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# Enhancement of human lymphocyte proliferative response to purified protein derivative by an anti-interleukin-2 receptor α chain antibody (CD25)

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## SUMMARY

While it is clear that the β subunit of interleukin-2 receptor (IL-2R) plays a pivotal role in IL-2-induced signal transduction, the function of the α subunit, other than modulating the association rate of IL-2, is still unknown. It has been reported that the interaction between IL-2 and the IL-2Rα subunit of several IL-2-dependent murine T-cell lines may result in a negative regulatory signal. To confirm this finding, we investigated the effect of an anti-IL-2Rα antibody, CD25-8D8, on the proliferative response of human peripheral blood lymphocytes. Lymphocytes from purified protein derivative (PPD)-positive donors were cultured with PPD and various concentrations of CD25-8D8 for up to 9 days, and [$^3$H]thymidine uptake was measured. Whereas the proliferative response of human lymphocytes to PPD was suppressed by high concentrations of CD25-8D8, subinhibitory amounts of CD25-8D8 enhanced lymphocyte proliferation by 3·5-fold (range 2·2–6·2-fold) on the second day after maximal [$^3$H]thymidine uptake had occurred. By itself, CD25-8D8 could not induce proliferation of washed 5-day PPD-activated lymphocytes during reculturing; instead, growth enhancement by CD25-8D8 was dependent on the presence of PPD-activated culture supernatant or moderate levels of exogenous IL-2. The enhancing effect of anti-IL-2Rα antibody, observed in both murine and human systems, reinforces the possibility that binding of IL-2 to the IL-2Rα chain plays a negative regulatory role in signal transduction.

## INTRODUCTION

Interleukin-2 (IL-2) is a polypeptide that functions as a growth factor for T cells.[1] IL-2 acts by binding to specific cell membrane receptor-designated IL-2 receptor (IL-2R).[2] Human IL-2R consists of at least two distinct, non-covalently associated receptor subunits: α and β chains. The IL-2Rα chain (p55, Tac or CD25 molecule) and IL-2Rβ chain (p70–75), which by themselves bind IL-2 with low affinity ($K_d$ of $\sim 10^{-8}$ M) and intermediate affinity ($K_d$ of $\sim 10^{-9}$ M), respectively, form a high-affinity receptor complex ($K_d$ of $\sim 10^{-11}$ M).[3–6] Recently, a third receptor subunit, designated IL-2Rγ (p64), has been identified and found to be associated with the β subunit in the presence of IL-2.[7,8] The high-affinity form of IL-2R exhibits the most favourable binding kinetics of individual subunits, i.e. it associates with IL-2 rapidly, like the IL-2Rα chain, but dissociates quite slowly, like the IL-2Rβ chain.[9,10]

Resting human T cells constitutively express a small number of IL-2Rβ chains.[5] Antigen or mitogen stimulation leads to a stronger induction of IL-2Rα gene expression than that of IL-2Rβ gene, resulting in excess surface α chain (10 000 or more) and the formation of approximately 2000–3000 high-affinity IL-2R molecules.[3,11,12] The exact mechanism by which IL-2R subunits interact with the ligand is unknown. Various reports indicate that the IL-2Rβ chain plays a pivotal role in IL-2-induced signal transduction.[13–15] The role of the IL-2Rα subunit appears to be limited to modulating IL-2 binding affinity of the IL-2R,[13,14] although there is a study which suggests a negative regulatory role of the IL-2Rα subunit.[16] Preliminary analysis of the IL-2Rγ subunit suggests that it also modulates the IL-2 binding affinity of the IL-2Rβ subunit by decreasing the IL-2 dissociation rate, and may be involved in ligand-induced signalling.[7,8]

In the present report, we examined the role of the IL-2Rα subunit on human lymphocyte proliferation. We found that, depending on the dose of antibody and the time of assay, a monoclonal antibody to the IL-2Rα chain (anti-CD25 mAb) either suppressed or enhanced the lymphocyte proliferative response to specific antigen (e.g. purified protein derivative;

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Abbreviations: FCS, fetal calf serum; IL-2, interleukin-2; IL-2R, interleukin-2 receptor; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; PPD, purified protein derivative of tuberculin.

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PPD). Enhancement of cell proliferation was observed only in the presence of antigen-activated culture supernatant or recombinant IL-2.

## MATERIALS AND METHODS

*Antibody and other reagents*

CD25-8D8, an IgG1 mAb which is specific for an epitope of the IL-2Rα molecule involved in IL-2 binding,[17] was generously provided by Dr O. Majdic (University of Vienna, Vienna, Austria). The murine isotype-matched IgG1 control was purchased from Sigma (St Louis, MO). The CD25-8D8 and IgG1 control were in the form of ascitic fluid without added azide or fetal calf serum, and were clarified by centrifugation and filtration before use. PPD of tuberculin was obtained from Connaught (Willowdale, Ontario, Canada). Recombinant human IL-2 was purchased from Genzyme (Boston, MA; Code RIL2). The unit of IL-2 in all experiments was the Biological Response Modifier Program (BRMP) unit, of which 1 U is the amount of IL-2 required for half maximal [$^3$H]thymidine uptake of 2000 CTLL-2 cells/well. One BRMP unit is equal to 1 NIH unit.

*Isolation of peripheral blood mononuclear cells*

Peripheral blood mononuclear cells (PMBC) were separated from heparinized blood of PPD-positive healthy donors by centrifugation over a Ficoll–Hypaque cushion. Cells were washed three times with RPMI-1640 medium and resuspended in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Grand Island, NY), 40 μg/ml gentamycin, 1 mM sodium pyruvate and $5 \times 10^{-5}$ M 2-mercaptoethanol (2-ME).

*Lymphocyte proliferation assay*

Each culture was set up in a microwell plate (Nunc, Roskilde, Denmark) in a final volume of 200 μl/well. Triplicate aliquots of $1 \times 10^5$ PBMC from PPD-positive donors were cultured with various concentrations of CD25-8D8 mAb or IgG1 control in the presence of PPD (30 μg/ml). The cultures were incubated in a humidified atmosphere with 5% $CO_2$ at 37°, and 0·4 μCi/well of [$^3$H]thymidine (Amersham, UK; specific activity 5 Ci/mmol) was added 18 hr before harvest. Incorporated radioactivity was counted in a Beckman LS3000 liquid scintillation counter. Values are given as mean c.p.m. of triplicate cultures.

*Cell activation and anti-CD25 mAb-induced cell proliferation assay*

PBMC were initially cultured at a cell density of $1 \times 10^6$ cells/ml in the presence of 30 μg/ml PPD for 5 days. PPD-activated PBMC were washed three times and recultured in the presence of 0·2 μg/ml of CD25-8D8 mAb, or IgG1 control with or without PPD-activated culture supernatant or recombinant IL-2. After an additional 4 days of culture, [$^3$H]thymidine was added and the incorporated radioactivity was counted.

PPD-activated culture supernatant was collected from the culture of PBMC at a cell density of $1 \times 10^6$ cells/ml in the presence of 30 μg/ml PPD for 48 hr, and stored in small aliquots at −20°.

*Immunofluorescence analysis*

PPD-activated cells were analysed for CD25 expression by indirect immunofluorescence using CD25-8D8 and FITC-conjugated sheep F(ab')$_2$ anti-mouse IgG plus IgM antibodies (Grup, Scandic, Vienna, Austria). To block non-specific Fc receptor binding, PBMC were incubated for 30 min at 4° with 10% human AB serum before staining. Membrane fluorescence was analysed on a FACSCAN flow cytometer (Becton Dickinson, Sunnyvale, CA).

*Statistical analysis*

The results are expressed in the figures as mean c.p.m. of triplicate cultures. The statistical significance of the results was assessed by the paired Student's *t*-test. A *P* value of less than 0·05 was considered to be significant.

## RESULTS

### Anti-IL-2Rα chain mAb, CD25-8D8, enhanced lymphocyte proliferation

Human PBMC cultured in the presence of 30 μg/ml of PPD for up to 9 days proliferated maximally on day 7 and became quiescent on day 9 (data not shown). In the presence of CD25-8D8 during the entire culture period, proliferation of PBMC on day 7 was slightly enhanced by 0·008–0·2 μg/ml of CD25-8D8 (Fig. 1a). At higher concentrations, the antibody suppressed cell proliferation. On day 9 of the culture, the suppressive

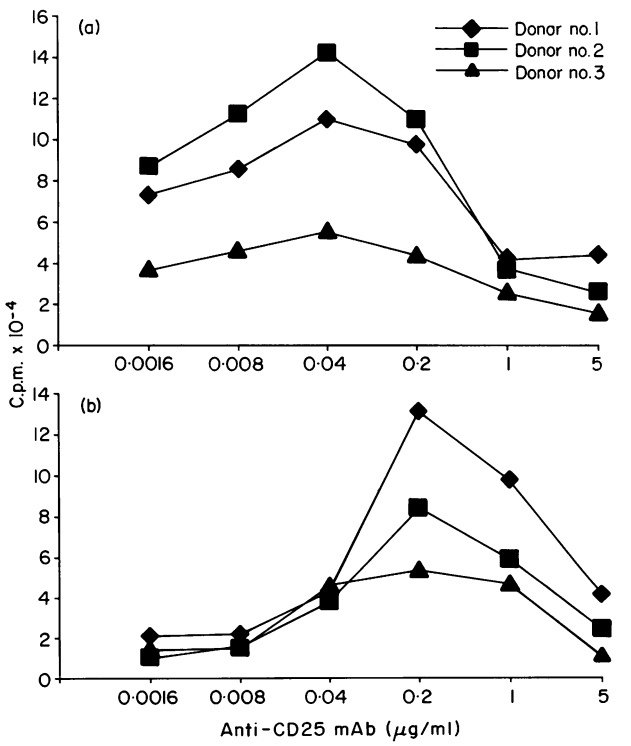


**Figure 1.** Effect of CD25-8D8 on lymphocyte proliferation. Human PBMC were stimulated with 30 μg/ml of PPD in the presence of various concentrations of CD25-8D8. [$^3$H]thymidine uptake was determined at 7 days (a) and 9 days (b) of activation. Each line represents different donors. Mean c.p.m. of PPD-stimulated PBMC of donor 1, 2 and 3 in the absence of CD25-8D8 at 7 days (a) and 9 days (b) were $7·6 \times 10^{-4}$, $9·9 \times 10^{-4}$, $2·5 \times 10^{-4}$ and $2·1 \times 10^{-4}$, $1·4 \times 10^{-4}$, $1·6 \times 10^{-4}$, respectively.

**Table 1.** Effect of 2-ME on the enhancement of lymphocyte proliferation by CD25-8D8

| Cultures* | With 2-ME | | Without 2-ME | |
|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| PBMC + PPD | 29473† | 16004 | 12134 | 6138 |
| PBMC + PPD + CD25-8D8 | 54718 | 25886 | 7652 | 4449 |
| PBMC + PPD + IgG1 | 25404 | 14788 | 7484 | 4152 |

* Human PBMC were stimulated with 30 μg/ml PPD in the presence of CD25-8D8 or isotype-matched IgG1 control 0·2 μg/ml, in 10% FCS–RPMI-1640 medium with or without $5 \times 10^{-5}$ M 2-ME. [$^3$H]thymidine uptake was determined 9 days after the initiation of culture.

activity of CD25-8D8 was not evident (Fig. 1b). However, CD25-8D8 strongly enhanced lymphocyte proliferation at the three doses employed (0·04, 0·2 and 1 μg/ml). The optimal enhancing concentration of CD25-8D8 mAb was 0·2 μg/ml. An isotype-matched IgG1 control did not affect cell proliferation at all concentrations tested (data not shown). Surprisingly, the enhancing effect of CD25-8D8 required the presence of 2-ME in the culture medium (Table 1).

In order to confirm the enhancement of lymphocyte proliferation by CD25-8D8, PBMC isolated from four additional donors were stimulated with PPD and cultured with 0·2 μg/ml of CD25-8D8 for 9 days. In all seven experiments, CD25-8D8 enhanced cell proliferation by a mean of 3·5-fold (range 2·2–6·2-fold; $P < 0·01$) (Fig. 2). Again, an IgG1 control showed no effect on cell proliferation ($P > 0·05$). Enhancement of cell proliferation by CD25-8D8 was not observed in the absence of PPD (Fig. 2).

### Anti-CD25 mAb-induced cell proliferation in the presence of activated culture supernatant

To investigate the requirement of accessory factor for the enhancement of cell proliferation by CD25-8D8 mAb, PBMC

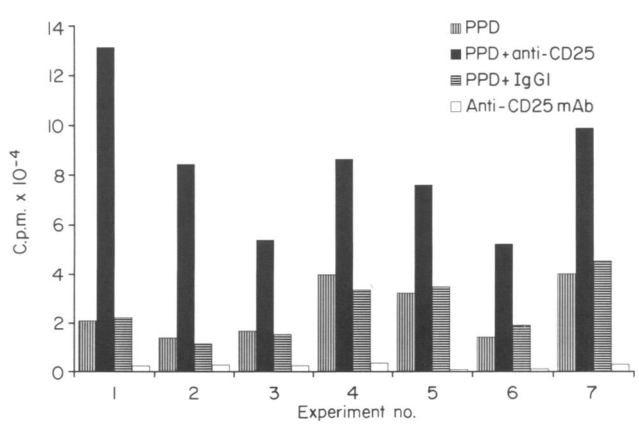


**Figure 2.** Costimulatory effect of CD25-8D8 and PPD. PBMC from seven different donors were cultured in 0·2 μg/ml of CD25-8D8 mAb or IgG1 control in the presence or absence of PPD. [$^3$H]thymidine uptake was determined 9 days after the initiation of culture.

**Table 2.** Induction of CD25 expression on human PBMC by PPD

| PPD conc. (μg/ml) | Days of activation | | |
|---|---|---|---|
| | 1 | 3 | 5 |
| 0 | 4·5* | 12·7 | 13·1 |
| 10 | 19·1 | 17·5 | 32·7 |
| 30 | 15·8 | 19·8 | 35·3 |
| 50 | 13·4 | 22·5 | 38·6 |
| 100 | ND | ND | 38·5 |

Human PBMC were stimulated with various concentrations of PPD. After 1, 3 and 5 days, cells were harvested and lymphocytes expressing CD25 were determined by indirect immunofluorescence and flow cytometry.

*Percentage of lymphocytes expressing surface CD25 molecules, as determined by flow cytometric analysis.

ND, not determined.

from PPD-positive subjects were initially activated with PPD for 1, 3 and 5 days. Analysis by indirect immunofluorescence assay revealed that more than 35% of activated lymphocytes expressed CD25 molecules after 5 days of activation with 30 μg/ml or more of PPD (Table 2). Activated PBMC were then washed after 5 days in culture and reincubated for another 4 days with CD25-8D8 mAb in the presence or absence of 48-hr PPD-activated culture supernatant. As shown in Fig. 3, CD25-8D8 mAb strongly enhanced proliferation of these PPD-activated cells only in the presence of PPD-activated culture supernatant ($P < 0·05$). Without the antibody, PPD-activated culture supernatant barely induced cell proliferation ($P > 0·05$).

Since PPD antigen might still be present in the PPD-activated culture supernatant, it was possible that PPD itself was involved in the enhancement effect of CD25-8D8. To address this question, PPD-activated PBMC were washed and cultured with added PPD and CD25-8D8. Under this condition, PPD or PPD plus CD25-8D8 did not alter cell

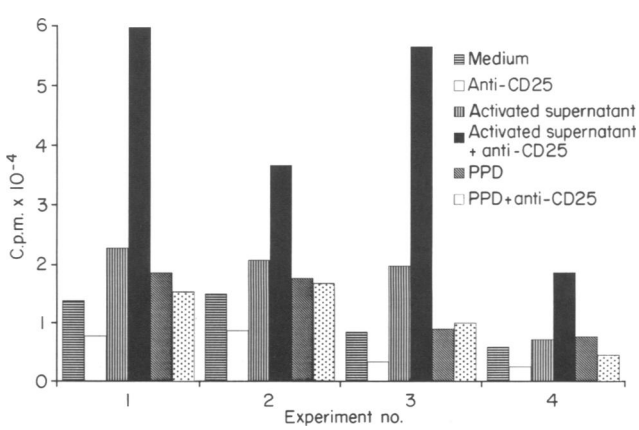


**Figure 3.** Enhancement of proliferation by CD25-8D8 requires PPD-activated culture supernatant. Human PBMC were stimulated with PPD for 5 days, washed, and reincubated with 0·2 μg/ml of CD25-8D8 under different conditions. [$^3$H]thymidine uptake was determined after 4 days.

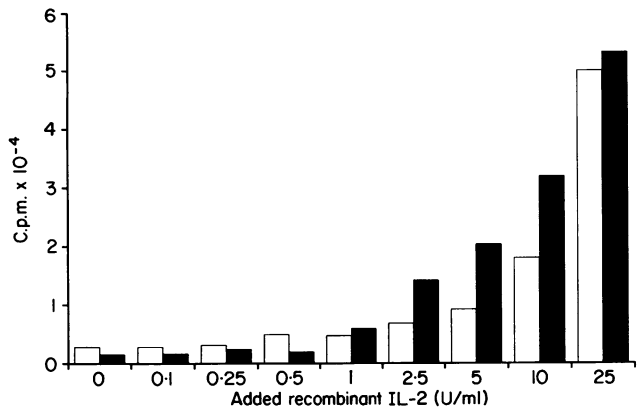


**Figure 4.** IL-2 is involved in the proliferation enhancement by CD25-8D8. Human PBMC were stimulated with PPD for 5 days, washed, and reincubated with various concentrations of recombinant IL-2 in the presence (filled bar) or absence (open bar) of 0·2 μg/ml of CD25-8D8. [$^3$H]thymidine uptake was determined after 4 days. Similar results were obtained with two additional experiments.

proliferation ($P > 0{\cdot}05$; Fig. 3). The results indicated that proliferation enhancement by CD25-8D8 required a factor(s) other than PPD to be present in PPD-activated culture supernatant.

### Interleukin-2 is involved in anti-CD25-mediated enhancement of cell proliferation

As PPD-activated culture supernatant contains a large number of cytokines, including IL-2, the role of IL-2 was investigated. Recombinant human IL-2 and CD25-8D8 were added to the culture of 5-day PPD-activated PBMC for another 4 days. Enhancement of cell proliferation by CD25-8D8 mAb could not be observed in the presence of too low (0·1–1 U/ml) or too high (25 U/ml) amounts of IL-2 ($P > 0{\cdot}05$; Fig. 4). Nevertheless, CD25-8D8 could exert the enhancing effect in the culture which contained 2·5–10 U/ml of IL-2 ($P < 0{\cdot}05$–$P < 0{\cdot}001$).

## DISCUSSION

The role of the IL-2Rα chain in the T-cell response to IL-2 is not clearly known at the molecular level. Several functional studies suggest that it is not sufficient for the transduction of the IL-2 signal, but it modulates IL-2 binding to high-affinity IL-2R, resulting in an increased IL-2 association rate.[13,14] In contrast to other studies, Kumar *et al.*[16] demonstrated that the relative predominance of low-affinity IL-2R on the cell surface of several IL-2-dependent murine T-cell lines coincided with a low level of responsiveness of those T cells to IL-2.[16] Their interpretation that IL-2α chain plays a negative regulatory role in T-cell activation by IL-2 is further supported by the enhancement of T-cell line proliferation by a moderate level of anti-IL-2Rα antibodies in the presence of a limited amount of IL-2.[16] Because the latter finding may be due to a peculiar property of cloned T-cell lines, we sought to confirm this by employing human peripheral blood lymphocytes in an antigen-stimulated proliferation study. We have found that a mAb specific for the IL-2-binding site of human IL-2Rα chain also enhanced proliferation of lymphocytes in response to PPD. The enhancement was evident at subinhibitory levels of antibody and was dependent on the availability of PPD-activated culture supernatant or added IL-2. Moreover, the enhancement was observed 2 days after the day of maximal [$^3$H]thymidine incorporation and also required the presence of 2-ME in the culture medium. To the best of our knowledge, such a late effect of CD25-8D8 on human lymphocytes has never been reported. This effect may have been overlooked either by employing a high dose of antibody, by looking for it at earlier points in time, or by culturing lymphocytes in the absence of 2-ME. It is quite intriguing as to why the enhancing effect of CD25-8D8 is observed with added 2-ME. One possible mechanism is that in long-term lymphocyte culture (9 days), oxygen-free radicals tend to form and the addition of 2-ME to the medium may lower the concentration of free radicals through reduction, thus damage to biomolecules and cells is reduced. It may also be possible that 2-ME influences the interaction between IL-2 and its receptor and/or subsequent signal transduction inside the cells in such a way that the enhancing effect of CD25-8D8 can be expressed. Although we have not yet tested other anti-CD25 antibodies, it is unlikely that such an effect will be unique to CD25-8D8, as Kumar *et al.*[16] were able to observe the same effect in all three mAb that they tested on murine cell lines.

Although Kumar *et al.*[16] reported that the enhancement of proliferation of murine IL-2-dependent T-cell lines by anti-IL-2Rα mAb was maximal on day 3 after activation with IL-2, their finding agrees with ours when the kinetics of growth and antibody enhancement are compared. In the murine system, IL-2 stimulates a rapid [$^3$H]thymidine uptake by quiescent T-cell lines for such a short duration that, by day 3 or 4, [$^3$H]thymidine uptake is at the minimum level. Similarly, human peripheral blood lymphocytes incorporate the highest amount of [$^3$H]thymidine on day 7 after PPD stimulation, and on day 9 only about 36% of that uptake remains in the culture which does not receive anti-IL-2Rα treatment. Thus, enhancement by anti-IL-2Rα in both systems was observed 1–2 days after the maximal [$^3$H]thymidine had taken place.

Because CD25-8D8 does not directly stimulate activated human lymphocytes to proliferate, the possibility that this mAb mimics IL-2 can be excluded. Conceivably, anti-IL-2Rα antibodies may just passively block the binding of IL-2 to excess IL-2Rα chain on the activated lymphocyte surface, making more IL-2 available for interacting with the high-affinity, but less abundant, IL-2R molecules. The fact that enhancement by these antibodies is not seen on the day of highest [$^3$H]thymidine uptake in regular cultures, and that the effect can also be observed with mAb that recognize epitopes distal to IL-2 binding,[16] make this possibility unlikely. Moreover, because the 75 000 MW intermediate-affinity receptor chain is expressed constitutively, in relatively large amounts, on most T cells, most low-affinity receptors (CD25) will, as they are expressed on the membrane, become complexed to form high-affinity receptor. The latter reason, together with the considerable difference in affinity between the high- and low-affinity receptors, would make CD25-8D8 blocking of IL-2 binding to low-affinity IL-2Rα chain even less likely. Kumar *et al.*[16] favour the idea that binding of IL-2 to the IL-2Rα chain transmits a true negative signal into the cytoplasm, and blocking of such interaction, or preferential modulation of the IL-2Rα chain by anti-IL-2Rα antibodies,

may shift the balance towards positive, stimulatory signalling from high-affinity IL-2R molecules. This idea appears to agree with a recent finding that an IL-2 analogue, which lacks the capacity to bind to the IL-2Rα chain, is a more effective agonist of growth of an IL-2Rα- and IL-2Rβ-transfected proB-cell line than IL-2 itself.[18]

Many authors have advocated the addition of anti-IL-2Rα antibody into lymphocyte cultures in order to reduce IL-2 consumption by activated cells.[19,20] Depending on the amounts of anti-IL-2Rα used and the time of assay, the addition may result in lower or higher levels of growth than that of untreated cultures. Erroneous measurement of lymphocyte proliferation may thus be caused by adding anti-IL-2Rα antibody into lymphocyte cultures.

## ACKNOWLEDGMENTS

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