLA-4 MAb (see Figure 3H), CTLA-4 accumulated very near the plane of the cell surface membrane. In other experiments, T cells were activated as in Figure 3, but stained with anti-CTLA-4 MAb without permeabilization. In this case, numerous cells showed CTLA-4 staining localized towards the coverslip (data not shown). We also compared the distribution of cell surface and intracellular CTLA-4 on the same cells. These cells showed heaviest accumulation of cell surface staining next to the coverslip, as in Figure 3H, with lower intensity cell surface staining up from the plane of the coverslip. Intracellular staining was similar to that shown in Figure 3. We conclude that both intracellular and cell surface CTLA-4 have an unusual distribution pattern, polarized towards the immobilizing/activating MAb. In contrast, the distribution of CD28 was identical whether permeabilized cells or intact cells (data not shown) were examined.

To determine whether the polarized distribution of CTLA-4 was dependent upon immobilization or the activation stimulus, we immobilized lymphocytes on chambered slides coated with a nonactivating anti-CD18 (β2 integrin) MAb. This did not lead to expression of CTLA-4 or the CD69 activation antigen. However, when anti-CD3 MAb-coated beads were added to immobilized cells, expression of CTLA-4 and CD69 was detectable within 48 hr. Examination of cells activated by adhesion to MAb-coated beads showed that CTLA-4 was not distributed towards the coverslip, but directed towards anti-CD3-coated beads (Figure 4). Anti-CD3-coated beads were attached to many of the activated cells, and could be visualized because of their faint fluorescence after staining with anti-CTLA-4 MAb; this staining was not seen with control immunoglobulin, and was perhaps due to association of membrane fragments containing CTLA-4 with the anti-CD3-coated beads. In activated cells bound by anti-CD3-coated beads, the distribution of CTLA-4 was clearly oriented towards attached anti-CD3-coated beads (Figure 5). Thus, CTLA-4 was localized preferentially towards the activating stimulus.

It was important to determine whether polarized distribution of CTLA-4 in anti-CD3-activated cells was also a feature of T cells activated in a more physiological manner. Therefore, we tested whether CD4+ cells activated with alloantigen showed a polarized distribution of CTLA-4. We previously have shown that the expression of CTLA-4 by these cells requires alloantigen stimulation, since without stimulation cells expressed <50 molecules/cell of CTLA-4; following stimulation, CTLA-4 expression increases >250-fold (Linsley et al., 1992). Thus, expression of CTLA-4 by these cells indicates that they have been activated by alloantigen. As shown in Figure 5, CTLA-4 in these cells was oriented towards alloantigen-bearing stimulator cells. The distribution of CTLA-4 towards activating stimuli was thus similar when T cells were activated with anti-CD3 MAb or with alloantigen. This indicates that the polarized expression of CTLA-4 during contact with APCs does not require interaction of CTLA-4 with CD80 or CD86 molecules on APCs. However, it remains possible that CD80 and CD86 expressed by the alloantigen-presenting cells used in Figure 5 may modify the kinetics or the extent of polarization.

**Reorientation of Polarized Expression of CTLA-4 and Up-Regulation of Cell Surface Expression**

We next asked whether intracellular CTLA-4 expression on previously activated T cells would become polarized
Immunity

538

Figure 3. Focal Localization of CTLA-4 Near Sites of Immobilization of Activated T Cells by Anti-CD3 MAb

Shown are confocal micrographs of T lymphocytes immobilized by anti-CD3 MAb and stained for various other T cell markers. Peripheral blood mononuclear cells were activated by binding to anti-CD3 MAb immobilized on glass coverslips. After 2 days of activation, immobilized cells were fixed in situ, permeabilized, and stained (red fluorescence) for expression of CD28 (A–D), CTLA-4 (E–J), CD2 (K), or CD5 (L); nuclei (green fluorescence) were counterstained with YO-PRO-1. (A–C) and (E–G) are serial horizontal (XY) optical sections (~50 nM thick) taken at 3 µM intervals beginning in the approximate plane of the coverslip (A and E). (D) and (H) are vertical (XZ) sections from the same cells shown in (A–C) and (E–G), respectively. (I–L) are vertical sections from different cells. CS, indicates plane of the coverslip.

When these cells were immobilized upon MAb-coated coverslips. Peripheral blood mononuclear cells were activated in solution with PHA, which leads to a randomly oriented distribution of CTLA-4 (Leung et al., 1995). Cells were then collected and immobilized on anti-CD3-coated or anti-CD18 MAb-coated coverslips (Figure 6). When cells were immobilized for 6 hr upon anti-CD3-coated coverslips, many showed intracellular CTLA-4 beneath the nucleus, oriented towards the plane of the coverslip (Figure 6A for a low power view and 6C for higher magnification of a vertical section of a typical cell). Cells immobilized upon anti-CD18-coated coverslips showed a different distribution of CTLA-4, with staining most prominent alongside the nucleus (Figures 6B and 6D). Thus, immobilized anti-CD3 MAb but not anti-CD18 MAb caused reorientation of intracellular CTLA-4 towards the plane of the coverslip within 6 hr.

In a similar experiment, we examined the kinetics of redistribution of cell surface CTLA-4 following immobilization on anti-CD3-coated coverslips. Initially, there was no particular orientation of CTLA-4 with respect to the coverslip, but after 2 hr of incubation, focal accumulation of CTLA-4 adjacent to the coverslip could be seen on some cells. Focal accumulation of CTLA-4 was greater after 4 hr (Figures 6E and 6G) and did not increase further after 6 hr. Cells incubated on anti-CD18 MAb-coated coverslips did not show focal accumulation of CTLA-4 beneath the nucleus, adjacent to the coverslip; most commonly, these cells showed patches of surface fluorescence away from the coverslip (Figures 6F and 6H). Thus, focal accumulation of cell surface CTLA-4 was observed within 2–6 hr after immobilization on anti-CD3 MAb-coated coverslips.

Induction of CTLA-4 Export to the Cell Surface

The rapid focal accumulation of cell surface CTLA-4 suggests mobilization of preexisting CTLA-4 from intracellular stores. To test whether intracellular CTLA-4 could be induced to accumulate on the cell surface, we tested various T cell stimuli for their abilities to increase cell surface expression of CTLA-4. Addition of anti-CD28 MAb 9.3 to PHA blasts did not increase cell surface expression of CTLA-4. We also tested pharmacological agents for their abilities to stimulate cell surface expression of CTLA-4. A more pronounced affect was obtained with the calcium ionophore ionomycin, which rapidly increased CTLA-4 cell surface expression at 37°C. This increase was maximal within 30 min, and began to decrease within 1–2 hr. In parallel experiments (data not shown), this increase was not observed when cells were
Focal Localization of CTLA-4

Figure 4. CTLA-4 Is Localized Towards Sites of T Cell Activation Rather than Adhesion to the Coverslip

Peripheral blood mononuclear cells were immobilized on anti-CD18 MAb-coated coverslips for 3 days, and activated with anti-CD3 MAb-coated beads. Cells were then fixed in situ, permeabilized, and stained for CTLA-4 (red fluorescence) and YO-PRO-1 (green fluorescence), as in Figure 3. (A–C) are serial horizontal optical sections (~50 nM thick) taken at 3 μM intervals beginning in the approximate plane of the coverslip (A). (D) is a vertical section from the same cell. Arrows indicate sites of attachment of 5 μM diameter anti-CD3-coated beads; CS, coverslip.

Figure 5. CTLA-4 in Activated CD4⁺ Cells Is Localized Towards Alloantigen-Presenting Cells

Lymphoblastoid cells were immobilized on anti-CD20-coated coverslips. Alloreactive CD4⁺ T cells were then added and cultured at 37°C for 24 hr. Cells were then fixed and permeabilized, and stained for CTLA-4 (red fluorescence) and nucleic acids (green fluorescence) as in Figure 3. Left, a horizontal optical section; right, a vertical section. Arrows indicate immobilized lymphoblastoid cells identified by their size and nuclear morphology.

incubated at 4°C. Other experiments showed this induction was blocked by addition of the calcium-specific chelator EGTA, indicating that induction was calcium dependent. Thus, raising intracellular calcium ion concentration leads to rapid induction of CTLA-4 expression at the cell surface.

The increase in cell surface CTLA-4 induced by ionomycin could result either from increased export or decreased rate of internalization. To distinguish between these possibilities, we measured the effects of PMA and ionomycin on uptake (or internalization) of BODIPY-conjugated anti-CTLA-4 MAb (Figure 7, bottom). Both agents increased the rate of uptake, with PMA causing a greater effect than ionomycin. Thus, the increased cell surface expression of CTLA-4 caused by ionomycin could not be attributed to decreased uptake, suggesting that the rate of export was increased by this treatment.

Expression of CTLA-4 in Perforin-Containing Vesicles

The release of perforin-containing granules by cytotoxic T cells is a Ca²⁺-dependent and directional process (Berke, 1994). The parallels between this directional release and the focal localization of CTLA-4 expression...
Immunity

Figure 6. Redirection of CTLA-4 Expression Following Attachment to Anti-CD3 MAb but Not Anti-CD18 MAb

Peripheral blood mononuclear cells were activated by PHA stimulation for 3 days, then immobilized on glass coverslips coated with anti-lymphocyte MAbs, and stained with biotinylated anti-CTLA-4 followed by PESA (red fluorescence) and with YO-PRO-1 (green fluorescence). In (A–D), cells were immobilized for 6 hr, fixed in situ, permeabilized and stained. (A–B) are horizontal nonconfocal images (pinhole fully open), and (C–D) are vertical confocal images of cells immobilized on anti-CD3 MAb (A, C) or anti-CD18 MAb (B, D). In (E–H), cells were immobilized for 4 hr and stained prior to permeabilization. (E) and (F) are horizontal nonconfocal images, while (G) and (H) are vertical confocal images of cells immobilized on anti-CD3 MAb (E, G) or anti-CD18 MAb (F, H). Scale bar on (A) is also appropriate for (B), (E), (F); the bar in (C) is appropriate for (D), (G), (H).

suggested a possible relationship between vesicles containing perforin and those containing CTLA-4. We examined this possibility by testing PHA blasts having reoriented localization of CTLA-4 (as in Figure 7) for colocalization of perforin and CTLA-4 intracellular staining. Vesicles containing both molecules tended to be oriented towards the plane of the coverslip, with CTLA-4-containing vesicles generally being more proximal to the plane of the coverslip and those containing perforin being more distal. As shown in Figure 8, numerous vesicles stained positive for both CTLA-4 and perforin (indicated by the arrows). Thus, vesicles containing internal stores of CTLA-4 and those containing perforin were adjacent and sometimes overlapped. We have also observed colocalization of CTLA-4-containing and perforin-containing vesicles in alloreactive T cell blasts (data not shown).

Discussion

We have shown that anti-CTLA-4 MAb or Fab fragments are endocytosed by activated T cells. In contrast, CD28 expression was not modulated by MAbs. MAbs to many cell surface antigens are internalized under the circumstances utilized here, so the uptake of anti-CTLA-4 MAbs was not necessarily unique. However several features of the uptake of anti-CTLA-4 MAb were noteworthy. The amount of anti-CTLA-4 MAb internalized exceeded the amount bound to the cell surface at any point in time (compare Figures 1C and 1D). This differs from the situation in which anti-CD3 MAb initially gave high levels of cell surface expression, which then decreased as MAb was taken up into endocytic compartments (Ledbetter et al., 1990). In addition, the amount of cell-associated anti-CTLA-4 MAb increased with time. This suggests continuous accumulation of MAb in a compartment where it was resistant to degradation. Moreover, the rate of uptake was slow and continuous, and did not readily show saturation with respect to time. Finally, Fab fragments of anti-CTLA-4 MAb were taken up equally as well as intact MAb, indicating that cross-linking of CTLA-4 by bivalent MAb was not required for uptake.

The uptake of anti-CTLA-4 MAb without accumulation at the cell surface suggests that in contrast with CD28, CD28 cycles continuously to and from the cell surface. Intracellular levels of anti-CTLA-4 MAb that accumulate over time are higher than those at the cell surface, suggesting that endocytosis from the cell surface is rapid compared with export of CTLA-4 to the cell surface. Other data also support cycling of CTLA-4 to and from the cell surface. The overlap of transferrin-containing endocytic vesicles with intracellular stores of CTLA-4 indicates that even in the absence of anti-CTLA-4 MAb, CTLA-4 is found in intracellular compartments (Peters et al., 1991). Thus, the distribution of intracellular CTLA-4 overlaps with both

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Focal Localization of CTLA-4

Figure 7. Calcium Ionophore Induces Increased Expression of CTLA-4 at the Cell Surface

PHA blasts were treated with medium alone (circles), PMA (10 ng/ml, squares), or ionomycin (1 µg/ml, triangles) for increasing periods of time at 37°C.

(Top) Cell surface staining for CTLA-4. Following incubation with the indicated agents, cells were washed and incubated with biotinylated MAb 10A8 at 4°C for 1 hr. Cells were then washed again, incubated with PESA at 4°C, and analyzed by flow cytometry. Cells stained with biotinylated-negative control MAb ME20 gave mean fluorescence intensities of ∼7.

(Bottom) Uptake of anti-CTLA-4 MAb. Cells were incubated with BODIPY-conjugated anti-CTLA-4 MAb 11D4 together with the indicated agents. After incubation for the indicated periods of time, cells were washed and uptake of anti-CTLA-4 MAb was measured as indicated in Figure 1. Each experiment was performed at least twice and gave identical results.

secretory and endocytic compartments. Taken together, these data suggest that CTLA-4 expression at the cell surface is dynamically regulated by its transit between intracellular stores and the cell surface.

Addition of intact anti-CTLA-4 MABs or Fab fragments to several in vitro and in vivo systems stimulates T cell activation (Linsley et al., 1992; Walunas et al., 1994; Krummel and Allison, 1995), whereas cross-linking these MABs inhibits T cell activation (Krummel and Allison, 1995). These studies have been interpreted as mimicking the interaction of cell surface CTLA-4 with its B7 ligands. However, we have shown here that rather than simply triggering CTLA-4 molecules on the cell surface, anti-CTLA-4 MABs and Fabs are efficiently and steadily internalized into endosomes. This is in contrast with anti-CD28 MABs, which are more readily retained at the cell surface. Since CTLA-4 and CD28 are differentially modulated by MABs, MAB-triggered signals through these molecules may have different relevance as mimics of cell surface stimulation by CD80/CD86 ligands. It will be important to determine whether the distribution of CTLA-4 is correlated with the differing effects of anti-CTLA-4 MABs upon T cell activation under different conditions (Krummel and Allison, 1995).

Under some circumstances, CD28-B7 interactions may regulate the effector phase of cell-mediated cytolyis (Ramarathinam et al., 1994; Azuma et al., 1992). The role of CTLA-4 in this process has not been determined. Our observation of colocalization of CTLA-4-containing and perforin-containing vesicles suggests a new avenue of investigation into whether CTLA-4 plays a role in the formation, release, or both, of perforin-containing vesicles.

Intracellular and cell surface CTLA-4 became focally localized towards sites of TCR cross-linking. Focal localization of cell surface CTLA-4 could result from its co-capping with components of the TCR during incubation with immobilized anti-CD3 MAb. We consider this unlikely because intracellular CTLA-4 was also focally localized, and there is no obvious direct connection between intracellular CTLA-4 and that on the cell surface.
Also arguing against a cocapping mechanism is the finding that CD2, CD4, and CD5, which may associate with the TCR during T cell activation, show a more uniform distribution.

An alternative explanation for focal localization of cell surface CTLA-4 is that lymphocytes may directionally regulate cell surface membrane protein organization. Polarization of intracellular CTLA-4 and that which is newly inserted into the cell membrane could be achieved by vectorial organization of the lymphocyte secretory apparatus during cell activation, analogous to directional cytokine release (Singer, 1992; Poo et al., 1988; Kupfer et al., 1991, 1994). Polarization of cell surface CTLA-4 would be maintained if the rate of internalization exceeded the rate of export as distance from the site of TCR engagement increased. This predicted rapid rate of internalization is consistent with our data showing steady uptake of anti-CTLA-4 MAb by activated T cells.

In contrast, CD28 (or many other lymphocyte cell surface proteins) does not show polarized distribution, nor does it show this apparent cycling from the cell surface. The molecular mechanisms involved in lymphocyte polarization are not well understood, although a recent report demonstrated the involvement of the Ras-related GTPase CDC42 in this process (Stowers et al., 1995).

Focal localization of CTLA-4 towards sites of TCR engagement may serve to regulate the specificity of CTLA-4–CD80/CD86 interactions. Since CTLA-4 has high avidity for CD80/CD86 molecules, its unregulated expression could lead to engagement by CD80/CD86 molecules on APCs not displaying appropriate antigenic peptides. This presents a problem for maintaining the antigen specificity of CTLA-4–CD80/CD86 interactions. With cytokines, the problem of functional specificity is solved by their focal release, thereby concentrating them near APCs interacting with antigen-specific T cells (Poo et al., 1988; Kupfer et al., 1991, 1994). In an analogous fashion, focal localization of CTLA-4 may limit its functional engagement to APCs bearing only specific peptides.

Cycling and focal localization of CTLA-4 may also help couple CTLA-4 engagement with the strength of an immune response. Data presented here show that CTLA-4 expression at the cell surface is a dynamic process and is directionally regulated by TCR engagement. Other recent evidence suggests that TCR/peptide-MHC interactions are also dynamic and reversible (Valitutti et al., 1995). These authors have suggested that a single peptide-MHC complex serially engages and triggers many TCRs, a process encouraged by the low avidity and fast kinetic off rates of TCR/peptide-MHC interactions.

Together, these findings lead us to speculate that CTLA-4 expression at the cell surface, and hence its ability to regulate immune responses, is directly related to the strength of TCR/peptide-MHC interactions. This in turn suggests that stronger TCR/peptide-MHC interactions would be most prone to productive signaling and, therefore, would be more likely to result in induction of CTLA-4 expression. The functional consequences of CTLA-4 engagement would thus be more pronounced for stronger TCR interactions driven either by higher TCR avidity (slower off rates) or by higher occupancy at elevated antigen concentrations.

**Experimental Procedures**

**Cell Culture**

Human peripheral blood mononuclear cells were activated by culture (Linsley et al., 1991; Leung et al., 1995) with 1 μg/ml PHA for 3 days, or by incubation for 2–3 days (at 2.5 × 10⁶/well) on anti-CD3 MAb G19-4-coated 4 cm² chambered coverslips (Nunc, Naperville, Illinois). Alloreactive CD4⁺ cells were cultured as described using Epstein-Barr virus–transformed B lymphoblastoid cells as stimulators (Linsley et al., 1991). Poly styrene beads (5 μM in diameter, IDC, Portland, Oregon) were coated with MAb G19-4 as described (Krummel and Allison, 1995).

**MAbs**

Anti-CTLA-4 MAbs, 11D4, 7F8, and 10A8 have been previously described (Linsley et al., 1992). Fab fragments of MAB 11D4 were prepared by M. Stebbins (Bristol-Myers Squibb Pharmaceutical Research Institute [BMSPRIS], Seattle, Washington). These were shown by gel permeation chromatography to contain undetectable amounts of aggregated material. CTLA-4 Ig was prepared by biologic al Process Research (BMSPRIS). Anti-CD3 MAb G19-4; anti-CD18 MAb 60.3; anti-CD20 MAb 1FS; and anti-CD28 MAb 9.3 were provided by Dr. J. Ledbetter (BMSPRIS). Negative control MAbs 52.4 (anti-decapeptide) and ME20 (anti-human melanoma) were provided by Dr. D. Yellon (BMSPRIS). Anti-CD25 MAb TOT14 was from AMAC (Westbrook, Maine). Anti-CD2, anti-CD4, anti-CD5, and anti-CD69 MAbs were purchased from Becton Dickinson (San Jose, California). Control murine immunoglobulin G1 was purchased fromPhar mingen (San Diego, California). Anti-perforin MAb 6G9 was from Ancell (Bayport, Minnesota). Biotinylated and fluorescein isothiocyanate–conjugated MAbs were purchased from the indicated suppliers or were prepared using standard techniques. BODIPY FL amine labeling reagent was purchased from Molecular Probes (Eugene, Oregon). Cy5 reactive dye was purchased from Biological Detection Systems (Pittsburgh, Pennsylvania). BODIPY- and Cy5-conjugated MAbs or Fab fragments, or both, were prepared according to the instructions of the manufacturers. Analysis by gel permeation chromatography of BODIPY-conjugated Fab fragments of MAb 11D4 showed that the conjugation procedure did not result in detectable formation of aggregates.

**Immunostaining**

MAbs were used at concentrations of 2–10 μg/ml. Staining of cell surface antigens was performed at 4°C in staining medium (DMEM containing 10% fetal bovine serum, 10 mM EDTA, 0.1% Na₂HPO₄, and 10 mM HEPES [pH 7.2]). For staining of intracellular antigens, cells were fixed for 5 min at 37°C in fixative solution (phosphate-buffered saline [PBS]) containing 4% EM grade paraformaldehyde (Poly sciences, Warrenington, Pennsylvania) and 0.1% saponin. Cells were then washed with PBS, blocked in binding medium containing 0.05% saponin, then stained in the same medium. Staining with biotinylated MAbs was followed by addition of R-phycocerythrin-conjugated streptavidin (PESA, Molecular Probes, Eugene, Oregon). After staining, cells were washed in PBS. Cells used for confocal microscopy were then incubated in fixative solution for 5 min before analysis. Nuclei, cytoplasm, or both, were counterstained by DNA binding dyes (from Molecular Probes, Eugene, Oregon), YO-PRO-1 (30 nM, green fluorescence), or propidium iodide (0.1 μg/ml, red fluorescence).

**Fluorescence Analysis**

Flow cytometry was performed using a 488 nM laser on a FACSscan (Becton Dickinson). BODIPY fluorescence was monitored in channel 1 and PESA fluorescence in channel 2. Confocal microscopy was performed on Leica CLSM, or Bio-Rad MRC-1024 model confocal microscopes. Green fluorescence was detected following excitation at 488 nM; red fluorescence, following excitation at 488 nM (PESA) or 565 nM (PESA and Texas Red); and blue fluorescence, at 464 nM. Images were recorded as TIF files and processed (Adobe Photoshop, Adobe Systems, Mountainview, California) to subtract background and to enhance lower and middle intensity fluorescence. Separate panels in each individual figure were processed identically.
Endocytosis Assays

To measure uptake of anti-CTLA-4 and anti-CD28 MAbs, cells were incubated with BODIPY- or FITC-conjugated MAbs for various periods of time at 37°C. Uptake of fluorescent MAb was then measured by flow cytometry or confocal microscopy. Initial experiments were performed using BODIPY-conjugated MAbs, since the emission of this fluorophore is not quenched at the acidic pH characteristic of some intracellular vesicular compartments. However, in later experiments, identical results were obtained using FITC-conjugated MAbs. Steady-state labeling of transferrin-containing endosomes was performed by incubating PHA blasts with TRIT (Molecular Probes) for 1 hr at 37°C. To colocalize TRIT-containing endocytic vesicles and intracellular CTLA-4, cells were then fixed and permeabilized, and intracellular CTLA-4 was stained with BODIPY-conjugated MAb 11D4. Colocalization of vesicles containing CTLA-4 with those containing perforin was performed by adding to fixed and permeabilized cells a mixture of anti-CTLA-4 MAb and anti-perforin MAb.

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