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# Antibodies directed at mouse IL-2-R α and β chains act in synergy to abolish T-cell proliferation in vitro and delayed type hypersensitivity reaction in vivo

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**Abstract** The anti-mouse IL-2-R β chain mAb TM-β1 which, by itself, does not affect IL-2-dependent proliferation through the high affinity mouse IL-2 receptor, was shown to cooperate in a synergistic way with a set of anti-IL-2-R α chain mAbs both in vitro and in vivo. In vitro, when associated at equimolar concentrations, the TM-β1/anti-α mAb association was four to ten times more efficient at inhibiting the proliferation of the CTL-L2 cell line than was a similar concentration of anti-α mAb alone. In addition, a bispecific antibody in which a Fab' fragment of TM-β1 was covalently linked to a Fab' fragment of one of the anti-α mAb (5A2) was shown to be as efficient as the TM-β1/5A2 association. The association of TM-β1 with 5A2 was also tested in vivo in a sheep red blood cell-induced delayed type hypersensitivity (DTH) model. TM-β1 which, by itself, had no effect on DTH, induced a two- to threefold decrease in the doses of 5A2 required to suppress this cell-mediated immune reaction.

**Key words** Monoclonal antibodies, mouse interleukin-2 receptor · Mouse, interleukin-2 receptor, monoclonal antibodies · Interleukin-2 receptor, monoclonal antibodies, mouse

## Introduction

Interleukin-2 receptors (IL-2-R) comprise at least three membrane anchored glycoproteins [10]. These chains can combine in various ways to form receptors with different affinities for IL-2: the α chain (or CD25, Tac antigen) [12] is a low-affinity receptor ($K_d = 10^{-8}$ M), the β/γ complex is an intermediate-affinity receptor ($K_d = 10^{-9}$ M), and the α/β/γ complex is a high-affinity receptor ($K_d = 10^{-11}$ M). The γ chain [18] alone or in the presence of α chain does not bind IL-2. The β chain [5] alone binds IL-2 with very low affinity ($K_d > 10^{-7}$ M) and the α/β complex can form a pseudo high-affinity receptor ($K_d = 10^{-10}$ M). Of these various receptor forms, only two are biologically functional – the β/γ and α/β/γ receptor complexes – as one might expect given the fact that heterodimerization of β and γ chains is required for signal transduction [11].

Various therapeutic strategies have been developed that involve agents that eliminate IL-2-R expressing cells and/or block IL-2 interaction with its receptors [14, 22]. Among them are anti-α chain monoclonal antibodies (mAbs), which have been successfully used in organ transplantation [8, 9, 15, 16]. Anti-β chain mAbs have more recently been obtained that inhibit IL-2 binding to the β/γ complex. However, none of these antibodies were able to affect IL-2 induced proliferation through high-affinity α/β/γ receptors [4, 6, 17, 20]. We previously showed that combining anti-human α and anti-human β mAbs resulted in a high synergistic blocking effect on IL-2 high affinity binding and IL-2 induced T cell proliferation of human T lymphocytes [1]. On the basis of these results, we then constructed a bispecific antibody carrying the anti-α and anti-β valencies. As a result of α and β chains crosslinking, the bispecific mAb displayed a high affinity binding that was specific

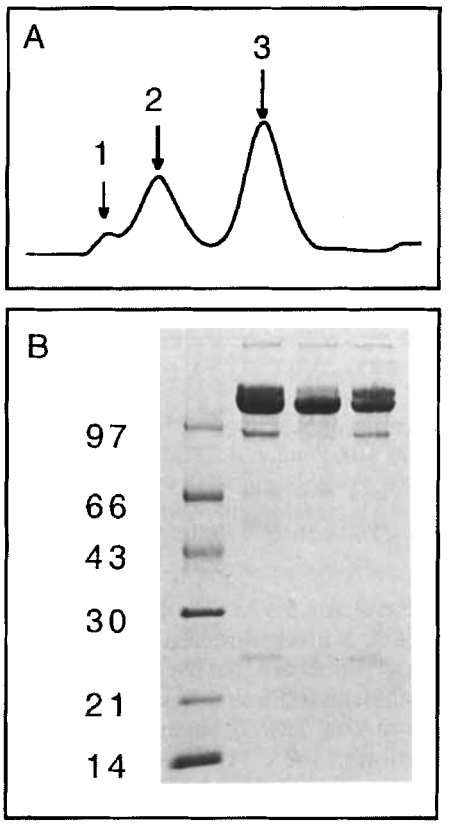


**Fig. 1** **A** Gel filtration chromatogram showing the separation of TM$\beta$1/5A2 bispecific antibody (*peak 1*) from undigested IgG (*peak 2*) and unreacted Fab' fragments (*peak 3*). **B** SDS-PAGE analysis (nonreducing conditions). *From left to right* molecular weight markers (in kDa), TM-$\beta$1 Fab'2 fragments, 5A2 Fab'2 fragments, and TM-$\beta$1/5A2 bispecific antibody

for $\alpha + /\beta +$ activated T cells as compared to $\beta +$ or $\alpha +$ cells. In addition, this bispecific antibody was shown to inhibit IL-2-induced proliferation at concentrations much lower than did the parental mAbs [4].

In this paper, using mAbs directed against the $\alpha$ and $\beta$ chains of the murine IL-2-R, we have analyzed whether anti-$\alpha$ and anti-$\beta$ mAb combinations and anti-$\alpha$/anti-$\beta$ bispecific mAbs could have interesting immunosuppressive properties both in vitro and in a delayed type hypersensitivity (DTH) model in vivo.

## Materials and methods

### Monoclonal antibodies (mAbs)

Hybridoma producing 125, 135, and 5A2 mAbs were a kind gift from J. Theze (Institut Pasteur, Paris, France) [2], and the mAbs were purified from ascitic fluids produced in nude mice. The TM-$\beta$1 mAb was prepared as described previously [19].

### Construction of the bispecific antibody

This was performed essentially as described previously [4]. Briefly, Fab'2 fragments of 5A2 and TM-$\beta$1 mAbs were prepared by pepsin treatment and purified by gel filtration on a prepacked Sephadex G200 column (Pharmacia, Uppsala, Sweden). They were then reduced by treatment with 2-mercaptoethylamine (MCEA) and sodium arsenite and reacted with 5–5'-dithiobis-2-nitrobenzoic acid (DTNB). The Fab'-TNB fragments obtained were separated by gel filtration and stored. The Fab'-TNB fragment obtained from 5A2 was reduced with MCEA to generate Fab'-SH. After removal of excess MCEA by gel filtration, it was mixed with an equimolar amount of Fab'-TNB prepared from TM-$\beta$1. The bispecific 5A2/TM-$\beta$1 antibody generated was purified from residual undigested IgG and unreacted Fab' fragments by gel filtration on a prepacked Sephadex G200 column (Fig. 1A). The purity of the bispecific antibody was further analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. A major band at 110 kDa was observed (Fig. 1B).

### CTL-L2 proliferation assays

These assays were performed in 96 multiwell plates. CTL-L2 cells were seeded at 10,000 cells/well in 100 µl culture medium containing 0.25 nM recombinant IL-2 (a kind gift of Roussel-Uclaf, Romainville, France) and various concentrations of anti-IL-2-R mAbs. After a 48-h incubation period at 37 °C, the cells were pulsed for 16 h with (3H)Tdr (0.25 uCi/well), harvested, and counted for thymidine incorporation. The 50 % inhibition points (IC50s) of mAb inhibition curves were determined using a nonlinear fit of the data (Grafit, Erithacus Software, Staines, UK).

### Delayed-type hypersensitivity (DTH)

Sheep red blood cells (SRBC) were collected and stored in Alsever's solution at 4 °C. Cells were washed three times before use and diluted to appropriate concentrations in sterile Hanks' balanced salt solution. The DTH reaction was performed as described previously [3]. Female BALB/c mice (Ets Janvier, Laval, France) were used at 8–10 weeks of age. An optimal immunizing dose of $5 \times 106$ SRBC was injected into the mouse's tail vein. Five days later, mice were tested for DTH with a subinflammatory challenging dose ($3 \times 108$ SRBC) injected into the right hind foot pad, the left pad being injected with Hanks' solution alone. Maximal (100 %) DTH reaction was determined 18 h after this challenge by the difference in size between the two foot pads. Mice were daily injected intraperitoneally with mAb for 5 days starting at the time of priming until the SRBC challenge.

Animal experiments were performed according to the "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985).

## Results

### Antiproliferative effects of the anti-murine IL-2-R mAbs

The antimurine IL-2-R $\alpha$ chain mAbs 5A2, 125, and 135 induced a dose-dependent inhibition of the IL-2-driven proliferation of the CTL-L2 cell line (Fig. 2). The equi-

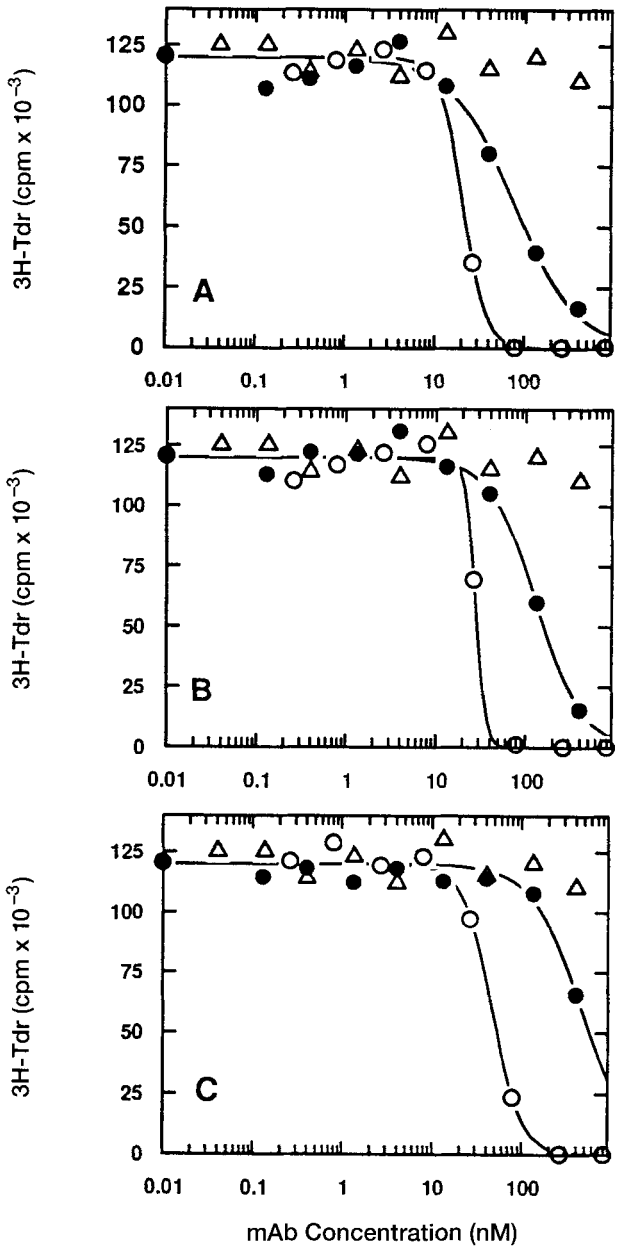


**Fig. 2A–C** Inhibition of IL-2-induced CTL-L2 proliferation by anti-IL-2-R mAbs: **A** effects of increasing concentrations of TM-$\beta$1 (△), 5A2 (●), and equimolar association of both antibodies (○); **B** effect of increasing concentrations of TM-$\beta$1 (△), 125 (●), and equimolar association of both antibodies (○); **C** effect of increasing concentrations of TM-$\beta$1 (△), 135 (●), and association of both antibodies (○)

librium dissociation constants ($K_d$) of these mAbs for the α chain on CTL-L2 cells are 1.1, 1.4 and 2.6 nM, respectively [3]. As expected, the more pronounced the inhibitory effects of these mAbs on CTL-L2 proliferation were, the higher their affinity for the α chain was. The IC50 describing these inhibitory effects were 75,

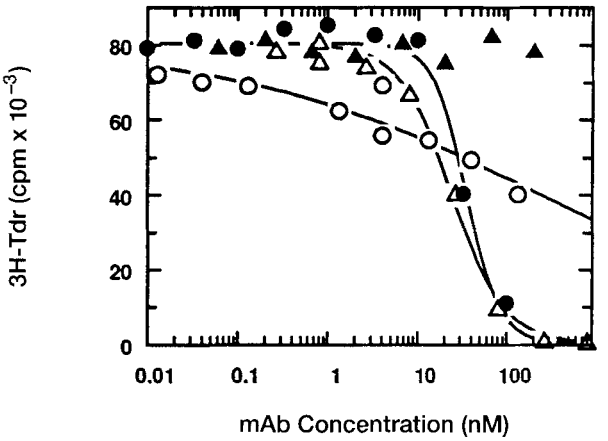


**Fig. 3** Inhibition of IL-2-induced CTL-L2 proliferation by the TM-$\beta$1/5A2 bispecific antibody: effects of increasing concentrations of TM-$\beta$1 (▲), 5A2 (○), equimolar association of both antibodies (●), and TM-$\beta$1/5A2 bispecific antibody (△)

131, and 450 nM for 5A2, 125, and 135 mAbs, respectively. TM-$\beta$1 is a mAb directed at the $\beta$ chain of the IL-2-R [19]. It binds to the $\beta$ chain on CTL-L2 cells with a $K_d$ of 10 nM (data not shown). In contrast to anti-α chain mAbs, TM-$\beta$1 has no inhibitory effect on the proliferation of the CTL-L2 cell line when tested at concentrations up to 500 nM (Fig. 2). However, when TM-$\beta$1 was associated at equimolar concentrations with any of the three anti-α chain antibodies, a marked improvement was observed in the anti-proliferative dose-response curves. When analyzing the total mAb concentrations giving 50 % inhibition of proliferation (IC50), the combination of TM-$\beta$1 with one anti-α chain turned out to be between five and ten times more potent than the anti-α chain mAb used alone. The IC50 for the TM-$\beta$1/5A2, TM-$\beta$1/125, and TM-$\beta$1/135 associations were 20, 28, and 46 nM, respectively.

In order to transfer this anti-α/anti-$\beta$ synergistic property onto a single molecule, a 5A2/TM-$\beta$1 bispecific mAb was chemically constructed and purified (Fig. 1). As shown in Fig. 3, this bispecific mAb was as potent in terms of molar concentrations as the association of the 5A2 and TM-$\beta$1 parental antibodies in inhibiting the proliferation of the CTL-L2 cell line.

## Effects of the anti-murine IL-2-R mAbs in a DTH model

In order to assess whether the in vitro synergistic properties of anti-α and anti-$\beta$ chain antibodies could be of interest for in vivo immunosuppression, the antibodies were tested in a DTH model in the mouse. The 5A2 mAb was chosen for these studies. This mAb, like the 135 and 125 mAbs, induced a dose-dependent inhibition of the DTH reaction with an IC50 of 1.3 μg/day (Fig. 4). TM-$\beta$1 alone had no effect when tested at doses up to

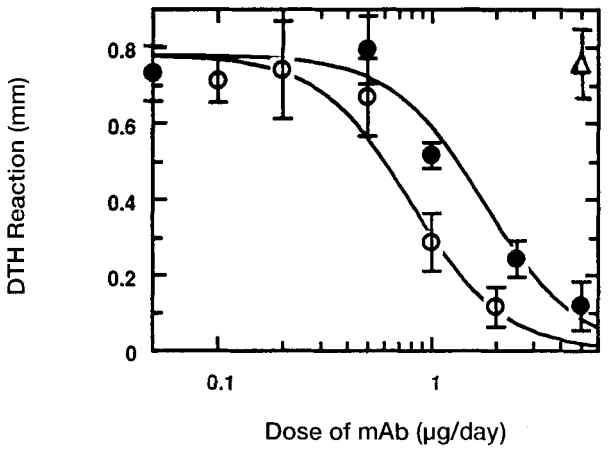


**Fig. 4** Effect of anti-IL-2-R mAbs on DTH-induced reaction in mouse: TM-$\beta$1, 5 μg/day, (△), increasing doses of 5A2 (●), or increasing doses of 5A2 in association with TM-$\beta$1, 5 μg/day (○)

5 μg/day. However, when TM-$\beta$1 at a dose of 5 μg/day was associated with 5A2, there was a clear shift of the 5A2 dose-response curve towards lower concentrations (IC50 = 0.6 μg/day; Fig. 4), demonstrating a synergistic effect of both antibodies in vivo.

## Discussion

Previous studies describing the anti-murine IL-2-R $\beta$ chain antibody TM-$\beta$1 have shown that it was able to inhibit the binding of IL-2 to the high-affinity receptors expressed by the CTL-L2 cell line at 4 °C. However, TM-$\beta$1 was unable to inhibit the IL-2-dependent growth of these cells at 37 °C [19]. Similar observations have been made by us [1, 4] and others [6, 17, 20] using anti-human IL-2-R $\beta$ chain mAbs. We have hypothesized that one mechanism responsible for this absence of inhibitory effect was a loss of affinity of the mAbs for the $\beta$ chain as a result of the association of the α chain with the $\beta/\gamma$ complex. Accordingly, anti-α mAbs that prevent the formation of high-affinity IL-2-R complexes were shown to restore the affinity of the anti-$\beta$ mAbs, and combining anti-α and anti-$\beta$ mAbs resulted in a high synergistic effect on IL-2-induced proliferation [1]. Subsequently, we showed that a chemically constructed anti-α/anti-$\beta$ bispecific mAb could reproduce and even enhance this synergistic property [4].

This study extends our earlier reports on the human IL-2-R system and shows that the anti-murine $\beta$ chain mAb TM-$\beta$1 does synergize with various anti-murine α chain mAbs to inhibit IL-2-induced proliferation of the CTL-L2 cell line. This is a good indication that the concept of targeting different receptor chains within a multi-subunit receptor to inhibit ligand action can be generalized, at least within the IL-2-R species.

In our previous study in humans, various anti-$\beta$ chain mAbs were combined with the anti-α chain mAb 33B3.1, and it was shown that the potency of the synergy was correlated with the affinity of the anti-$\beta$ mAb tested. In this study, various anti-α chain mAbs have been tested in combination with the anti-$\beta$ mAb TM-$\beta$1 and it is shown that the potency of the synergy observed is correlated with the affinity of the anti-α mAbs tested. Together, these results indicate that, for a combination of anti-α and anti-$\beta$ mAbs directed at epitopes interfering with the binding of IL-2 to the α and $\beta$ chains, respectively, the potency of the anti-α/anti-$\beta$ synergy is a direct function of both the affinity of the anti-α mAb and that of the anti-$\beta$ mAb. In this context, the efficiency of the synergy will be limited by the antibody that has the lower affinity. The relatively moderate synergistic effect observed in this study on CTL-L2 proliferation (a four to tenfold decrease in IC5O when comparing the anti-α/anti-$\beta$ combination to a corresponding equal concentration of anti-α alone) is very likely due to the low affinity of TM-$\beta$1 ($K_d \sim 10$ nM). Similar results have been obtained in humans. An anti-$\beta$ mAb (Tic-1) of low affinity ($K_d \sim 10$ nM) as compared to its anti-α partner 33B3.1 ($K_d \sim 0.2$ nM) was shown to synergize moderately [4].

Our previous in vitro study in humans has shown that a bispecific antibody constructed on the basis of anti-α and anti-$\beta$ mAbs could reproduce on a single molecule the synergistic property of the anti-α/anti-$\beta$ association. This bispecific antibody was even more efficient (more than tenfold) than the combination of both parental antibodies [4]. In the present study, the synergistic action of TM-$\beta$1 and 5A2 mAbs was also reproduced with a TM-$\beta$1/5A2 bispecific antibody. Contrary to what was found with anti-human IL-2-R mAbs [4], the TM-$\beta$1/5A2 bispecific antibody was not better than the TM-$\beta$1/5A2 association. As discussed above, this is also likely related to the relatively low affinity of TM-$\beta$1.

Other studies from our laboratory have shown that anti-α mAbs belonging to cluster I (inhibition of IL-2 binding and IL-2-induced proliferation) were efficient in suppressing a delayed type hypersensitivity (DTH) reaction induced by sheep red blood cells in mice [2, 3]. The magnitude of their effects was related to their respective $K_d$s but no synergistic effect between anti-α mAbs could be observed. Using a similar in vivo model, and in agreement with the in vitro data, we show that the TM-$\beta$1 mAb alone at doses up to 5 μg/day is not able to affect this DTH reaction. However, and again in agreement with the in vitro data, it synergizes at high doses (5 μg/day) with the anti-α mAb 5A2 to suppress the DTH response (a two to threefold shift in the dose-response curve of 5A2).

A number of IL-2-R-directed bioreagents have already been developed as immunosuppressive compounds that may be potentially useful in organ trans-

plantation. These include xenogenic anti-α mAbs [8, 9, 15, 16], chimerized or humanized anti-α mAbs [13], IL-2 bacterial toxin [7], or IL-2-IgM fusion proteins [21]. Our previous reports in vitro indicated that associating anti-α and anti-β IL-2-R mAbs, as well as using anti-α/anti-β bispecific antibodies might represent powerful immunosuppressive strategies [1, 4]. This study strengthens this hypothesis by showing that anti-α and anti-β mAbs can also synergize in vivo. This observed synergistic effect was limited, due to the low affinity of one of the antibody partners (TM-β1). We anticipate that the availability of an anti-β mAb with an affinity similar to that of 5A2 should be able to enhance the synergy.

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