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CD45RO$^+$ memory T cells but not CD45RA$^+$ naive T cells can be efficiently activated by remote co-stimulation with B7

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Abstract

Co-stimulatory signals are absolutely required for T cell activation after TCR – MHC-peptide interaction. The most important co-stimulatory signal known so far is mediated by the interaction of CD28 on T cells with B7 on APC. Here we demonstrate that the co-stimulatory signal from the B7 molecule does not necessarily have to come from the same cell which presents antigen. Titration curves obtained by limiting the amount of anti-CD3 mAb suggests that the same amount of TCR – CD3 cross-linking is required for full T cell activation whether B7 is present on the same or on another cell, but that the kinetics of T cell activation is slower when B7 is present on a separate cell from the primary signal. Finally and most importantly we also show that CD45RO$^+$ memory T cells, but not CD45RA$^+$ naive T cells, can be efficiently activated when B7 is expressed on bystander cells. These findings imply that co-stimulatory activation requirements of B7 are more stringent for naive than for memory T cells, which could be an important mechanism involved in the maintenance of self-tolerance.

Under physiological conditions, T cells are activated when their TCR – CD3 complex binds to antigenic peptides presented on MHC molecules of antigen presenting cells (APC). The function of the TCR – CD3 complex is 2-fold: recognition of the specific antigen in the context of the appropriate MHC molecule, and transmission of an activation signal across the plasma membrane (1,2). However, to induce proliferation and maturation into effector cells, T cells need a second signal in addition to the one transmitted through the TCR – CD3 complex (3). A number of accessory molecules present on the cell surface of T cells with known ligands on the APC have been implicated in providing the co-stimulatory signal in T cell activation: CD2 and CD58 (LFA-3), CD11a/CD18 (LFA-1) and CD54 (ICAM-1), CD28 or CTLA-4 and B7, and CD29/CD49d (VLA-4) and VCAM-1 (4 – 9). Intercellular signaling after TCR – MHC – peptide interaction in the absence of the co-stimulatory signal results in T cell inactivation in the form of clonal anergy (10).

So far, the best candidate co-stimulatory signal that determines whether TCR stimulation leads to full T cell activation, or to T cell anergy, is generated by interaction of CD28 on the T cells with B7 on APC. *In vivo*, the B7 molecule is constitutively expressed on dendritic cells in both lymphoid and non-lymphoid tissue, whereas monocytes/macrophages are only positive under inflammatory conditions (11). It has been demonstrated *in vitro* that cross-linking of the CD28 molecule can rescue mouse T cell clones from becoming anergic (12). In addition, co-stimulation of T cells with mAb to the TCR – CD3 complex and CD28 results in greatly enhanced activation (13 – 15). This effect apparently involves two mechanisms, stabilization of mRNA for IL-2 (14,15) and enhanced gene transcription, mediated by a CD28-responsive element in the enhancer of the IL-2 gene (16). It has also been demonstrated by others and ourselves that cross-linking CD28 can be replaced by presentation of the B7 molecule (17 – 19).

Although it is well accepted that the B7 molecule on professional APC can deliver the co-stimulatory signal needed for T cell activation (17 – 19), it is not well established whether the two signals needed for T cell activation, being TCR – CD3 ligation and B7 – CD28 interaction, need to be delivered by the same cell. Experiments in which mouse T cell clones were used

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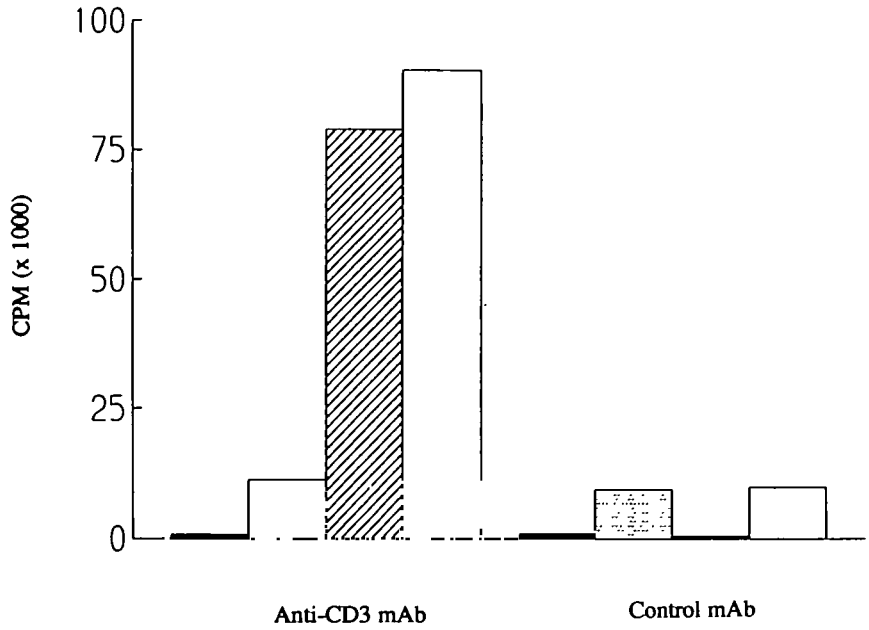

Fig. 1. Co-stimulation of anti-CD3-induced T cell proliferation can be mediated by B7 on the same or on separate cells. Proliferation of purified T cells (4×10^4 cells/well) co-cultured for 5 days with 3T6-CD32 cells (black bar), 3T6-B7 cells (grey bar), 3T6-CD32/B7 cells (hatched bar), or 3T6-CD32 cells together with 3T6-B7 cells (open bars) in the presence of anti-(CD3) mAb CLB-T3/4.1 or control mAb E4 at 100 ng/ml. Proliferation was measured by [^{3}H]thymidine incorporation and represents the mean of triplicate wells. The data is representative of four experiments using T cells from different donors.

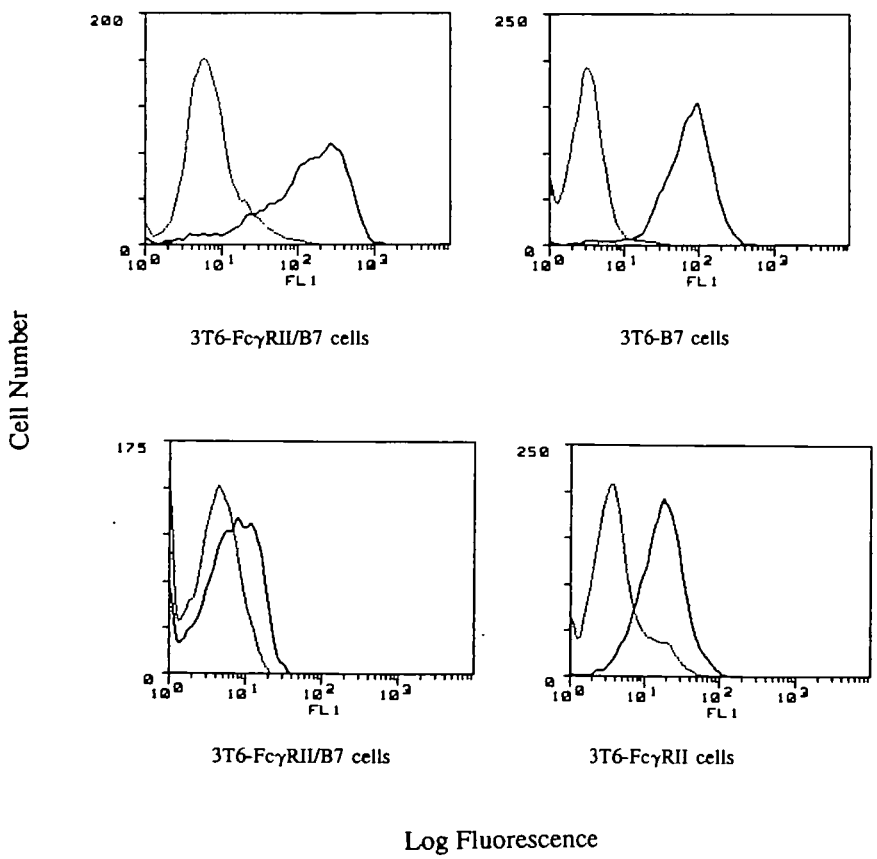

Fig. 2. FACS analysis of B7 expression (staining with anti-B7 mAb B7-24, top panel) or CD32 expression (staining with anti-CD32 mAb AT10, bottom panel) on different 3T6 cell lines. The dotted lines represent staining with the secondary antibody alone, the solid line in the presence of specific antibody.

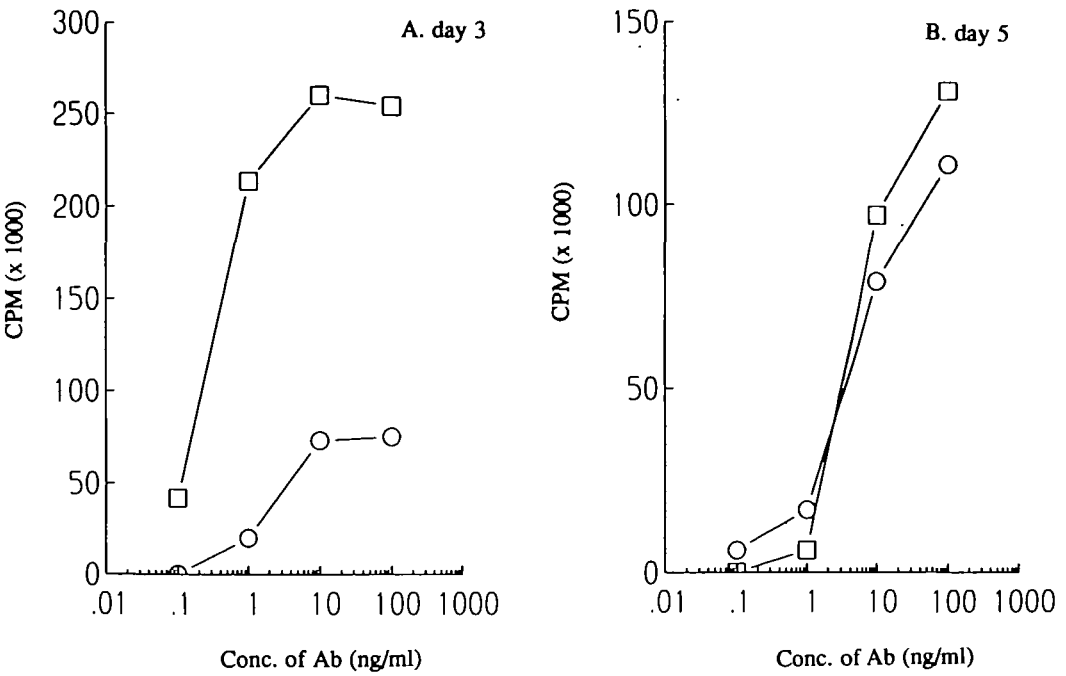

Fig. 3. Co-stimulation of anti-CD3-induced T cell proliferation under suboptimal conditions. Proliferation of purified T cells (4×10^4 cells/well) co-cultured with 3T6-CD32/B7 cells (squares) or 3T6-CD32 cells together with 3T6-B7 cells (circles) in the presence of different concentrations of anti-(CD3) mAb CLB-T3/4.1. (A) Culture period of 3 days (B) Culture period of 5 days Proliferation was measured by [^{3}H]thymidine incorporation and represents the mean of triplicate wells The data is representative of four experiments using T cells from different donors

suggested that the first and second signal can be delivered by separate cells (20,21). However, it was recently demonstrated that for the activation of naive mouse CD4$^+$ T cells, the signal for the TCR – CD3 complex and the co-stimulatory signal provided by B7 needed to come from the same cell (22,23).

For the study presented here we have used peripheral blood mononuclear cells isolated from heparinized blood. T cells were purified as previously described (19). Purified T cells were further separated into CD45RA$^+$ and CD45RO$^+$ cells by immunomagnetic depletion using mAbs and Dynal beads. Purified T cells were cultured with the mouse fibroblast cell line 3T6 expressing human FcγRIIa high responder allele (CD32) (3T6-CD32 cells), 3T6 cells expressing human B7 (3T6-B7 cells), or 3T6 cells expressing CD32 and the B7 molecule (3T6-CD32/B7 cells) as previously described (19). T cells were cultured with irradiated (2500 rad) or mitomycin C-treated (10 μg/ml for 5 h) 3T6 cells in 96-well flat-bottom tissue culture plates in 200 μl/well complete Iscove's modified Dulbecco's medium with or without anti-CD3 mAb CLB-T3/4.1. Proliferation of the T cells was measured by pulsing the cultures with 1 μCi/well [^{3}H]thymidine. T cell proliferation is expressed as the mean c.p.m. of triplicate wells. Expression of cell surface molecules was measured by indirect immunofluorescence. All reagents were diluted in PBS supplemented with 1% BSA and 0.02% NaN_3, and all steps were performed on ice. Cells (10^5/sample) were incubated with 10% heat-inactivated normal goat serum for 30 min. Subsequently, the cells were incubated for 30 min with 0.1 μg mAb in the presence of 10% heat-inactivated normal goat serum. The cells were washed and incubated with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse antibodies. After extensive washing, the cells were analyzed using a FACStar flow cytometer.

In order to analyze the effect of accessory signaling by B7 presented on APC or on bystander cells with human T cells, we used 3T6 mouse fibroblasts transfected with the human FcγRIIa high responder allele (3T6-CD32), with human B7 (3T6-B7), or with both these molecules (3T6-CD32/B7). As previously demonstrated (19), Fig 1 shows that in this system, purified resting T cells cannot be induced to proliferate with mAb to the TCR – CD3 complex alone, but that B7 is able to provide the necessary co-stimulatory signal. Furthermore, we find that under conditions for optimal stimulation of resting human T cells through the TCR – CD3 complex, B7 can provide the second signal, whether present on the same cell as the primary signal or not. Flow cytometry showed that the level of B7 expression on the 3T6-B7 and 3T6-B7/CD32 cells is comparable, and the level of CD32 on the 3T6-CD32/B7 cells is somewhat lower than on the 3T6-CD32 cells (Fig. 2). Figure 3(A) shows that co-stimulation by B7 on a separate cell from the primary signal in a 3 day culture period is possible, but clearly less efficient. The titration curves obtained by limiting the amount of anti-CD3 mAb during a 5 day culture period suggests that the same amount of TCR – CD3 cross-linking is required for full T cell activation whether B7 is present on the same or on another cell. However, it is suggested from the comparison of Fig. 3(A and B) that the kinetics of T cell activation is slower when B7 is present on a separate cell from the primary signal.

If the bystander help via B7 would work *in vivo* for all mature T cells, this could impose problems for the maintenance of self-tolerance. Indeed, the results above imply that T cells recognizing allogeneic or self antigens on cells lacking co-stimulatory molecules can be activated via B7/CD28 interaction with bystander APC such as dendritic cells, which have been shown

to constitutively express the B7 molecule *in vivo* (11). In the next experiments we tested whether B7 co-stimulation on the same or on a different cell as the CD3 – TCR stimulus would give different results when naive or memory T cells are used. Figure 4 demonstrates that under the experimental conditions used, memory T cells can be activated when small numbers of bystander cells provide the co-stimulatory signal of B7. The optimal proliferation in this experiment was observed at day 4. However, the naive T cells could not be induced to proliferate when B7 was presented on bystander cells, not even after 5 days.

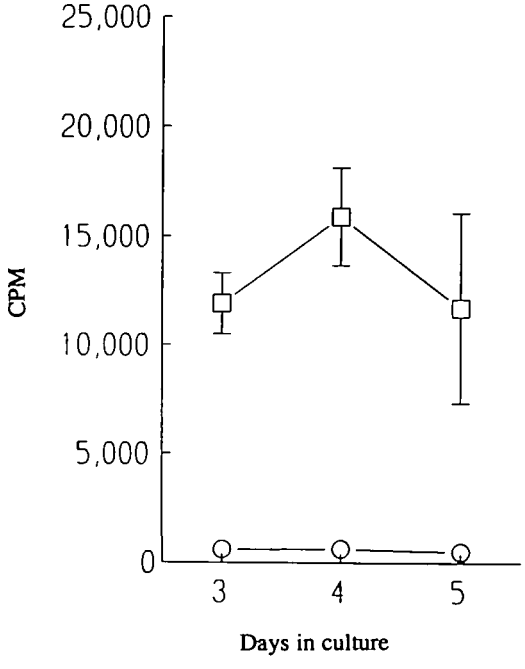

Fig. 4. Co-stimulation of T cells with B7 presentation on bystander cells is only possible for CD45RO$^+$ memory cells. Proliferation of purified T cells (10^4 cells/well) co-cultured with 3T6-CD32 cells (10^4 cells/well) together with 3T6-B7 cells (10^3 cells/well) in the presence of 100 ng/ml of anti-(CD3) mAb CLB-T3/4.1. Open circles represent CD45RA$^+$ naive T cells and open squares represent CD45RO$^+$ memory T cells Proliferation was measured by [^{3}H]thymidine incorporation and represents the mean of triplicate wells. The data is representative of two experiments using T cells from different donors.

Similar data was obtained in a different experiment. Table 1 shows that when the anti-CD3 mAb was presented on 3T6-CD32 cells without B7, T cells are not activated at all. When the anti-CD3 mAb was presented on the same cell as the B7 molecule, both T cell subsets were stimulated to proliferate. In contrast, when the anti-CD3 mAb and B7 were presented on separate cells, only the CD45RO$^+$ memory T cells were induced to proliferate, albeit less efficiently than when the anti-CD3 stimulus and B7 were on the same cell. In this experiment parallel cultures were set up to measure IL-2 production after 16 h. It was found that when the anti-CD3 mAb and B7 were presented on the same cell, both subpopulations were induced to secrete IL-2, with the CD45RO$^+$ memory T cells producing much more. When the anti-CD3 mAb and B7 were presented on separate cells, only in the cultures with CD45RO$^+$ memory T cells, small amounts of IL-2 could be detected. These experiments show that under the experimental conditions used here, only the CD45RO$^+$ memory T cells can be co-stimulated via B7 – CD28 interaction on bystander APC. This suggests that it is very unlikely that *in vivo* naive CD45RA$^+$ T cells recognizing self antigens could be co-stimulated via B7 – CD28 interaction on bystander APC. This could be an important mechanism involved in the maintenance of self-tolerance. Although our findings seem to contradict what has been reported in mouse models, it does not, since in mice the majority of the CD4$^+$ T cells will be of the CD45RA$^+$ phenotype. The fact that we find that under suboptimal conditions only the CD45RO$^+$ memory T cells can be co-stimulated via B7 – CD28 interaction on bystander APC correlates well with recently published data obtained with purified B7. It has been demonstrated that a fusion protein of B7 is very efficient in co-stimulating antigen-primed CD4$^+$ CD45RO$^+$ T cells, but was unable to co-stimulate resting naive CD4$^+$ CD45RA$^+$ T cells (24). However, despite the fact that it is generally accepted that B7 is the ligand for CD28, it cannot be excluded that the CTLA-4 molecule is involved in the B7-mediated co-stimulation. It was recently shown that the CTLA-4 molecule is upregulated after T cell activation and seems to be restricted to the CD45RO$^+$ subpopulation of T cells (25). Furthermore, it was demonstrated that cross-linking CTLA-4 with mAbs resulted in synergistic activation with cross-linking of CD28 with mAb.

In conclusion, we propose that under physiological conditions, only the CD45RO$^+$ memory T cells, which have been educated to discriminate between self and non-self, can be co-stimulated with B7 on bystander APC. In addition, it could very well be that

Table 1. Only CD45RO$^+$ memory T cells can be co-stimulated via B7 – CD28 interaction with bystander APC

Stimulator cells	Responder T cells	IL-2 production after 16 h (U/ml)[b]	Proliferation of T cells (CPM)[a]	
			day 3	day 4
3T6-CD32	CD45RO-depleted	<0.02	1249 ± 32	1955 ± 178
3T6-CD32 + 3T6-B7	CD45RO-depleted	<0.02	1802 ± 167	2022 ± 198
3T6-CD32/B7	CD45RO-depleted	0.069	10,321 ± 1535	15,590 ± 623
3T6-CD32	CD45RA-depleted	<0 02	1384 ± 72	1538 ± 287
3T6-CD32 + 3T6-B7	CD45RA-depleted	0.07	3075 ± 406	5675 ± 201
3T6-CD32/B7	CD45RA-depleted	23.5	10,323 ± 1069	20,391 ± 1568

[a]Proliferation of T cells, depleted of either CD45RO$^+$ cells or CD45RA$^+$ cells, was measured by incorporation. The T cells (10^4/well) were stimulated with 100 ng/ml of the anti-CD3 mAb CLB-T3/4.1 presented on CD32-expressing mouse 3T6 cells (10^4 cells/well).

[b]For IL-2 production, T cells (10^5/well) were stimulated as described for the proliferation. IL-2 production was measured using the CTLL assay using recombinant human IL-2 as standard.

this co-stimulation of memory T cells is, at least to some extent, mediated by interaction with CTLA-4.

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Abbreviation

APC	antigen presenting cell

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