Immunology 1998 **93** 366–375

Non-responsiveness of antigen-experienced CD4 T cells reflects more stringent co-stimulatory requirements

M. E. HAMEL,* E. NOTEBOOM† & A. M. KRUISBEEK* *Divisions of *Immunology and †Cell Biology, the Netherlands Cancer Institute, Amsterdam, the Netherlands*

SUMMARY

We recently reported that previously activated T cells, irrespective of the nature of the first stimulus they encountered, are unable to respond to Staphylococcal enterotoxin B (SEB), nor to soluble anti-CD3 monoclonal antibody (mAb) presented by splenic antigen-presenting cells (APC). Such previously activated T cells are, however, fully capable of responding to plate-bound anti-CD3 plus splenic APC. These data suggest differential integration of the T-cell receptor (TCR) and co-stimulatory signalling pathways in naive versus antigen-experienced T cells. Consistent with this hypothesis, anti-CD28 mAb restores the proliferative capacity of resting *ex vivo* $CD45RB^{lo}$ $CD4^+$ T cells (representing previously activated T cells) to both soluble anti-CD3 mAb and SEB. Interestingly, mAb-mediated engagement of cytotoxic T-lymphocyte antigen-4 (CTLA-4) completely negates the rescue effects mediated by anti-CD28 mAb in $CD45RB^{lo}$ cells. Nevertheless, the non-responsiveness of $CD45RB^{lo}$ $CD4^+$ T cells cannot be reversed by anti-CTLA-4 Fab fragments, indicating that it is not related to negative regulatory effects of CTLA-4 engagement itself. Interestingly, the addition of interleukin-2 (IL-2) restores the proliferative capacity of $CD45RB^{lo}$ $CD4^+$ T cells to SEB and soluble anti-CD3 mAb. Moreover, when rescued by IL-2, the cells are less susceptible to the negative regulatory effects of CTLA-4 engagement. Together, these findings suggest that the non-responsiveness of $CD45RB^{lo}$ $CD4^+$ T cells to certain stimuli may be related to inadequate TCR signalling, primarily affecting IL-2 production.

INTRODUCTION

Peripheral T-cell non-responsiveness can be induced by several different types of interactions, through yet to be fully defined molecular mechanisms. One model of non-responsiveness is based on the observation that T helper cell type 1 (Th1) clones, triggered via their T-cell receptor (TCR) in the absence of co-stimulation, are non-responsive to subsequent restimulation. Such cells display a strongly reduced capacity to proliferate, due to an inability to produce interleukin-2 (IL-2).[1–3] Another model uses the observation that *in vivo* exposure to either bacterial or retroviral superantigens (SA) initially leads to proliferation of T cells bearing the pertinent Vβ TCR, followed by specific apoptotic death in some of the responding T cells and long-lasting proliferative non-responsiveness to rechallenge with superantigens in the remaining population. Non-responsiveness in the superantigen model is also related to an intrinsic inability to produce IL-2.[4–9]

Received 16 September 1997; revised 30 November 1997; accepted 30 November 1997.

Abbreviations: SEB, Staphylococcal enterotoxin B.

Correspondence: Prof. Dr A. M. Kruisbeek, Division of Immunology, the Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands.

The current view on the requirements for T-cell activation states that T lymphocytes need to integrate at least two signals provided by antigen-presenting cells (APC).[10] The first signal originates from the peptide–major histocompatibility complex (MHC)/TCR interaction and can also be induced by superantigens and antibodies to the TCR–CD3 complex. Signal 2 is a so-called co-stimulatory signal, which is antigen-independent and can be transmitted by soluble factors and by the interaction of a number of membrane-bound molecules expressed on APC with their respective receptors on T cells.[10] Co-stimulation via the CD28 receptor on T cells has been studied most extensively.[11–15] CD28 expressed on T cells can interact with at least two counter receptors, CD80 and CD86.[13,15–17] CD28 triggering has been shown to enhance IL-2 production by inducing IL-2 transcription[18] and by stabilizing IL-2 mRNA transcripts.[19,20] Furthermore, CD28 co-stimulation is able to promote T-cell survival by enhancing the expression of the cell survival factor Bcl-x_L.[21] Recently, CD28–CD80/CD86 interactions were shown to decrease the threshold number of triggered TCR required for the induction of T-cell activation.[22] Currently, only CD28-derived co-stimulation during the initial TCR engagement has been shown to prevent the induction of non-responsiveness in Th1 clones when stimulated under anergizing conditions.[23]

It has been postulated that the phenomenon of non-

responsiveness observed in the superantigen model is also related to TCR triggering in the absence of co-stimulation via CD28, i.e., by presentation on non-professional APC *in vivo*,[7] since superantigens do not need to be processed before they bind to MHC II molecules and engage TCR (reviewed in[24]). However, we have shown in previous experiments[25] that T cells pre-stimulated *in vitro* with Staphylococcal enterotoxin B (SEB) presented by dendritic cells, which constitutively express high levels of the CD28 counter receptor CD86,[26] are as non-responsive to subsequent SEB encounter as T cells pre-stimulated by any other APC.[25] Furthermore, we documented that, irrespective of the nature of the first stimulus, all previously *in vitro*-activated resting T-cell populations display a non-responsive phenotype to SEB rechallenge.[25] Together, these data suggest that this type of non-responsiveness is the inevitable result of the maturation of unprimed T cells to antigen-exposed T cells.

In the present study, we investigated the mechanisms of non-responsiveness of previously activated T cells, using resting *ex vivo* $CD4^+$ T cells. Isolation of $CD4^+$ T cells expressing low levels of CD45RB enriches for a population of antigen-experienced cells.[27–37] These cells are regarded as less dependent on co-stimulation than their counterparts expressing high levels of CD45RB.[29,38] However, we visualize here that $CD45RB^{lo}$ $CD4^+$ T cells are not able to proliferate in response to certain stimuli presented by APC, in contrast to $CD45RB^{hi}$ $CD4^+$ T cells. We also examine the co-stimulatory requirements of $CD45RB^{lo}$ $CD4^+$ T cells stimulated under these conditions, and address the possible contribution of the recently identified negative regulator CTLA-4[39,40] to this type of non-responsiveness.

MATERIALS AND METHODS

Mice

Adult BALb/c and C57BL/6 mice (6–10 weeks old) were obtained from our breeding facilities and housed under specific pathogen-free conditions.

Monoclonal antibodies and cytokines

The following mAb were used in this study: 145-2C11 (anti-CD3ε), GK1·5, RL172·4 (both anti-CD4), 2·43, 53-6·7 (both anti-CD8), M5/114 (anti-MHC class II), PC 61 5·3 (anti-IL-2Rα), J1J10 (anti-Thy-1·2), RG7/7·6 (mouse-anti-rat immunoglobulin κ, also reactive with hamster immunoglobulin κ) [all obtained from American Type Culture Collection (ATCC), Rockville, MD]. The following mAb were purchased from Pharmingen, San Diego, CA: 16A (anti-CD45RB), 37·51 (anti-CD28),[23] 4F10 (anti-CTLA-4).[39] LG.3A10 (anti-murine CD27)[41] was obtained from L. A. Gravestein (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

Human recombinant IL-2 (Cetus Corporation, Emeryville, CA) was used at a final concentration of 50 U/ml, unless otherwise indicated.

Fab fragments

Total hamster IgG (Pierce Chemical Co, Rockford, IL) and 4F10 were cleaved into Fab fragments using papain-immobilized agarose beads (Sigma Chemical Co, St Louis, MO) for 18 hr at 37°. Purity of the Fab fragments was confirmed by non-reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Functionality of 4F10 Fab fragments was tested by flow cytometry using the endocytosis assay described by Linsley *et al.*[42] Spleen cells were activated for 2 days with soluble anti-CD3 mAb. Dead cells were removed by Ficoll gradient centrifugation (Lympholyte-M, Cedarlane). Activated T cells (2×10^5) were incubated in round-bottom wells in 200 µl complete medium at 37° with 1 µg/ml phycoerythrin (PE)-labelled 4F10 mAb (Pharmingen) in the presence of saturating concentrations of anti-FcR mAb (2.4G2), 10 µg/ml unlabelled 4F10 mAb or hamster IgG or varying concentrations of 4F10 or hamster IgG Fab fragments. After 6 hr, cells were washed once with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide. Samples were analysed by flow cytometry as described.[25] Fab fragments were used in proliferation assays at a final concentration of 40 µg/ml.

T-cell preparation: isolation of $CD45RB^{hi}$ and $CD45RB^{lo}$ $CD4^+$ T cells

$CD4^+$ T cells were obtained from lymph nodes of unprimed mice as described before[25] to a purity level of >98% $CD3^+CD4^+$. To obtain $CD45RB^{hi}$ and $CD45RB^{lo}$ $CD4^+$ cells, $CD4^+$ cells were incubated on ice with saturating amounts of biotinylated anti-CD45RB mAb in medium plus 5% fetal calf serum (FCS), washed twice and incubated with avidin–fluorescein isothiocyanate (FITC; Sigma). After washing, cells were sorted on the basis of their CD45RB expression into $CD45RB^{hi}$ and $CD45RB^{lo}$ cells using a Becton-Dickinson FACSTAR PLUS sorter (Becton-Dickinson, San Jose, CA).

Proliferation assays

Cultures were performed in round-bottomed microtitre wells in complete medium as described.[25] Twenty thousand to fifty thousand purified T cells were incubated with 1×10^5–2×10^5 irradiated (20 Gy) syngeneic T-cell-depleted spleen cells as a source of APC in a total volume of 200 µl per microtitre well. Cultures were incubated in a 37°, 5% CO_2 humidified incubator. SEB (Sigma) was used at 10 µg/ml. For immobilized, plate-bound antibody-induced proliferation, purified anti-CD3ε (145-2C11) was diluted in PBS to 10 µg/ml and 50 µl was added per microtitre well. Plates were incubated for 4–5 hr at 37° and washed three times with PBS before use. Alternatively, anti-CD3 mAb was used in soluble form at 10 µg/ml. IL-2, anti-CD27, anti-CD28 and anti-CTLA-4 mAb were added to the cultures as indicated in the Figs. Purified total hamster IgG (Pierce) was used as a negative control for hamster antibodies. After 72 hr, cultures were pulsed with 0·4 µCi/well [^{3}H]thymidine ([^{3}H]TdR) and harvested 12–18 hr later. [^{3}H]TdR incorporation was determined using liquid scintillation counting. Determinations were performed in triplicate and standard deviations are as indicated in the figures.

RESULTS

Anti-CD28 mAb restores the proliferative response of $CD45RB^{lo}$ $CD4^+$ T cells to SEB

We reported recently that previously activated T cells exhibit an intrinsic inability to respond to SEB.[25] Since it is unclear to what extent T-cells that have been previously activated *in vitro*, as used in our previous report,[25] resemble a physiologi-

cally occurring population of T cells that has developed *in vivo*, we determined whether the same phenomena hold true for resting CD4$^+$ T cells present in the CD45RB^{lo} population.

Sorting CD4$^+$ T cells by flow cytometry based on the level of CD45RB expression results in separation of cells with a CD45RB^{lo} and CD45RB^{hi} phenotype. Both subsets were small in size and expressed no or dim levels of IL-2 receptors (IL-2R; data not shown). The CD45RB^{hi} phenotype has been reported to include both authentic naive[27–29] and long-term memory T cells[30–33] in a resting state, while the CD45RB^{lo} population is enriched for more recently activated T cells,[31–34] in addition to memory cells.[29,35–37] Importantly, in young mice, as used in this study, most CD45RB^{hi} CD4 T cells are likely to represent naive CD4 T cells.

Using these populations, we tested whether our earlier observation that recently antigen-exposed T cells respond differently to superantigen stimulation than unprimed CD4$^+$ T cells holds true. Since we noted that even after 48 hr of culture, CD45RB^{hi} CD4$^+$ T cells still are highly fluorescent due to bound FITC-labelled anti-CD45RB mAb, unsorted and unlabelled CD4$^+$ T cells which consist of approximately 85% CD45RB^{hi} and 15% CD45RB^{lo} CD4$^+$ T cells are included in most Figs as controls. Generally, responses of these unsorted CD4$^+$ T cells are in arithmetic accordance with the relative contributions of CD45RB^{hi} and CD45RB^{lo} CD4$^+$ T cells (see Figs), indicating that no artefacts are introduced by flow cytometry sorting or the presence of bound anti-CD45RB mAb. [^{3}H]TdR incorporation values for unsorted, CD45RB^{hi} and CD45RB^{lo} CD4$^+$ T cells responding to plate-bound anti-CD3 mAb and SEB are shown in Fig. 1(a). All populations of CD4$^+$ T cells respond equally well to plate-bound anti-CD3 mAb in the presence of T-cell-depleted spleen cells as a source of APC (Fig. 1a), confirming data reported by Croft *et al.*[29] Unsorted CD4$^+$ T cells and CD45RB^{hi} CD4$^+$ T cells also respond to SEB when presented by splenic APC. In contrast, CD45RB^{lo} CD4$^+$ T cells do not respond at all under these conditions (Fig. 1a), in agreement with our earlier data on *in vitro*-activated T cells[25] and with data reported by Lee and Vitetta.[43] Kinetic analysis reveals no [^{3}H]TdR incorporation by CD45RB^{lo} CD4$^+$ T cells after 24 and 48 hr either (data not shown), showing that the lack of proliferation after 72 hr is not the result of accelerated kinetics of CD45RB^{lo} CD4$^+$ T cells responding to SEB presented by splenic APC.

One explanation for these results could be that the co-stimulatory requirements of CD45RB^{lo} CD4$^+$ T cells responding to SEB are different from those of CD45RB^{hi} CD4$^+$ T cells. Importantly, the APC in this experimental setting are fully competent with respect to providing co-stimulation to CD45RB^{hi} CD4$^+$ T cells. Co-stimulation to CD45RB^{lo} CD4$^+$ T cells may therefore be qualitatively or quantitatively inadequate. Cross-linking of the CD28 receptor through mAb addition reversed the non-responsiveness of the CD45RB^{lo} CD4$^+$ T cells (Fig. 1a, b), resulting in [^{3}H]TdR incorporation levels comparable to those of CD45RB^{hi} CD4$^+$ T cells. The increase in [^{3}H]TdR incorporation observed in unsorted CD4$^+$ T cells (Fig. 1a, b) is probably related to the rescue from non-responsiveness of CD45RB^{lo} CD4$^+$ T cells. The proliferation of CD45RB^{hi} CD4$^+$ T cells is only marginally influenced by the addition of anti-CD28 mAb (Fig. 1a, b), suggesting optimal co-stimulation for CD45RB^{hi} CD4$^+$ T cells by splenic APC.

Other interventions aimed at provision of co-stimulatory activity could not rescue the non-responsiveness of CD45RB^{lo}

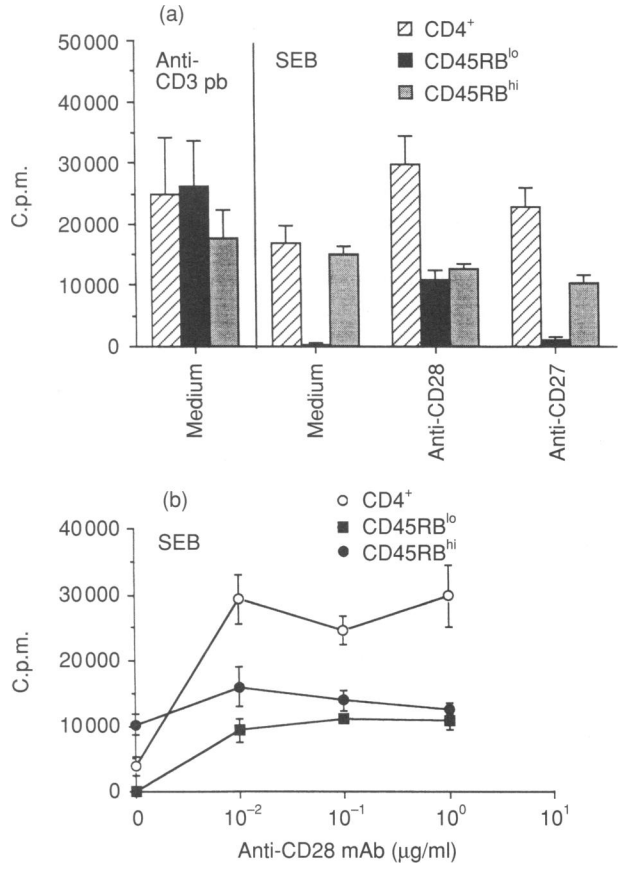

Figure 1. CD45RB^{lo} CD4$^+$ T cells are able to respond to plate-bound anti-CD3 antibody, but not to SEB; anti-CD28 mAb restores the proliferative capacity of CD45RB^{lo} T cells responding to SEB. Fifty thousand unseparated, total CD4$^+$ T cells, or CD45RB^{hi} or CD45RB^{lo} CD4$^+$ T cells were incubated with 2×10^5 T-cell-depleted syngeneic irradiated spleen cells and (a) anti-CD3 mAb (plate-bound) or SEB in the absence or presence of 1 μg/ml anti-CD28 mAb or 1 μg/ml anti-CD27 mAb; (b) SEB in the presence of increasing concentrations of anti-CD28 mAb. The dose of 10 μg/ml SEB was optimal for all conditions (see ref. 25). ^{3}H-Thymidine incorporation in the presence of anti-CD28 mAb or anti-CD27 mAb in the absence of SEB did not exceed 500 c.p.m. Data are representative of four experiments.

CD4$^+$ T cells. For instance, a mixture of IL-1 and IL-6 has no effect on the proliferative capacity of CD45RB^{lo} CD4$^+$ T cells to SEB (data not shown), in spite of being co-stimulatory for all CD4$^+$ T-cell populations tested when plate-bound anti-CD3 is used in the absence of APC (data not shown; see also ref. 44). Also, anti-CD27 mAb cannot rescue the CD45RB^{lo} CD4$^+$ T cells from non-responsiveness to SEB (Fig. 1a), showing the specificity of the CD28-mediated effects. In sharp contrast, in the absence of splenic APC, antibody-mediated cross-linking of CD27 is as effective as CD28 in co-stimulating a proliferative response in CD45RB^{lo} CD4$^+$ T cells when platebound anti-CD3 mAb is used as a stimulus (Fig. 2a, b). While future experiments will address the different means through which CD28 and CD27 engagement induce co-stimulatory activity, the point to be derived from these results is that CD28, but not CD27, induces rescuing events in CD45RB^{lo} cells, when stimulated with SEB in the presence of splenic APC.

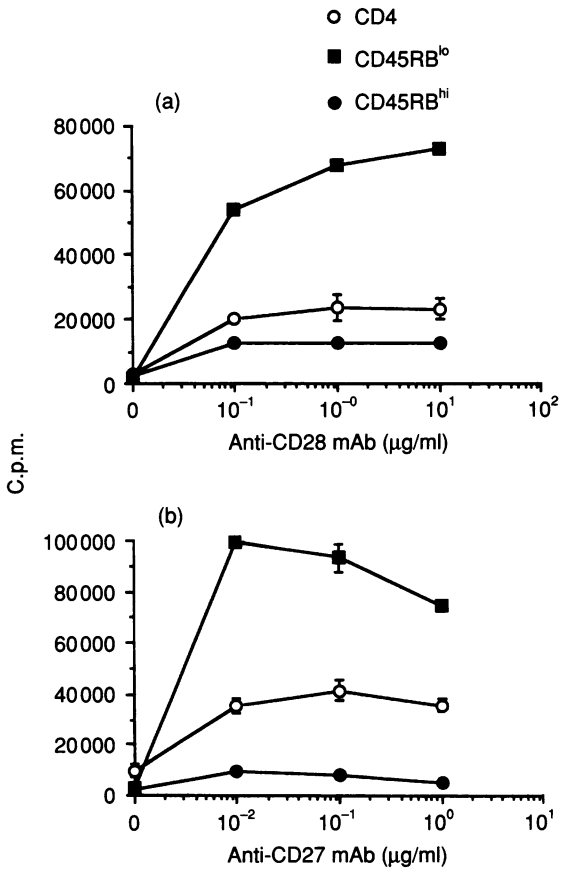

Figure 2. Both anti-CD28 and anti-CD27 mAb co-stimulate CD45RB^{lo} CD4^{+} T cells when responding to plate-bound anti-CD3. (a) Fifty thousand unseparated, CD45RB^{hi} and CD45RB^{lo} CD4^{+} T cells were stimulated with plate-bound anti-CD3 mAb and increasing doses of anti-CD28 mAb, in the presence of 10 μg/ml cross-linking mAb (RG7/7.6). (b) Cells were stimulated as described above in the presence of increasing doses of anti-CD27 mAb and 10 μg/ml RG7/7.6). Proliferation in the absence of plate-bound anti-CD3 mAb was <1500 c.p.m. for all CD4^{+} T-cell subsets in the presence or absence of anti-CD28 and anti-CD27 mAb. Data are representative of two experiments.

Taken together, these data show that CD45RB^{lo} CD4^{+} T cells, in contrast to CD45RB^{hi} CD4^{+} T cells, are unable to mount a proliferative response to SEB presented by splenic APC. The finding that mAb-mediated CD28 engagement is sufficient to rescue the cells from non-responsiveness suggests that co-stimulatory signalling in CD45RB^{lo} CD4^{+} T cells as provided by splenic APC is quantitatively or qualitatively insufficient.

CD45RB^{lo} CD4^{+} T cells do not respond to soluble anti-CD3 mAb presented by splenic APC; anti-CD28 mAb restores the proliferative capacity of these cells

A recent report by Farber *et al.*[27] documented that CD45RB^{lo} CD4^{+} T cells, in contrast to CD45RB^{hi} CD4^{+} T cells, are non-responsive to soluble anti-CD3 mAb presented by splenic APC. As this type of non-responsiveness resembles the one described for SEB by Lee & Vitetta[43] and by us[25] (Fig. 1) in being confined to CD45RB^{lo} CD4^{+} T cells, we investigated

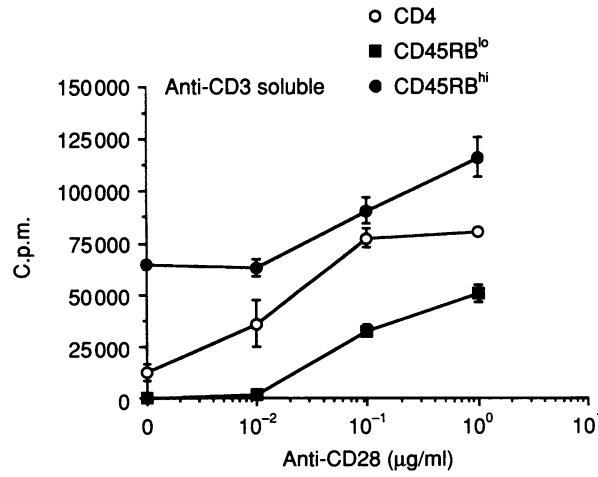

Figure 3. CD45RB^{lo} CD4^{+} T cells are non-responsive to soluble anti-CD3 mAb presented by T-cell-depleted spleen cells; anti-CD28 mAb restores proliferative capacity of CD45RB^{lo} CD4^{+} T cells to soluble anti-CD3 mAb. Twenty thousand total CD4^{+} T cells, CD45RB^{hi} or CD45RB^{lo} T cells were incubated with 1×10^{5} T-cell-depleted spleen cells and soluble anti-CD3 mAb in the presence of increasing doses of anti-CD28 mAb. Data are representative of five experiments.

whether anti-CD28 mAb rescues CD45RB^{lo} CD4^{+} T cells from non-responsiveness in this experimental setting as well. Indeed, as shown in Fig. 3, while no [^{3}H]TdR incorporation is observed when CD45RB^{lo} CD4^{+} T cells are stimulated with anti-CD3 mAb presented by splenic APC, the addition of anti-CD28 mAb restores the proliferative capacity of CD45RB^{lo} CD4^{+} T cells. In contrast, the addition of IL-1/6 or anti-CD27 mAb did not reverse the non-responsiveness of CD45RB^{lo} CD4^{+} T cells to soluble anti-CD3 (data not shown). These data suggest a common underlying mechanism for the phenomena of non-responsiveness of CD45RB^{lo} CD4^{+} T cells to SEB and soluble anti-CD3 mAb presented by splenic APC.

The susceptibility to inhibitory signals derived from CTLA-4 is largely restricted to the CD45RB^{lo} CD4^{+} T cells subset

CTLA-4 is a receptor on T cells which recently has been shown to regulate negatively T-cell responses.[39,40,45,46] CTLA-4, like CD28, can use CD80 and CD86 as ligands.[38] Given the non-responsiveness of CD45RB^{lo} CD4^{+} T cells to SEB and soluble anti-CD3 mAb, we hypothesized that this non-responsiveness might be a consequence of the dominant effects of interaction of their CTLA-4 with CD80/CD86 molecules present on splenic APC. A variant of this hypothesis states that engagement of CTLA-4 on CD45RB^{lo} CD4^{+} T cells induces qualitatively or quantitatively different signal transduction pathways which, together with TCR engagement and despite CD28 engagement, result in non-responsiveness. The basic premise of both hypotheses is that the balance between positive regulatory effects of CD28 and the negative regulatory effects of CTLA-4 is different between CD45RB^{hi} and CD45RB^{lo} CD4^{+} T cells. To test these hypotheses, we compared total CD4^{+} T cells, purified CD45RB^{hi} and CD45RB^{lo} CD4^{+} T cells for their sensitivity to the effects of CD28 and CTLA-4 engagement when activated with soluble anti-CD3 mAb in the presence of splenic APC.

As shown in Fig. 4(a), the addition of anti-CD28 mAb in the absence of anti-CTLA-4 mAb restores (see also Figs 1 and 3) the proliferative capacity of CD45RB^{lo} CD4$^+$ T cells stimulated with soluble anti-CD3 mAb. Strikingly, the addition of anti-CTLA-4 mAb completely obliterates, in a dose-dependent fashion, the anti-CD28-supported proliferation. This significant block in proliferation is not related to competition for availability of Fc receptors, as 10 μg/ml of irrelevant hamster mAb does not exert this effect (data not shown). In contrast, CD45RB^{hi} CD4$^+$ T cells (Fig. 4b) display a different sensitivity to mAb-mediated engagement of CD28 and CTLA-4 than CD45RB^{lo} CD4$^+$ T cells. First, in agreement with data shown in Figs 1 and 3, [^{3}H]TdR incorporation is observed in cultures of CD45RB^{hi} CD4$^+$ T cells responding to soluble anti-CD3 mAb, implying sufficient co-stimulation by splenic APC for this T-cell population to induce proliferation. In Fig. 4, no enhancing effect on the proliferation of CD45RB^{hi} CD4$^+$ T cells is observed upon addition of anti-CD28 mAb. In some experiments, such as shown in Fig. 3 and Fig. 5 (to be discussed below), we observed an enhancing effect of CD28-mediated co-stimulation on the proliferative capacity of this CD4 T-cell subset. These discrepancies are likely to be related to experimental variations. More importantly, the overall effect of the addition of anti-CTLA-4 mAb to soluble anti-CD3 mAb cul-

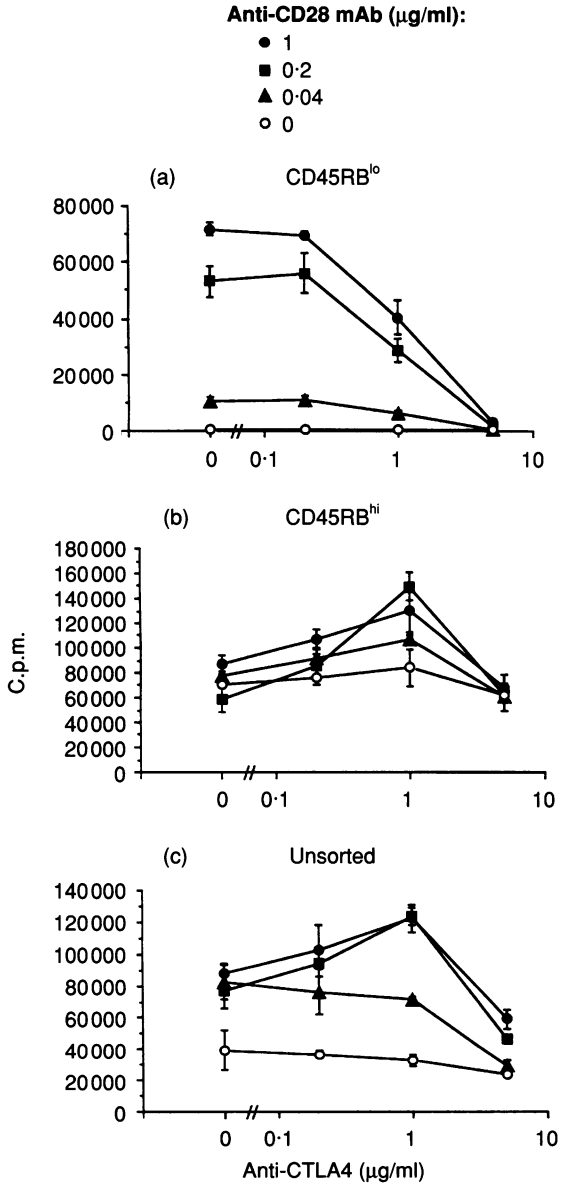

Figure 4. Susceptibility to inhibitory effects of CTLA-4 engagement is restricted to CD45RB^{lo} CD4$^+$ T cells. Twenty thousand CD45RB^{lo} (a), CD45RB^{hi} (b) or unsorted CD4$^+$ T cells (c) were incubated with soluble anti-CD3 mAb in the presence of 1×10^5 T cell depleted spleen cells as APCs, increasing doses of anti-CTLA-4 mAb and varying concentrations of anti-CD28 mAb, as described in the Materials and Methods. The addition of 10 μg/ml irrelevant hamster mAb did not exert any effect on [^{3}H] TdR incorporation levels (data not shown). Data are representative of three experiments.

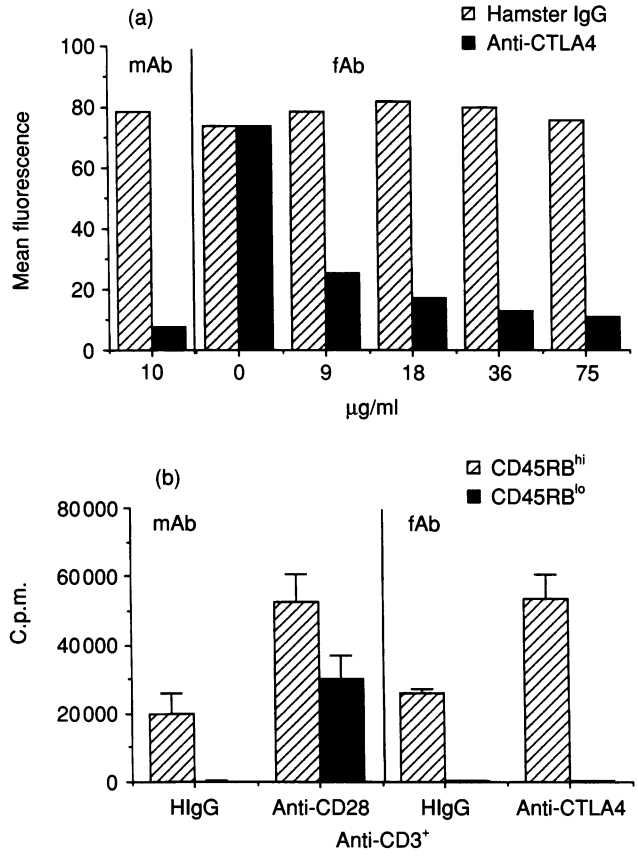

Figure 5. Non-responsiveness of CD45RB^{lo} CD4$^+$ T cells to soluble anti-CD3 mAb is not related to CTLA-4. (a) Anti-CTLA-4 Fab fragments effectively block CTLA-4 staining of 2-day-activated splenic T cells. Spleen cells were activated for 2 days with anti-CD3 mAb, after which period all living cells were T cells, based on Thy1-staining (data not shown). Subsequently, 2×10^5 activated T cells were incubated as described in the Materials and Methods with 1 μg/ml PE-labelled anti-CTLA-4 mAb in the presence of 10 μg/ml unlabelled hamster IgG or anti-CTLA-4 mAb (left panel) or varying doses of Fab fragments derived from hamster IgG or anti-CTLA-4 mAb (right panel). Mean fluorescence was determined by flow cytometry. Mean fluorescence of these cells incubated with PE-labelled irrelevant mAb (background) was 4·8. (b) Twenty thousand CD45RB^{lo} or CD45RB^{hi} CD4$^+$ T cells were incubated with 5 μg/ml anti-CD3 mAb in the presence of 1×10^5 irradiated splenic APCs, in the presence of 1 μg/ml hamster IgG or anti-CD28 mAb (left panel) or 40 μg/ml Fab fragments derived from hamster IgG or anti-CTLA-4 mAb (right panel).

tures of CD45RB^{hi} CD4$^+$ T cells is modest, as shown in Fig. 4(b). In fact, with increasing anti-CTLA-4 mAb concentrations a slight but reproducible increase in [^{3}H]TdR incorporation is observed, followed by a slight decrease in [^{3}H]TdR incorporation for the highest anti-CTLA-4 mAb concentration used. The differential susceptibility of CD45RB^{lo} and CD45RB^{hi} CD4$^+$ T cells to CD28- and CTLA-4-mediated effects is not simply a consequence of differences in CD28 and CTLA-4 expression levels, since CD28 expression levels are identical in both subsets and neither CD45RB^{hi} nor CD45RB^{lo} CD4$^+$ T cells display detectable levels of CTLA-4 when resting (data not shown).

Total CD4$^+$ T cells display an intermediate phenotype with respect to the effects of anti-CD28 mAb and anti-CTLA-4 mAb. Addition of anti-CD28 mAb to soluble anti-CD3 mAb cultures results in an increase in [^{3}H]TdR incorporation by these cells, probably primarily reflecting the induction of proliferation of CD45RB^{lo} CD4$^+$ T cells within this population (Fig. 4c). Also, the addition of anti-CTLA-4 mAb to these cultures results in a more pronounced reduction in [^{3}H]TdR incorporation than in cultures of CD45RB^{hi} CD4$^+$ T cells (Fig. 4c). In conclusion, these data document that among CD4$^+$ T cells, under these experimental conditions, susceptibility to inhibitory signals initiated by CTLA-4 engagement is largely confined to the CD45RB^{lo} CD4$^+$ T-cell subset. It is recognized that in other studies progression of primary T-cell responses can be negatively regulated by anti-CTLA-4 mAb as well. Walunas *et al.*[39] reported that proliferative responses of both unprimed total T cells as well as of unprimed class I-restricted 2C TCR transgenic T cells can be inhibited by anti-CTLA-4 mAb, when provided with suboptimal signal 1 stimulation using anti-CD3 mAb, but co-stimulated optimally with anti-CD28 mAb. Also, Krummel & Allison[40] reported a complete block in proliferation of total unprimed T cells upon CTLA-4 engagement, when stimulated under conditions in which proliferation is dependent on co-stimulation via CD28. Together, these and our data are in agreement with a model in which CTLA-4 exerts its negative regulatory effects only under conditions in which T-cell proliferation is dependent on CD28-mediated co-stimulation.

Non-responsiveness of CD45RB^{lo} CD4$^+$ T cells to soluble anti-CD3 mAb is not related to CTLA-4

Since CD45RB^{lo} CD4$^+$ T cells display a relatively high susceptibility to the negative regulatory effects of CTLA-4 engagement compared to CD45RB^{hi} CD4$^+$ T cells under the experimental conditions used in this report, we tested whether the non-responsiveness of CD45RB^{lo} CD4$^+$ T cells to soluble anti-CD3 mAb is a consequence of interaction of their CTLA-4 with CD80/CD86 molecules on splenic APC. Fab fragments were isolated from anti-CTLA mAb and control hamster IgG. Binding ability of these Fab fragments to CTLA-4 was tested using the endocytosis assay described by Linsley *et al.*[42] (Fig. 5a). Activated T cells were incubated with phycoerythrin (PE)-labelled anti-CTLA-4 mAb in the presence of whole anti-CTLA-4 mAb or whole hamster IgG (Fig. 5a, left panel), or increasing doses of Fab fragments derived from anti-CTLA-4 mAb or hamster IgG (Fig. 5a, right panel). Whole anti-CTLA-4 mAb as well as anti-CTLA-4 Fab fragments effectively competed for endocytosis of PE-labelled anti-CTLA-4 mAb, in contrast to hamster IgG mAb and Fab fragments. Subsequently, these Fab fragments were tested for their ability to rescue CD45RB^{lo} CD4$^+$ T cells from non-responsiveness to soluble anti-CD3 mAb. As shown in Fig. 5(b), CD45RB^{hi} and CD45RB^{lo} CD4$^+$ T cells were cultured with soluble anti-CD3 mAb presented by splenic APC, in the presence of hamster IgG mAb or anti-CD28 mAb (Fig. 5b, left panel) or Fab fragments derived from hamster IgG or anti-CTLA-4 (right panel). Again, CD45RB^{lo} CD4$^+$ T cells were non-responsive to soluble anti-CD3 mAb, and responded when provided with extra co-stimulation by anti-CD28 mAb (Fig. 5b, left panel). CD45RB^{hi} CD4$^+$ T cells, in contrast, respond to soluble anti-CD3 mAb, and in the assay shown, the response could be further enhanced by anti-CD28 mAb (Fig. 5b, left panel). The addition of anti-CTLA-4 Fab fragments to cultures of CD45RB^{hi} CD4$^+$ T cells resulted in a twofold increase in proliferation (Fig. 5b, right panel), when compared to hamster IgG Fab fragments, showing the functionality of the anti-CTLA-4 Fab fragments. However, no rescue from non-responsiveness could be observed in cultures of CD45RB^{lo} CD4$^+$ T cells (Fig. 5b, right panel), indicating that the non-responsiveness of CD45RB^{lo} CD4$^+$ T cells is unlikely to be related to CTLA-4.

Responsiveness of CD45RB^{lo} CD4$^+$ T cells to SEB can be restored by IL-2

It was shown above that CD28 engagement can rescue responses of CD45RB^{lo} cells, while CD27 engagement cannot. It is not clear through which pathways CD27 exerts its co-stimulatory effect, but it has been suggested that it does not act by enhancing IL-2 production.[18] In contrast, among the downstream effects of co-stimulation via CD28 are enhancement of IL-2 production by induction of IL-2 mRNA transcription[19] and by stabilization of IL-2 mRNA transcripts[19,20]. In agreement with our earlier published work,[25] no IL-2 can be measured in 24-hr cultures of CD45RB^{lo} CD4$^+$ T cells responding to SEB, in contrast to cultures of unsorted and CD45RB^{hi} CD4$^+$ T cells (data not shown). This suggests that the lack of proliferation upon SEB encounter observed in CD45RB^{lo} CD4$^+$ T cells is due to an inability to produce IL-2. Indeed, as shown in Fig. 6, the addition of IL-2 restored in a dose-dependent manner the proliferative capacity of these cells to respond to SEB. The addition of IL-2 to CD45RB^{hi} CD4$^+$ T cells responding to SEB did not result in higher [^{3}H]TdR incorporation levels (Fig. 6). The increase in [^{3}H]TdR incorporation upon addition of IL-2 observed in unsorted CD4$^+$ T cells (Fig. 6) is therefore probably due to the induction of proliferation in CD45RB^{lo} CD4$^+$ T cells within this population. Thus, an exogenous source of IL-2 can mimic the rescuing effects of anti-CD28 mAb on the responsiveness of CD45RB^{lo} CD4$^+$ T cells to SEB, probably reflecting the enhancing effects of CD28 engagement on IL-2 production. This hypothesis is supported by our earlier report[25] on lack of IL-2 production by previously activated T cells triggered under certain conditions.

IL-2 restores proliferation of CD45RB^{lo} CD4$^+$ T cells to soluble anti-CD3 mAb and reverses the susceptibility to the inhibitory effects of CTLA-4 engagement

We next examined whether the non-responsiveness to soluble anti-CD3 mAb displayed by CD45RB^{lo} CD4$^+$ T cells can be

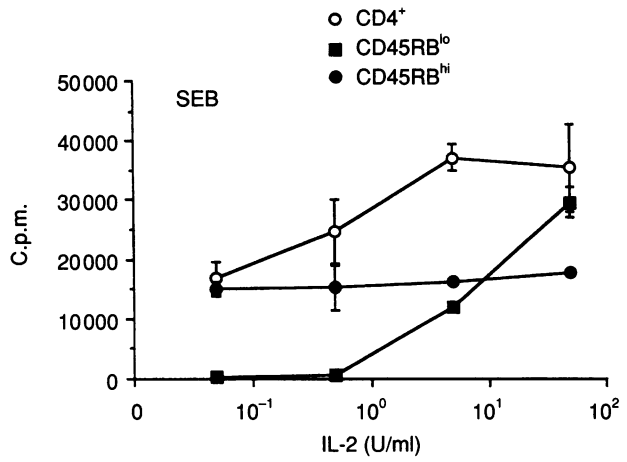

Figure 6. IL-2 restores the proliferative capacity of CD45RB^{lo} CD4$^+$ T cells responding to SEB. Fifty thousand 10^4 unseparated, total CD4$^+$ T cells, or CD45RB^{hi} or CD45RB^{lo} CD4$^+$ T cells were incubated with 2×10^5 T-cell-depleted syngeneic irradiated spleen cells, SEB and increasing concentrations of IL-2. ^{3}H-Thymidine incorporation values for cultures containing IL-2 in the absence of SEB were 3717 c.p.m., 3589 c.p.m. and 1988 c.p.m., for unsorted CD4$^+$ T cells, CD45RB^{lo} and CD45RB^{hi} T cells, respectively. Data are representative of four experiments.

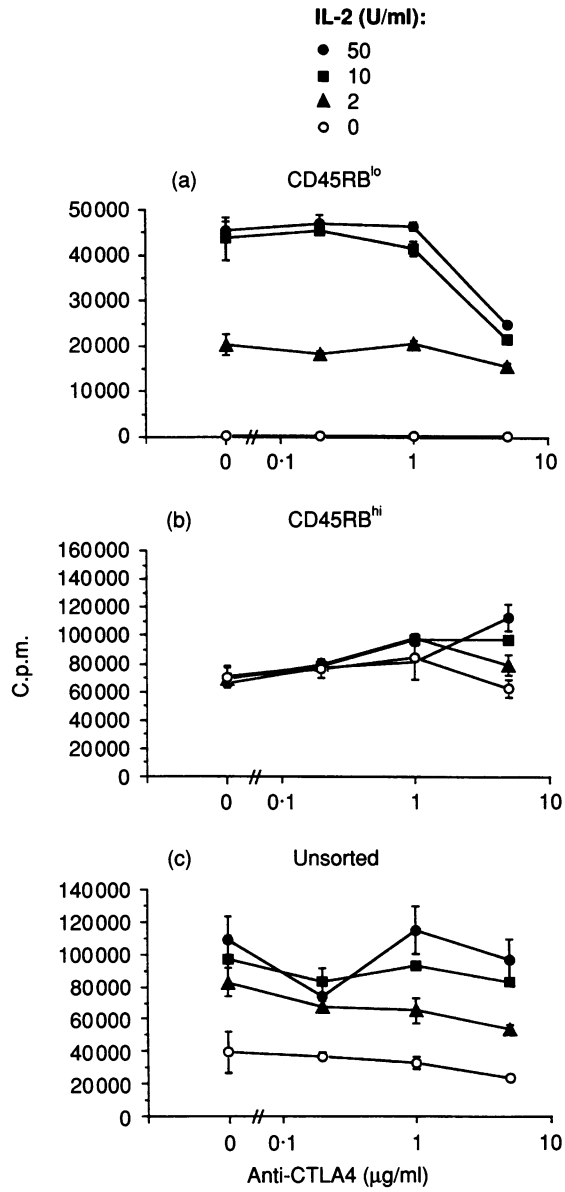

Figure 7. IL-2 restores proliferation of CD45RB^{lo} CD4$^+$ T cells to soluble anti-CD3 mAb and reverses the susceptibility to the inhibitory effects of CTLA-4 engagement. Twenty thousand 10^4 CD45RB^{lo} (a), CD45RB^{hi} (b) or unsorted CD4$^+$ T cells (c) were incubated with soluble anti-CD3 mAb in the presence of 1×10^5 T-cell-depleted spleen cells as APC, increasing doses of IL-2 and varying concentrations of anti-CTLA-4 mAb, as described in the Materials and Methods. The addition of 10 μg/ml irrelevant hamster mAb did not exert any effect on [^{3}H]TdR incorporation levels (data not shown). Data are representative of three experiments.

reversed as well by the addition of IL-2. Indeed, in analogy to the rescuing effects of IL-2 on SEB responses, also soluble anti-CD3 responses are rescued by IL-2 (Fig. 7a; data without anti-CTLA-4). No enhancing effect of IL-2 on CD45RB^{hi} CD4$^+$ T cells was observed (Fig. 7b; data without anti-CTLA-4). Total CD4$^+$ T cells again display an intermediate phenotype, reflecting responses of both CD45RB^{hi} and CD45RB^{lo} CD4$^+$ T cells in this population (Fig. 7c; data without anti-CTLA-4). Together, these findings indicate that the non-responsiveness of CD45RB^{lo} CD4$^+$ T cells to SEB and soluble anti-CD3 mAb reflects an inability to produce IL-2, in agreement with earlier findings.[25]

In Fig. 4, we showed that the non-responsiveness of CD45RB^{lo} CD4$^+$ T cells can be modulated depending on the balance between signalling via CD28 and CTLA-4. This latter molecule was reported to regulate negatively T-cell responses by blocking IL-2 production and cell cycle progression.[39,40,45,46] Thus, CTLA-4 might counterbalance the positive regulatory effects of CD28 triggering in CD45RB^{lo} CD4$^+$ T cells by interfering with IL-2 production. If that hypothesis were true, the prediction is that addition of IL-2 should render these cells resistant to the negative regulatory effects of CTLA-4 engagement. Indeed, in the presence of exogenously added IL-2, CTLA-4 engagement has only a modest effect (Fig. 7a), resulting in at best a 50% reduction in responses only at the highest anti-CTLA-4 mAb concentration used. In fact, no reduction is observed when 1 μg/ml of anti-CTLA-4 mAb is added to IL-2 containing cultures (Fig. 7a), while this dose of anti-CTLA-4 mAb inhibits responses of CD45RB^{lo} CD4$^+$ T cells up to 50% when no exogenous IL-2 is added (Fig. 4a). No negative effects of CTLA-4 engagement are visualized in CD45RB^{hi} CD4$^+$ T cells (Fig. 7b) and only minor effects are visualized in unsorted CD4$^+$ T cells (Fig. 7c). These data are consistent with the notion that CTLA-4-induced non-responsiveness of CD45RB^{lo} CD4$^+$ T cells is at least in part a consequence of interference with IL-2 production.

DISCUSSION

Antigen-experienced T cells are believed to be less dependent on co-stimulation when compared to naive T cells.[29,38] However, we here document that antigen-experienced CD45RB^{lo} CD4$^+$ T cells, in contrast to CD45RB^{hi} CD4$^+$

T cells, display a non-responsive phenotype, when their TCR is triggered by SEB or soluble anti-CD3 mAb, in agreement with other reports.[25,27,43] On the other hand, the response of CD45RB^{lo} CD4$^+$ T cells to plate-bound anti-CD3 mAb is unimpaired, and in fact superior to that of CD45RB^{hi} CD4$^+$ T cells. This non-responsiveness is related to an intrinsic inability to produce IL-2 under these experimental conditions and can be reversed by providing the cells with mAb-mediated CD28 co-stimulation. Importantly, we document that the defect in IL-2 production is unlikely to be related to CTLA-4, despite the fact that CTLA-4 engagement completely negates the CD28 supported proliferation. This notion is reinforced by our unpublished observation that CD45RB^{lo} CD4 T cells are able to respond to soluble anti-CD3 presented by P815 cells transfected with either CD80 or CD86, although these cells require significantly higher numbers of CD80/86 transfectants for the induction of proliferation compared to CD45RB^{hi} CD4 T cells. Furthermore, when titrating anti-CD28 mAb to cultures of CD45RB^{lo} CD4 T cells responding to soluble anti-CD3 mAb in the presence of splenic APC, no shift is observed in the dose–response curve of CD28 mAb, when the interaction of CTLA-4 with CD80/86 is blocked by CTLA-4Ig or a combination of anti-CD80 and CD86 mAb, again arguing against a role of CTLA-4 in the non-responsiveness described in this report (our unpublished observations). We therefore contend that it is highly unlikely that the non-responsiveness of CD45RB^{lo} CD4 T cells to SEB and soluble anti-CD3 mAb is related to CTLA-4. Whether the non-responsiveness of CD45RB^{lo} CD4$^+$ T cells is related to other (unidentified) interactions that negatively regulate T-cell activation or whether TCR signalling is affected remains to be elucidated. It is clear, however, that neither the non-responsiveness nor the CTLA-4 susceptibility of CD45RB^{lo} cells is related to modulations in apoptotic mediators.[47]

Differential responsiveness of naive and antigen-exposed T cells has been attributed to their differential expression of isoforms of the protein tyrosine phosphatase CD45 (reviewed in ref. 48). This molecule is a crucial regulator of the activities of the protein tyrosine kinases (PTK) p56 lck and p59 fyn (reviewed in ref. 49). The importance of CD45 is illustrated by the phenotype of T cells that lack cell surface expression of CD45; such cells are not able to respond to antigen.[50,51] Furthermore, mice deficient in CD45[52] show severe abnormalities in T-cell development and no T-cell proliferation in response to anti-CD3 mAb.[52] Also, p56 lck and p59 fyn are hyperphosphorylated in CD45-deficient mice, leaving p56 inactive. Furthermore, abnormalities in ζ phosphorylation, related to lack of ZAP-70-ζ-association have been reported in CD45-deficient mice.[52]

Alternative splicing of exons 4, 5 and 6 encoding for the extracellular domain of CD45 results in the generation of different isoforms of CD45 (reviewed in ref. 48). Naive T cells express high molecular weight forms of CD45, while memory T cells predominantly express a low molecular weight isoform of CD45.[35] Studies by Novak *et al.*[53] showed that the different isoforms of CD45 differentially affect the sensitivity to MHC class II ligands, with the low molecular isoform found on memory cells conferring the highest sensitivity. Moreover, several studies reported that the low molecular weight forms of CD45 are physically associated with CD4 and the TCR complex, while the high molecular weight forms are not.[54–56] Thus, in memory CD4 T cells, the accessibility of the CD4-associated p56 lck is likely to be higher than in naive CD4 T cells. This might explain why memory T cells are more efficient in responding to peptide presented by APC. The expression of different isoforms of CD45 might represent a way of regulating the efficacy of signalling through a receptor without altering its specificity.[53]

The differential stoichiometry of the TCR in naive and antigen-exposed T cells might also explain the observed non-responsiveness of the latter cells to certain stimuli. The TCR-stimuli involved in this type of non-responsiveness, SA and soluble anti-CD3 mAb, are not presented as small peptide fragments, but bind as a complete protein to components of the TCR-signalling module. Thus, these stimuli might affect the conformation of the complex that is formed by the TCR–CD3 complex, CD4 and CD45, resulting in differential signalling via the TCR. This notion is reinforced by data reported by Patarca *et al.*,[57] which suggest that triggering of the TCR of a CD4$^+$ T cell clone with the Mls^{1a} superantigen leads to a signal transduction pathway that differs from the one induced upon triggering with the pertinent peptide antigen or alloantigen. Possibly, soluble anti-CD3 mAb and SA prevent association of CD45 with TCR/CD3 and CD4 when the low molecular weight form of CD45 is expressed. Indeed, the downstream effects of SA-restimulation of previously SA-exposed T cells reveal decreased ζ-phosphorylation and ζ-ZAP-70 association when compared to naive T cells,[58] resembling the phenotype of cells derived from the CD45-null mice described above.[52]

Non-responsiveness of CD45RB^{lo} CD4$^+$ T cells to soluble anti-CD3 mAb has previously been connected to inhibitory signals delivered via CD4 upon MHC class II interaction selectively in CD45RB^{lo} CD4$^+$ T cells by Farber *et al.*[27] These authors showed that CD45RB^{lo} CD4$^+$ T cells were not able to proliferate in response to soluble anti-CD3 mAb presented by wild-type splenic APC, in agreement with our data. It was further shown that these cells were functional when anti-CD3 mAb was presented by splenic APC derived from MHC class II-deficient mice.[27] In our view, these data are also compatible with the hypothesis postulated above in which soluble anti-CD3 mAb and SEB perturb the stoichiometry of the TCR complex with CD3, CD4 and CD45. CD4 is the only protein known to specifically bind to MHC class II molecules.[59] When SA or soluble anti-CD3 mAb are integrated in the TCR signalling module, MHC class II-CD4 interactions might hamper the accessibility of p56 lck by CD45. The absence of MHC class II in turn might facilitate the CD4–CD45 interaction and thus negate the negative effects related to the binding of SA or anti-CD3 mAb in the TCR-signalling complex. Biochemical analysis of the signalling events in the panel of T-cell transfectants, expressing individual CD45 isoforms,[53] constructed by Bottomly and colleagues, might provide important information concerning this issue.

Interestingly, Novak *et al.*[53] reported that immobilized anti-TCR antibody activates T cells irrespective of their CD45 isoform expression, in agreement with data shown in Figs 1 and 2. These results imply that immobilized anti-CD3 mAb is not a suitable TCR stimulus to study the differential responsiveness of naive and antigen-exposed T cells, since it negates the important role of differential CD45 isoform expression. The non-responsiveness of CD45RB^{lo} CD4$^+$ T cells visualized

here may reflect yet another mechanism for the evolutionary developed self-limiting nature of the T-cell response. Ultimately, attempts at manipulating T-cell-mediated immune responses will benefit from a better understanding of the differential regulation of activation of naive and antigen-exposed T cells.

ACKNOWLEDGMENTS

The authors wish to thank M.A. van Halem for preparing the manuscript, A. J. Schrauwers for biotechnical assistance, K. Kieboom for her enthusiastic and expert technical contribution to this work, and D. Amsen, C. Revilla Calvo, H. Spits, M. Oosterwegel and G. van Bleek for helpful discussions.

REFERENCES

1. Jenkins M.K. & Schwartz R.H. (1987) Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J Exp Med* **165,** 302.
2. Quill H. & Schwartz R.H. (1987) Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J Immunol* **138,** 3704.
3. Mueller D.L. & Jenkins M.K. (1995) Molecular mechanisms underlying functional T-cell unresponsiveness. *Curr Opin Immunol* **7,** 375.
4. White J., Herman A., Pullen A.M., Kubo R., Kappler J.W. & Marrack P. (1989) The Vβ-specific superantigen Staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell* **56,** 27.
5. Rammensee H.G., Kroschewski R. & Frangoulis B. (1989) Clonal anergy induced in mature $V\beta6^+$ T lymphocytes on immunizing Mls-1^b mice with Mls-1^a expressing cells. *Nature* **339,** 541.
6. Kawabe Y. & Ochi A. (1990) Selective anergy of $V\beta8^+$, $CD4^+$ T cells in Staphylococcus enterotoxin B-primed mice. *J Exp Med* **172,** 1065.
7. Rellahan B.L., Jones L.A., Kruisbeek A.M., Fry A.M. & Matis L.A. (1990) *In vivo* induction of anergy in peripheral $V\beta8^+$ T cells by Staphylococcal enterotoxin B. *J Exp Med* **172,** 1091.
8. Webb S., Morris C. & Sprent J. (1990) Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell* **63,** 1249.
9. Kawabe Y. & Ochi A. (1991) Programmed cell death and extrathymic reduction of $V\beta8^+$ $CD4^+$ T cells in mice tolerant to Staphylococcus aureus enterotoxin B. *Nature* **349,** 245.
10. Chambers C.A. & Allison J.P. (1997) Co-stimulation in T cell responses. *Curr Opin Immunol* **9,** 396.
11. Gimmi C.D., Freeman G.J., Gribben J.G. *et al.* (1991) B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc Natl Acad Sci USA* **88,** 6575.
12. Linsley P.S., Brady W., Grosmaire L., Aruffo A., Damle N.K. & Ledbetter J.A. (1991) Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* **173,** 721.
13. Freeman G.J., Borriello F., Hodes R.J. *et al.* (1993) Uncovering of functional alternative CTLA-4 counter-receptor in B7-deficient mice. *Science* **262,** 907.
14. Hathcock K.S., Laszlo G., Dickler H.B., Bradshaw J., Linsley P.S. & Hodes R.J. (1993) Identification of an alternative CTLA-4 ligand costimulatory for T cell activation. *Science* **262,** 905.
15. Azuma M., Ito D., Yagita H. *et al.* (1993) B70 antigen is a second ligand for CTLA-4 and CD28. *Nature* **366,** 76.
16. Freeman G.J., Freedman A.S., Segil J.M., Lee G., Whitman J.F. & Nadler L.M. (1989) B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. *J Immunol* **143,** 2714.
17. Engel P., Gribben J.G., Freeman G.J. *et al.* (1994) The B7-2 (B70) costimulatory molecule expressed by monocytes and activated B cells is the CD86 differentiation antigen. *Blood* **84,** 1402.
18. Hintzen R.Q., Lens S.M.A., Lammers K. *et al.* (1995) Engagement of CD27 with its ligand (CD70) provides a second signal for T-cell activation. *J Immunol* **154,** 2612.
19. Fraser J.D., Irving B.A., Crabtree G.R. & Weiss A. (1991) Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* **251,** 313.
20. Lindsten T., June C.H., Ledbetter J.A., Stella G. & Thompson C.B. (1989) Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* **244,** 339.
21. Boise L.H., Minn A.J., Noel P.J. *et al.* (1995) CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-xL. *Immunity* **3,** 87.
22. Viola A. & Lanza Vecchia A. (1996) T cell activation determined by T cell receptor number and tunable threshold. *Science* **273,** 104.
23. Harding F.A., McArthur J.G., Gross J.A., Raulet D.H. & Allison J.P. (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356,** 607.
24. Woodland D.L. & Blackman M.A. (1993) How do T-cell receptors, MHC molecules and superantigens get together? *Immunol Today* **14,** 208.
25. Hamel M.E., Eynon E.E., Savelkoul H.F.J., Van Oudenaren A. & Kruisbeek A.M. 1995. Activation and re-activation potential of T cells responding to staphylococcal enterotoxin B. *Int Immunol* **7,** 1065.
26. Inaba K., Witmer-Pack M., Inaba M. *et al.* (1994) The tissue distribution of the B7-2 costimulator in mice: Abundant expression on dendritic cells *in situ* and during maturation *in vitro*. *J Exp Med* **180,** 1849.
27. Farber D.L., Luqman M., Acuto O. & Bottomly K. (1995) Control of memory CD4 T cell activation: MHC class II molecules on APCs and CD4 ligation inhibit memory but not naive CD4 T cells. *Immunity* **2,** 249.
28. Croft M., Duncan D.D. & Swain S.L. (1992) Response of naive antigen-specific $CD4^+$ T cells *in vitro*: characteristics and antigen-presenting cell requirements. *J Exp Med* **176,** 1431.
29. Croft M., Bradley L.M. & Swain S.L. (1994) Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J Immunol* **152,** 2675.
30. Ericsson P.-O., Orchansky P.L., Carlow D.A. & Teh H.-S. (1996) Differential activation of phospholipase C-γl and mitogen-activated protein kinase in naive and antigen-primed CD4 T cells by the peptide/MHC ligand. *J Immunol* **156,** 2045.
31. Zimmermann C., Brduscha-Riem K., Blaser C., Zinkernagel R.M. & Pircher H. (1996) Visualization, characterization, and turnover of CD8+ memory T cells in virus-infected hosts. *J Exp Med* **183,** 1367.
32. Powrie F., Correa-Oliveira R., Mauze S. & Coffman R.L. (1994) Regulatory interactions between $CD45RB^{high}$ and $CD45RB^{low}$ $CD4^+$ T cells are important for the balance between protective and pathogenic cell-mediated immunity. *J Exp Med* **179,** 589.
33. Hayden K.A., Tough D.F. & Webb S.R. (1996) *In vivo* response of mature T cells to Mls a antigens: long-term progeny of dividing cells include cells with a naive phenotype. *J Immunol* **156,** 48.
34. Birkeland M.L., Johnson P., Trowbridge I.S. & Pure E. (1989) Changes in CD45 isoform expression accompany antigen-induced murine T-cell activation. *Proc Natl Acad Sci USA* **86,** 6734.
35. Bradley L.M., Croft M. & Swain S.L. (1993) T-cell memory: new perspectives. *Immunol Today* **14,** 197.

36. Bradley L.M., Duncan D.D., Yoshimoto K. & Swain S.L. (1993) Memory effectors: a potent, IL-4 secreting helper T cell population that develops *in vivo* after restimulation with antigen. *J Immunol* **150,** 3119.
37. Swain S.L. (1994) Generation and *in vivo* persistence of polarized Th1 and Th2 CD45RB^{lo} cells. *Immunity* **1,** 543.
38. Lenschow D.J., Walunas T.L. & Bluestone J.A. 1996. CD28/B7 system of T-cell costimulation. *Annu Rev Immunol* **14,** 233.
39. Walunas T.L., Lenschow D.J., Bakker C.Y. *et al.* Immunity **1,** 405.
40. Krummel M.F. & Allison J.P. (1995) CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182,** 459.
41. Gravestein L.A., Nieland J.D., Kruisbeek A.M. & Borst J. (1995) Novel mAbs reveal potent co-stimulatory activity of murine CD27. *Int Immunol* **7,** 551.
42. Linsley P.S., Bradshaw J., Greene J.A., Peach R., Bennett K.L. & Mittler R.S. (1996) Intracellular trafficking of CTLA-4 and focal localization towards sites of TCR engagement. *Immunity* **4,** 535.
43. Lee W.T. & Vitetta E.S. (1992) Memory T cells are anergic to the superantigen Staphylococcal Enterotoxin B. *J Exp Med* **176,** 575.
44. Holsti M.A. & Raulet D.H. (1989) IL-1 and IL-6 synergize to stimulate IL-2 production and proliferation of peripheral T cells. *J Immunol* **143,** 2514.
45. Krummel M.F. & Allison J.P. (1996) CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* **183,** 2533.
46. Walunas T.L., Bakker C.Y. & Bluestone J.A. (1996) CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med* **183,** 2541.
47. Hamel M.E., Leupers, T.J.M. & Kruisbeek A.M. (1997) Nonresponsiveness and susceptibility to CTLA-4 of antigen-exposed CD4 T cells are not regulated by the Bcl-2 family of apoptotic mediators, but can be restored by IL-2. *Thymus*, **24,** 259–277.
48. Trowbridge I.S. & Thomas M.L. (1994) CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu Rev Immunol* **12,** 85–116.
49. Sefton B.M. & Taddie J.A. (1994) Role of tyrosine kinases in lymphocyte activation. *Curr Opin Immunol* **6,** 372.
50. Pingel J.T. & Thomas M.L. (1989) Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell* **58,** 1055.
51. Weaver C.T., Pingel J.T., Nelson J.O. & Thomas M.L. (1991) CD8$^+$ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T-cell receptor stimuli. *Mol Cell Biol* **11,** 4415.
52. Stone J.D., Conroy L.A., Byth K.F. *et al.* (1997) Aberrant TCR-mediated signaling in CD45-Null thymocytes involves dysfunctional regulation of Lck, Fyn, TCR-zeta, and ZAP-70. *J Immunol* **158,** 5773.
53. Novak T.J., Farber D., Leitenberg D., Hong S., Johnson P. & Bottomly K. (1994) Isoforms of the transmembrane tyrosine phosphatase CD45 differentially affect T cell recognition. *Immunity* **1,** 109.
54. Dianzani U., Luqman M., Rojo J. *et al.* (1990) Molecular associations on the T cell surface correlate with immunological memory. *Eur J Immunol* **20,** 2249.
55. Dianzani U., Redoglia V., Malavasi F. *et al.* (1992) Isoform-specific associations of CD45 with accessory molecules in human T lymphocytes. *Eur J Immunol* **22,** 365.
56. Leitenberg D., Novak T.J., Smith B.R. & Bottomly K. (1996) The extracellular domain of CD45 controls association with the CD4-T cell receptor complex and the response to antigen-specific stimulation. *J Exp Med* **183,** 249.
57. Patarca R., Wei F.Y., Irequi M.V. & Cantor H. (1991) Differential induction of Interferon γ gene expression after activation of CD4$^+$ T cells by conventional antigen and Mls superantigen. *Proc Natl Acad Sci USA* **88,** 2736.
58. Migita K., Eguchi K., Kawabe Y., Tsukada T., Ichinose Y. & Nagataki S. (1995) Defective TCR-mediated signaling in anergic T cells. *J Immunol* **155,** 5083.
59. Doyle C. & Strominger J.L. (1987) Interaction between CD4 and class II MHC molecules mediates cell adhesions. *Nature* **330,** 256.