Phenotypic analysis of CTLA-4 and CD28 expression during transient peptide-induced T cell activation \textit{in vivo}

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Abstract

The T cell co-stimulatory receptors CD28 and CTLA-4 appear to have opposite effects on T cell activation, mediating augmentation and inhibition of T cell responses respectively. Since these two receptors use the same ligands, CD80 (B7-1) and CD86 (B7-2), the co-ordinate timing of CD28 and CTLA-4 expression has a major impact on the regulation of immune responses. While the kinetics of co-stimulatory molecules have been established for T cell stimulation \textit{in vitro}, little is known about CD28 and CTLA-4 expression in response to T cell activation \textit{in vivo}. In this study we have investigated the kinetics of CD28 and CTLA-4 expression upon CD4\(^+\) T cell activation in response to soluble peptide \textit{in vivo}. Using mice transgenic for a T cell receptor specific for the I-A\(^u\)-restricted N-terminal peptide of myelin basic protein MBP Ac1–9, we show maximal up-regulation of both CD28 and CTLA-4 2 days after peptide administration. CTLA-4 expression correlated positively with early activation markers on the same cells and was high on blast cells. Administration of peptide analogs with higher affinity for I-A\(^u\) MHC class II revealed a higher increase in CTLA-4 than in CD28 expression in response to improved TCR ligation. Further, a small population of CD4\(^+\) T cells expressing CTLA-4, CD25 and CD45RB\(^{low}\) was identified in mice that had not been treated with specific peptide. The implications of these observations for immune regulation are discussed.

Introduction

T cell activation through the T cell receptor (TCR)–CD3 complex is necessary but not sufficient for initiating and controlling an immune response. While specific ligands for the TCR define the target, a range of additional components determine the appropriate reaction to an antigenic challenge. Among co-stimulatory molecules, the CD28/CTLA-4–B7 system plays an important role (1,2). Although CD28 and CTLA-4 (CD152) share some structural features and bind to the same ligands, CD80 (B7-1) and CD86 (B7-2), their functional properties are strikingly different. CD28 synergizes with TCR–CD3 to provide positive co-stimulation while several lines of evidence have suggested a role for CTLA-4 as an essential attenuator of immune responses (3). Blockade of CTLA-4 potentiates T cell activation, while CTLA-4 cross-linking prevents T cell cycle progression \textit{in vitro} (4–9). The fundamental inhibitory function of CTLA-4 has been most dramatically demonstrated with CTLA-4-deficient mice that suffer from a lymphoproliferative disorder (10–12).

The central role of co-stimulation for immunoregulation has made the CD28/CTLA-4–B7 system a favourite target for immune intervention (13–19). Depending on the model and phase of disease, however, CD80/CD86 blockade may result in differential outcomes, either ameliorating or exacerbating disease. Insight into the kinetics of expression of co-stimulatory molecules \textit{in vivo} is therefore a prerequisite for rational therapeutic manipulations (20,21). In contrast to productive immunity, T cell activation by aqueous antigens does not usually result in full effector functions but, depending on the frequencies of treatment and specific T cells, may instead lead to peripheral T cell tolerance. This has been demonstrated for various models and methods such as i.v., i.p., oral or intranasal (i.n.) administration of soluble antigens (22–25). Unlike anergy
in vivo induced by TCR-CD3 triggering in the absence of co-stimulation, however, blockade of CD80/CD86 does not appear to lead to, and blocking CD28 and CTLA-4 may interfere with a tolerogenic outcome of initial T cell activation in vivo (26–29). Understanding the role of co-stimulation in this context as well as optimal manipulation of the system will require insight into the kinetics and expression patterns of co-stimulation. In general, CD28 is constitutively expressed at moderate levels on murine T cells and modulated upon activation, whereas CTLA-4 expression is dependent on T cell activation and involves a characteristic pattern of intracellular trafficking. Levels of cell surface CTLA-4 are small compared to CTLA-4 in intracellular vesicles even at maximal CTLA-4 protein expression. Both cell surface and vesicular CTLA-4 are polarized towards the site of TCR-CD3 engagement and characterized by dynamic recycling between both compartments (30–34). Current knowledge of the kinetics of CTLA-4 and CD28 is largely based on T cell activation in vitro, often in response to non-physiological activation stimuli. Little is known, however, about the kinetics and regulation of positive and negative co-stimulation during T cell activation induced by aqueous physiological TCR ligands in vivo.

Previous studies from this laboratory have explored i.p. and i.n. administration of soluble antigenic peptides to induce T cell tolerance and protect conventional and TCR transgenic (Tg) H-2^d mice from experimental autoimmune encephalomyelitis (EAE), a CD4^+ T cell-mediated inflammatory disease with similarities to multiple sclerosis in humans (23,35–37 and Burkhart et al., submitted). For the myelin basic protein (MBP) H129.19; Sigma, St Louis, MO) and co-stained with FITC-labeled anti-CD45RB (clone 16A, PharMingen), Rat IgG2a–FITC or –biotin (clone R35-95; PharMingen), hamster IgG–biotin (clone G235-2356; PharMingen), and rat IgM–biotin (clone R4-22; PharMingen) were used as isotype controls. After red blood cell lysis and fixation in FACS lysing solution (Becton Dickinson, Mountain View, CA), cells were washed in 0.03% saponin/ PBS and stained for CTLA-4 (clone UC10-4F10-11, hyphy-cryptin (PE) conjugate; PharMingen) or with a PE-conjugated hamster IgG control (clone G235-2356; PharMingen) in the presence of 5% normal mouse serum while transiently permeabilized in 0.3% saponin/PBS. After washing in 0.03% saponin/ PBS and FACS buffer (HBSS (Gibco, Paisley, UK), 2% FCS (Sigma) and 0.01% NaN3), data were acquired on a FACScan (Becton Dickinson) and analyzed using Lysys II software (Becton Dickinson). Logarithmically amplified fluorescence data were collected for 10,000 intact lymphocytes as determined by scatter gates.

Results

The majority of peptide-specific (CD4^+) T cells express CTLA-4 2 days after 4Y peptide i.n.

For sensitive detection and analysis of phenotypic changes of T cells during antigen-specific activation in vivo, we used Tg mice expressing a TCR (V_α4 V_β8.2) specific for the N-terminal acetylated peptide of MBP Ac1–9, the dominant I-A^d-restricted CD4^+ T cell epitope in this model (40). Peptide analogs with tyrosine or alanine substitutions in place of the original lysine at position 4 displayed higher affinity for the I-A^d MHC class II and its tolerogenicity (35,36). Recent work in the TCR Tg model revealed a short phase of T cell activation following i.p. or i.n. administration of MBP Ac1–9 (23 and Burkhart et al., submitted). In the present study we have used this Tg model and higher-affinity peptide analogues to analyze TCR ligation-induced changes in CTLA-4 and CD28 expression in response to i.p. administered peptide. We have further addressed the correlation between CTLA-4 expression and other activation markers in response to specific peptide and for a minor population of CTLA-4^+ cells in control animals.

Methods

Mice

The generation of TCR Tg mice and analysis of the Tg4 and Tg3 lines was described previously (23,38). Transgenic T cells express the V_α4 V_β8.2 TCR of the MBP Ac1–9–specific T cell hybridoma 1934.4 derived from the encephalitogenic T cell clone PJR-25 (39). Mice were bred under specific pathogen-free conditions in isolators and subsequently maintained in conventional facilities in the School of Medical Sciences, Bristol University.

Peptides and i.n. administration

The acetylated N-terminal peptide of MBP Ac1–9 (AcASQKRPSQR), and the analogues with alanine (4A) and tyrosine (4Y) substitutions at position 4 and MBP 89–101 (VHFFKINVTPRTP), were synthesized using Fmoc chemistry on an AMS 422 multiple peptide synthesizer (Abimed, Langenfeld, Germany). Mice were given 100 μg peptide in 25 μl PBS under light ether anesthesia as described (35).

Reagents and Flow cytometry analysis

Following peptide i.n. administration, blood samples and cell suspensions from spleens and cervical lymph nodes (CLN) were surface stained with FITC-conjugated anti-CD4 (clone H129.19; Sigma, St Louis, MO) and a biotinylated antibody against either V_β8.2 (clone F23.1), CD28 (clone 37.51; PharMingen, San Diego, CA), CD25 (clone 7D4; PharMingen), CD69 (clone H1.2F3; PharMingen), CD80 (clone 16-10A1; PharMingen), CD86 (clone GL1; PharMingen) or Fas (clone Jo2; PharMingen) followed by staining with streptavidin–TriColor (Caltag, Burlingame, CA). Alternatively, CD4^+ cells were identified with Quantum Red-conjugated antibody (clone H129.19; Sigma) and co-stained with FITC-labeled anti-CD45RB (clone 16A, PharMingen), Rat IgG2a–FITC or –biotin (clone R35-95; PharMingen), hamster IgG–biotin (clone G235-2356; PharMingen), and rat IgM–biotin (clone R4-22; PharMingen) were used as isotype controls. After red blood cell lysis and fixation in FACS lysing solution (Becton Dickinson, Mountain View, CA), cells were washed in 0.03% saponin/PBS and stained for CTLA-4 (clone UC10-4F10-11, phycoerythrin (PE) conjugate; PharMingen) or with a PE-conjugated hamster IgG control (clone G235-2356; PharMingen) in the presence of 5% normal mouse serum while transiently permeabilized in 0.3% saponin/PBS. After washing in 0.03% saponin/PBS and FACS buffer (HBSS (Gibco, Paisley, UK), 2% FCS (Sigma) and 0.01% NaN3), data were acquired on a FACScan (Becton Dickinson) and analyzed using Lysys II software (Becton Dickinson). Logarithmically amplified fluorescence data were collected for 10,000 intact lymphocytes as determined by scatter gates.
Fig. 1. CTLA-4 and CD28 expression after i.n. peptide administration. Tg4 mice received 100 µg Ac1–9[4Y] i.n. or MBP 89–101 as control peptide. Two days later cell suspensions from spleen and CLN were surface stained with anti-CD4–FITC and biotin-conjugated anti-CD28/streptavidin-TriColor (solid lines, B) or hamster control IgG (dotted lines, B) followed by either anti-CTLA-4–PE (solid lines, A) or control hamster IgG staining (dotted lines, A) of transiently permeabilized cells. (A) CTLA-4 expression of CD4^+ gated cells after MBP 89–101 i.n. (left) and Ac1–9[4Y] (right). (B) Specific increase in CD28 expression by CD4^+ gated cells from CLN (left) and spleen (right) after Ac1–9[4Y] i.n. (thick solid line) compared to controls (MBP 89–101 i.n., thin solid lines). The numbers indicate median channel fluorescence. Data are representative of cell samples from eight mice.
CD4+ lymphocytes, typically 70–78% for blood and CLN, and 80–85% of splenic CD4+ cells. These levels of positive staining required cell permeabilization to include the intracellular pool of CTLA-4. Only ~2–8% of CD4+ cells showed significant cell surface staining, confirming that the majority of the CTLA-4 protein is localized intracellularly (not shown). CTLA-4 expression was not observed for CD4– cells. A small but significant population of ~3–6% of CD4+ cells from control-treated mice also expressed CTLA-4, as will be discussed below. CD28 levels were also examined. Unlike CTLA-4, CD28 expression was increased ~2-fold 2 days after 4Y peptide i.n. administration (Fig. 1B).

**Kinetics of CTLA-4 and CD28 expression after 4Y i.n.**

To compare changes in CTLA-4 and CD28 expression in response to peptide in vivo, CD4+ cells from Tg4 mice were analyzed at various times after i.n. peptide administration (Fig. 2). CTLA-4 expression was readily detectable 1 day after 4Y i.n., with ~7–20% of CD4+ cells staining positive. CD28 was transiently down-regulated 1 day after 4Y i.n. Expression of both CD28 and CTLA-4 was maximal 2 days after 4Y i.n. and declined thereafter. The fluorescence intensity of anti-CTLA-4 staining of CD4+ gated cells changed with the same kinetic pattern as the total CTLA-4 expression (not shown). Interestingly, a significant proportion of CD4+ cells (10–20%) expressed CTLA-4 4 days after 4Y i.n. while CD28 had returned to basal constitutive levels by day 6 after 4Y i.n.. The expression pattern and kinetics of expression of CD28 and CTLA-4 were similar on T cells from spleen, CLN and blood. Although the overall levels of expression were lower, the kinetics of CTLA-4 and CD28 expression after i.n. administration of the lower-affinity peptides 4A and 4K were similar to the kinetics observed after 4Y i.n. (not shown).

**CTLA-4 expression correlates with activation marker expression following i.n. peptide administration.**

It has been shown that CTLA-4 expression requires T cell activation. Yet, early CTLA-4 cross-linking inhibits T cell activation in vitro as demonstrated by impaired CD69 and CD25 expression, IL-2 production and cell cycle progression (8,9). Since 4Y peptide i.n. induces specific T cell proliferation in vivo (C. Burkart, unpublished data) as well as high CTLA-4 expression (Fig. 1A), we wanted to address how CTLA-4 expression correlated with indicators of T cell activation. When CD4+ cells were analyzed for CD25 and CD69 expression 16 h after 4Y i.n., the population of CTLA-4+ cells coincided with indicators of T cell activation. When CD4+ cells were analyzed for CD25 and CD69 expression 16 h after 4Y i.n., the population of CTLA-4+ cells coincided with CD25+ T cells that expressed the highest levels of these early activation markers (Fig. 3). Under these conditions, therefore, CTLA-4 expression correlated positively with rather than inhibiting CD25 and CD69 expression.

As observed before (Figs 1a and 2), most CD4+ T cells expressed CTLA-4 2 days after 4Y i.n. (Fig. 4c and d). While the majority of CLN cells was of small or intermediate size, as determined by forward scatter (Fig. 4a and b), the small population of blast cells also expressed high levels of CTLA-4 (Fig. 4b and c). Virtually all of the blasting CD4+ T cells in the spleen were also CTLA-4+ (not shown). In addition to CTLA-4, CD4+FSC+ cells expressed high levels of other T cell surface molecules associated with activation (Table 1). Compared to CTLA-4+CD4+ T cells, the CD4+CTLA-4+ population as a whole (Table 1) or when FSC+ cells were excluded (not shown) showed a positive correlation with up-regulated CD4, CD28, CD69 and CD86 expression. Elevated levels of CD86 on CTLA-4+ versus CTLA-4- cells and significant CD69 expression by ~30% of CTLA-4+CD4+ cells were observed up to day 6 after 4Y i.n..
Fig. 3. CTLA-4 expression correlates with early activation markers. Sixteen hours following 100 µg Ac1–9[4Y] i.n. spleen suspensions from Tg4 mice were stained for CD4, CTLA-4 and either CD25 or CD69. CD4⁺ gated cells were further divided into CTLA-4⁺ (filled histograms) and CTLA-4⁻ (open histograms) populations, and analyzed for CD25 and CD69 expression. Histograms are representative of data from four mice.

Fig. 4. 4Y-induced blast cells mainly consist of CD4⁺CTLA-4⁺ cells. Two days after i.n. administration of 100 µg peptide, CD4–FITC and CTLA-4–PE-stained CLN cell suspensions prepared from Tg4 mice were analyzed for size-dependent CTLA-4 expression. The SSC/FSC plots (a and b) show one representative of eight CLN samples. (c and d) CD4 and CTLA-4 expression on FCS high cells only (c) and on all other lymphocytes (d), as gated in (b).
Phenotypic analysis of CTLA-4 and CD28 expression in vivo

Table 1. Correlation of CTLA-4 expression with other T cell ligands/receptors

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<th>89–101 i.n.</th>
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<th>4Y i.n.</th>
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<th>FSC&lt;sup&gt;high&lt;/sup&gt; cells only</th>
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<td></td>
<td>CD4&lt;sup&gt;+&lt;/sup&gt;CTLA-4&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>CD4&lt;sup&gt;+&lt;/sup&gt;CTLA-4&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>TCR (V&lt;sub&gt;B&lt;/sub&gt;2.2) (MFI)</td>
<td>105 ± 1</td>
<td>68 ± 4</td>
<td>75 ± 10</td>
<td>85 ± 11</td>
<td>130 ± 4</td>
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<tr>
<td>CD4 (MFI)</td>
<td>156 ± 3</td>
<td>123 ± 2</td>
<td>164 ± 3</td>
<td>197 ± 4</td>
<td>306 ± 6</td>
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<td>CD28 (MFI)</td>
<td>26 ± 3</td>
<td>38 ± 2</td>
<td>34 ± 1</td>
<td>73 ± 2</td>
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<td>CD25 (% positive)</td>
<td>9 ± 4</td>
<td>86 ± 7</td>
<td>7 ± 7</td>
<td>9 ± 6</td>
<td>38 ± 6</td>
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<td>CD69 (% positive)</td>
<td>7 ± 3</td>
<td>60 ± 18</td>
<td>15 ± 11</td>
<td>41 ± 13</td>
<td>82 ± 2</td>
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<td>CD45RB (MFI)</td>
<td>52 ± 5</td>
<td>16 ± 5</td>
<td>64 ± 13</td>
<td>72 ± 15</td>
<td>230 ± 80</td>
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<td>CD80 (MFI)</td>
<td>3</td>
<td>9 ± 2</td>
<td>3</td>
<td>4 ± 2</td>
<td>1 ± 1</td>
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<tr>
<td>CD86 (MFI)</td>
<td>4</td>
<td>26 ± 13</td>
<td>4</td>
<td>8 ± 2</td>
<td>29 ± 1</td>
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<td>Fas (MFI)</td>
<td>15 ± 2</td>
<td>33 ± 1</td>
<td>28</td>
<td>32</td>
<td>44 ± 2</td>
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MFI, median fluorescence intensity.

Two days after i.n. administration of 100 µg peptide, CLN suspensions from Tg4 mice were triple stained for CD4, CTLA-4 and one of the indicated markers in the table. Gated CD4<sup>+</sup>CTLA-4<sup>+</sup> and CD4<sup>+</sup>CTLA-4<sup>+</sup> populations were analyzed separately. High FCS cells as shown in Fig. 4 were analyzed without further gating. Data represent means ± SD of four to seven samples.

Differences in CTLA-4 and CD28 expression with lower avidity TCR–MHC class II–peptide complex formation in vivo

The two transgenic lines, Tg4 and Tg3, differ only in their TCR expression levels. TCR expression is similar between peripheral Tg4 and T cells from conventional mice or non-Tg littermates of the same strain, whereas peripheral T cells from Tg3 mice express ~50% of physiological TCR levels at the cell surface (38). The 4A and 4Y peptides display ~10- and 1000-fold higher affinities for I-A<sup>2</sup> respectively compared to the wild-type 4K peptide (41,42). Splenocytes from both Tg lines show similar patterns of responsiveness to these peptides in vitro with the dose response being shifted towards lower peptide concentrations with Tg4 splenocytes (23). To determine if CTLA-4 and CD28 expression are influenced by the strength of TCR ligation in vivo, the 4Y, 4A and 4K peptides were given i.n. to Tg3 and Tg4 mice 2 days prior to analysis. In general, CTLA-4 expression correlated with peptide affinity in both Tg lines, with the proportion of CTLA-4<sup>+</sup> among CD4<sup>+</sup> cells being the greatest after 4Y i.n. (Fig. 5a and b). The levels of CTLA-4 in the CTLA-4<sup>+</sup>CD4<sup>+</sup> cells also correlated with peptide affinity (not shown). This difference was most pronounced and reproducible for 4Y- and 4K-induced CTLA-4 expression, whereas the degree of CTLA-4 expression after 4A i.n. was more variable. With all of the three peptides, there was only a small tendency for reduced CTLA-4 expression with the lower TCR expressing Tg3 line.

Up-regulation of CD28 expression above constitutive levels appeared to be more sensitive to increases in peptide affinities with Tg3 compared to Tg4 CD4<sup>+</sup> cells (Fig. 5c and d). Constitutive basal levels of CD28 were comparable in both Tg strains. Maximal increases in CD28 expression following 4Y i.n. were ~25% higher in Tg3 compared to Tg4 mice. Treatment with 4A peptide induced similar CD28 expression in both Tg lines which did not exceed 4K-induced CD28 up-regulation in Tg4 mice and was marginally higher than with 4K-treated Tg3 mice.

Phenotype of CTLA-4<sup>+</sup> cells in control animals

CD4<sup>+</sup> lymphocyte populations from control mice consistently contained a low but significant proportion (3-6%) of CTLA-4<sup>+</sup> cells (Fig. 1A). This population did not arise non-specifically as a result of peptide i.n. since its size and phenotype did not change with the frequency of i.n. treatment, the time...
patterns during T cell activation induced by soluble peptide

In this report we examine CTLA-4 and CD28 expression

CD4

Discussion

In this report we examine CTLA-4 and CD28 expression patterns during T cell activation induced by soluble peptide in vivo. Most peptide-specific T cells expressed high levels of CTLA-4 2 days after i.n. administration of the high-affinity 4Y peptide. The cell surface expression of CTLA-4 was low compared to the levels of intracellular CTLA-4, as described earlier for T cells activated in vitro (34). However, recent reports have shown parallel kinetic changes of surface and intracellular CTLA-4 levels, and indicate that the proportion of intracellular to surface CTLA-4 remains unchanged, suggesting that CTLA-4 in both loci may reflect similar activation-induced changes in expression (34, 43). At the same time after 4Y peptide i.n. CD28 levels were increased only ~2-fold compared to basal constitutive levels. This appears to be similar to CD28 up-regulation during T cell activation in vitro and is consistent with a high increase in the ratio of CTLA-4 to CD28 (31).

T cell activation in vitro induced maximal CTLA-4 expression after 2-3 days (4, 6, 34). We found very similar kinetics for CD4+ T cells ex vivo after peptide i.n.. Changes in CD28 expression occurred with similar kinetics compared to CTLA-4 except for an early transient decrease. Transient down-regulation of surface CD28 has been observed previously after antibody-mediated CD28 ligation and for mitogen-activated T cells in the presence but not in the absence of B7+ CHO cells, indicating that it requires CD28 ligation (44, 45). It was conceivable that down-regulation of CD28 could favour an interaction between CTLA-4 and CD80/86. Since it has been shown that early CTLA-4 cross-linking in vitro can inhibit cell cycle progression as well as CD69 and CD25 expression (8, 9), we examined the correlation of CTLA-4 expression with these phenotypic indicators of T cell activation. We found that CTLA-4 expression after peptide i.n. correlated positively with CD25 and CD69 expression and blast cell formation, suggesting that it did not result in a net negative stimulus at this stage of activation. In a different system, in vitro stimulation of purified T cells alone or mixed cell populations also revealed higher CTLA-4 expression on activated T cells (31). This does not imply, of course, that CTLA-4 expression during the early phase of activation is of no consequence. Since CD28 does not appear to be required for early cell cycle progression (46, 47), however, an antagonistic effect of CTLA-4 at this stage may not be so obvious in the absence of strong cross-linking. It is noteworthy that a significant proportion of T cells still expressed CTLA-4 at a time (days 6-10) when CD28 was no longer elevated above constitutive levels. This constellation might support fast termination of the response following peak activation. One could further speculate that high CTLA-4 expression might reduce the ability of T cells to become activated by future encounters with antigen. CTLA-4-mediated inhibition may be facilitated by up-regulation of CD86 on T cells which, as suggested previously, may not be high enough to substantially contribute to positive co-stimulation but may suffice for significant CTLA-4 ligation (6). Future work will now address the significance of these observations for tolerance induction and examine CD80/86 expression patterns by different types of APC after peptide i.n.

While the overall kinetics were similar for changes in CD28 and CTLA-4 expression, changes in peak expression levels in response to TCR-ligand modulation were different. CTLA-4 expression appeared to correlate positively with TCR ligation although a 2-fold difference in surface TCR levels made little difference compared to a 1000-fold difference in peptide affinity. Unlike highly reproducible 4Y- and 4K-induced CTLA-4 expression with little variability in both Tg lines, there was substantial variation after 4A i.n.. This could be interpreted as a closer to sigmoidal than linear positive correlation between peptide–MHC class II affinity and CTLA-4 expression, with 4Y and 4K falling within plateau ranges, and 4A within a linear range more sensitive to small differences in the uptake and/or response to 4A peptide between mice. In contrast to modulation of CTLA-4 expression, CD28 up-regulation was far less sensitive to improved peptide–MHC class II ligation, at least in Tg4 mice which express approximately physiological levels of TCR on peripheral T cells. It appeared that lower TCR expression levels by Tg3 T cells improved CD28 up-regulation in response to increases in peptide affinity. It is possible that this observation may reflect a higher requirement for CD28-mediated co-stimulation at lower TCR expression levels. In the H-2^d EAE model, the affinity of peptides for I-A^d correlates with their tolerogenicity (35, 36). It will be important to establish whether the superior sensitivity to increases in peptide affinity of CTLA-4 over CD28 up-regulation may play a role in this respect.

We also detected a small population of CTLA-4+ cells with an unusual phenotype in mice that had not received a specific peptide. The most characteristic features of these CTLA-4+ cells were CD25 expression and low levels of CD45RB. It is possible that CTLA-4 expression could be induced upon activation by endogenous peptides. Although low CD45RB expression could indicate both effector or memory functions, the unique phenotype of these cells and other preliminary data do not support either possibility. These cells were not larger than the CTLA-4- population and also differed from CD45RBlow CTLA-4+ cells in 4Y-treated mice (our unpublished data). It remains to be shown, however, whether CTLA1+ CD4+ cells in control animals represent a single homogeneous population with variable expression of some molecules or whether they constitute two or more distinct populations. The small proportion of ~10% CD25+ cells, for example, might represent memory cells. Considering the intriguing phenotype of the majority of these CTLA-4+ T cells, it is tempting to hypothesize links with CD4+ CD45RB/RClow and CD4+ CD25+ T cells previously identified as vital regulators in normal unimmunized animals. The former protected rats from wasting disease and mice from colitis (48–50). Other investigators emphasized the presence of CD25 as an indicator of regulatory T cells within the normal T cell repertoire (51–55). Failure to mimic regulator...
functions by activation-induced CD25 expression suggested that a particular phenotype including CD25 expression rather than the presence of CD25 per se characterized these regulator cells (54). Since CD45RB<sub>low</sub> and CD25<sup>+</sup>CD4<sup>+</sup> T cells in normal unimmunized mice represent overlapping yet non-identical populations and depletion of either population is sufficient to abrogate regulation, the question remains as to how these markers relate to the functionally relevant phenotype of such regulator T cells. If CTLA-4 constitutes a common denominator, depletion of CD45RB<sub>low</sub> or CD25<sup>+</sup> cells would remove most CTLA-4<sup>+</sup> cells. Although CTLA-4 blockade of CD25<sup>+</sup> T cells did not interfere with regulation in an in vitro model (54), a possible function of CTLA-4 expression could be size control of this cell population itself. The combination of CTLA-4, CD25 and Fas might enable survival while avoiding expansion of this population. CD69 expression, observed for ~60% of CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells although the functional significance of this observation remains unclear (54). It will be important to determine the functional properties of the minor population of CD4<sup>+</sup>CD45RB<sub>low</sub> CTLA-4<sup>+</sup>CD45RB<sub>low</sub> cells. If constitutive CTLA-4 expression were linked to vital regulation in this context, it would add another note to the repertoire of CTLA-4 for immune homeostasis.

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Abbreviations

Ac acetylated
CLN cervical lymph node
i.n. intranasal
MBP myelin basic protein
Tg transgenic

References


