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Research article

Differential clinical efficacy of anti-CD4 monoclonal antibodies in rat adjuvant arthritis is paralleled by differential influence on NF-κB binding activity and TNF-α secretion of T cells

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Abstract

The aim of this study was to analyze the differential effects of three anti-CD4 monoclonal antibodies (mAbs) (with distinct epitope specifities) in the treatment of rat adjuvant arthritis (AA) and on T-cell function and signal transduction. Rat AA was preventively treated by intraperitoneal injection of the anti-CD4 mAbs W3/25, OX35, and RIB5/2 (on days –1, 0, 3, and 6, i.e. 1 day before AA induction, on the day of induction [day 0], and thereafter). The effects on T-cell reactivity *in vivo* (delayed-type hypersensitivity), *ex vivo* (concanavalin-A-induced proliferation), and *in vitro* (mixed lymphocyte culture) were assessed. The *in vitro* effects of anti-CD4 preincubation on TCR/CD3-induced cytokine production and signal transduction were also analyzed. While preventive treatment with OX35 and W3/25 significantly ameliorated AA from the onset, treatment with RIB5/2 even accelerated the onset of AA by approximately 2 days (day 10), and ameliorated the arthritis only in the late phase (day 27). Differential clinical effects at the onset of AA were paralleled by a differential influence of the mAbs on T-cell functions, i.e. in comparison with OX35 and W3/25, the 'accelerating' mAb RIB5/2 failed to increase the delayed-type hypersensitivity to *Mycobacterium tuberculosis,* increased the *in vitro* tumor necrosis factor (TNF)-α secretion, and more strongly induced NF-κB binding activity after anti-CD4 preincubation and subsequent TCR/CD3-stimulation. Depending on their epitope specificity, different anti-CD4 mAbs differentially influence individual proinflammatory functions of T cells. This fine regulation may explain the differential efficacy in the treatment of AA and may contribute to the understanding of such treatments in other immunopathologies.

Keywords: adjuvant arthritis, anti-CD4 monoclonal antibody, TNF-alpha, NF-kappaB

AA = rat adjuvant arthritis; AP-1 = activator protein-1; ConA = concanavalin A; DC = dendritic cells; DTH = delayed-type hypersensitivity; ELISA = enzyme-linked immunosorbent assay; FACS = flowcytometry; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; HEPES = N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); IFN = interferon; IL = interleukin; K_A = affinity constant; k_{ass} = association rate constant; k_{diss} = dissociation rate constant; mAb = monoclonal antibody; MFI = mean fluorescence intensity; MHC = major histocompatibility complex; NF-AT = nuclear factor of activated T cells; PBS = phosphate-buffered saline; PE = phycoerythrin; PMA = phorbol myristoyl acetate; PMSF = phenylmethylsulfonyl fluoride; RA = rheumatoid arthritis; RPMI = Roswell Park Memorial Institute [medium]; SEM = standard error of the mean; Shc = src-homology-domain-containing-protein; TCR = T-cell receptor; TNF = tumor necrosis factor.

Introduction

CD4+ T cells and their cytokine products play an important role in rheumatoid arthritis (RA) and experimental models of arthritis, therefore representing potential therapeutic targets [1]. A specific therapeutic approach is the direct targeting of CD4+ T cells by use of monoclonal antibodies (mAbs) against the CD4 coreceptor. Anti-CD4 mAbs induce either cell depletion [2] or functional inactivation of T cells [3,4], although activation of T-cell functions has also been reported [5]. These contrasting effects may explain the variability in the clinical efficacy of different anti-CD4 mAbs in the treatment of RA, i.e. promising initial efficacy in open anti-CD4 trials [6,7], subsequent disappointing double-blind clinical trials (reviewed in [8]), and, finally, a revival of the anti-CD4 treatment principle with new, humanized anti-CD4 mAbs [9,10].

The focus of the present study was to analyze the effects of the anti-CD4 mAbs W3/25, OX35, and RIB5/2 in rat adjuvant arthritis (AA), a well-known, clearly CD4+ T-cell-dependent experimental arthritis model [11–13]. These mAbs target different epitopes of the CD4 molecule and do not compete for CD4 binding [14]. They are equally effective in suppressing AA upon treatment of established disease [11,12] (and authors own unpublished observations). In contrast, preventive treatment with the anti-CD4 mAbs W3/25, OX35, and RIB5/2 (on days –1, 0, 3, and 6, i.e. 1 day before AA induction, on the day of induction [day 0], and thereafter) had opposite effects in the induction phase of AA. In order to explain this differential efficacy, several parameters were analyzed.

Methods

For animals, arthritis model, antibodies, and affinity determination (surface plasmon resonance), see Supplementary material.

T-cell reactivity was measured *in vivo* by delayed-type hypersensitivity (DTH) and *in vitro* by proliferation assay or mixed lymphocyte culture, and cytokines were measured by bioassay or ELISA (see Supplementary material; for tumor necrosis factor (TNF)-α [15]).

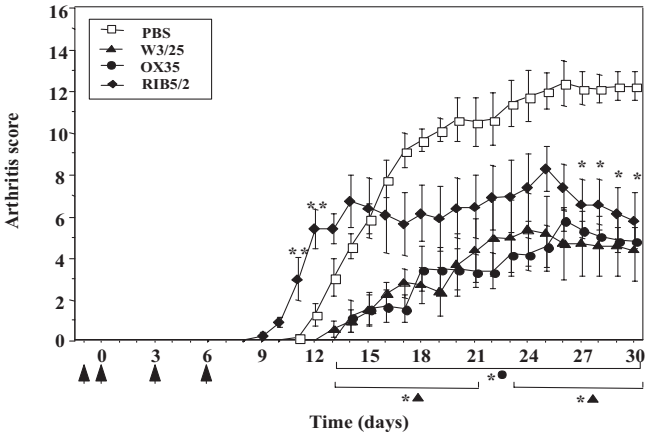
Cells were stimulated by preincubation with anti-CD4 mAbs and subsequent stimulation of TCRs. Electrophoretic mobility shift assay (EMSA) was as described in the Supplementary material. For statistical analysis, we used the Mann–Whitney (*U*) test/Spearman rank correlation ($P \leq 0.05$; see Supplementary material).

Results

Clinical effects

Preventive treatment with the anti-CD4 mAbs W3/25 and OX35 led to a marked, significant suppression of the arthritis score from day 13 to 30 in comparison with phosphate-buffered saline (PBS)-treated animals ($P \leq 0.05$; Fig. 1). In contrast, the anti-CD4 mAb RIB5/2 significantly accelerated the onset of the arthritis by approximately 2 days ($P \leq 0.01$; days 11, 12; see Fig. 1), resulting in an aggravated clinical score on these days, and ameliorated clinical signs only from day 27 ($P \leq 0.05$; see Fig. 1). The accelerating effect of the mAb RIB5/2 was reproduced in two additional treatment experiments, and this effect was observed despite a variable onset of AA in the PBS-treated animals (day 9 to 11); i.e. in all experiments, the onset of AA occurred 2 days earlier than in the controls. In order to identify potential mechanisms for these differential effects, the molecular properties of the mAbs and their influence on T-cell effector functions *in vivo* and *in vitro* were investigated. For the sake of simplicity, we refer to the mAb RIB5/2 as 'accelerating' (although this term is applicable only to the onset of AA) and the mAbs W3/25 and OX35 as 'ameliorating'.

Figure 1



Arthritis score after preventive treatment of rat adjuvant arthritis (AA) with various anti-CD4 mAbs or PBS (controls) (means ± SEM; $n = 6$ for all groups). Arrows show the days of treatment (days –1, 0, 3, 6). Treatment with W3/25 and OX35 significantly suppressed AA from day 13 until day 30. In contrast, RIB5/2 accelerated the onset of AA by 2 days and led to significant improvement only in the late phase (from day 27). $^{**}P \leq 0.01$, $^{*}P \leq 0.05$, in comparison with PBS-treated rats. One representative of three experiments is shown.

Affinity of the monoclonal antibodies

Calculation of the affinity constant (K_A) resulted in comparable values for OX35 and RIB5/2 (see Supplementary Table 1). In contrast, the affinity of W3/25 was 50-fold that for the two other mAbs. While the association rate constants (k_{ass}) for all three mAbs were within the same order of magnitude, striking differences (up to 40-fold) were observed for the dissociation rate constants (k_{diss}). Thus, although differences in overall affinity did not match differential clinical efficacy, the accelerating mAb RIB5/2 displayed the highest k_{diss}.

T-cell reactivity

T-cell reactivity was investigated on day 13 of AA, i.e. when the clinical differences between the accelerating and ameliorating anti-CD4 mAbs were maximal.

In vivo

Compared to the PBS-treated control group, the ameliorating mAb OX35 induced a significant increase of the DTH in response to the arthritogen *Mycobacterium tuberculosis* (Supplementary Fig. 1). The other ameliorating anti-CD4 mAb, W3/25, also induced an increase, but statistical significance was not reached. In contrast, treatment with the accelerating anti-CD4 mAb, RIB5/2, had virtually no influence on the DTH.

In vitro

Upon stimulation with concanavalin A (ConA), total T cells from RIB5/2-treated animals showed lower proliferation rates than those from W3/25- or OX35-treated rats (Supplementary Fig. 2). In the mixed lymphocyte culture, RIB5/2 was also the most potent inhibitor of T-cell activation in the case of total T cells; however, this was not observed in purified $CD4^+$ T cells (Supplementary Fig. 4).

Production of TNF-α after anti-CD4 preincubation

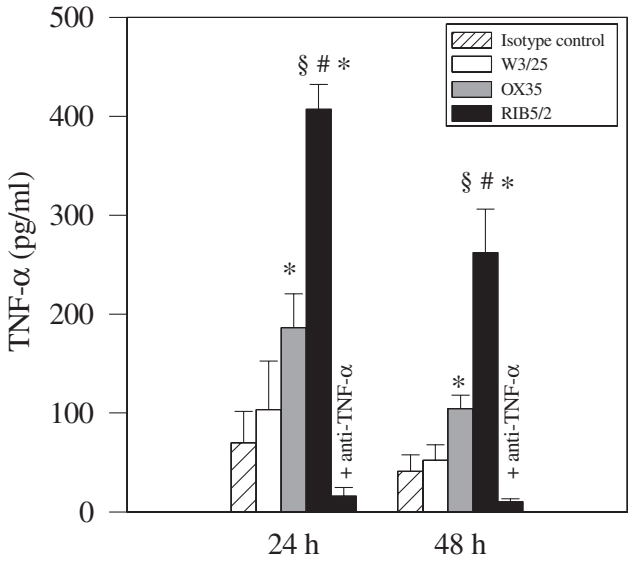
To simulate the preventive therapeutic application in AA, i.e. the activation of $CD4^+$ T cells in the presence of anti-CD4 mAbs, purified spleen $CD4^+$ T cells were incubated with anti-CD4 mAb and, after cross-linking, stimulated with plate-bound anti-TCRα/β mAb (so called anti-CD4 preincubation).

Interestingly, anti-CD4 preincubation with the accelerating anti-CD4 mAb RIB5/2 led to a significantly higher TNF-α secretion, in comparison both with the isotype control and with the two other anti-CD4 mAbs, W3/25 and OX35 (Fig. 2). Differential induction of TNF-α by RIB5/2 was also seen after CD4-cross-linking on the $CD4^+$ T-cell clone A2b or on TCR-stimulated $CD4^+$ T-cell blasts (both data sets not shown). For IFN-γ, IL-10, and IL-4, see Supplementary material.

Signal transduction

Because the three anti-CD4 mAbs recognize distinct epitopes of the CD4 molecule, which is involved in signaling cascades, the influence of these mAbs on early signaling events was also investigated. No influence at all of the anti-CD4 mAbs was found for calcium influx (primary $CD4^+$ spleen T cells), phosphorylation of src-homology-domain-containing protein (Shc), or the translocation of nuclear factor of activated T cells (NF-AT) (both primary $CD4^+$ T cells and clone A2b), and the activity of $p59^{fyn}$ (A2b). The activity of the CD4-associated $p56^{lck}$ after CD4 cross-linking (A2b) was comparably increased by all three anti-CD4 mAbs (all the data not shown).

Figure 2



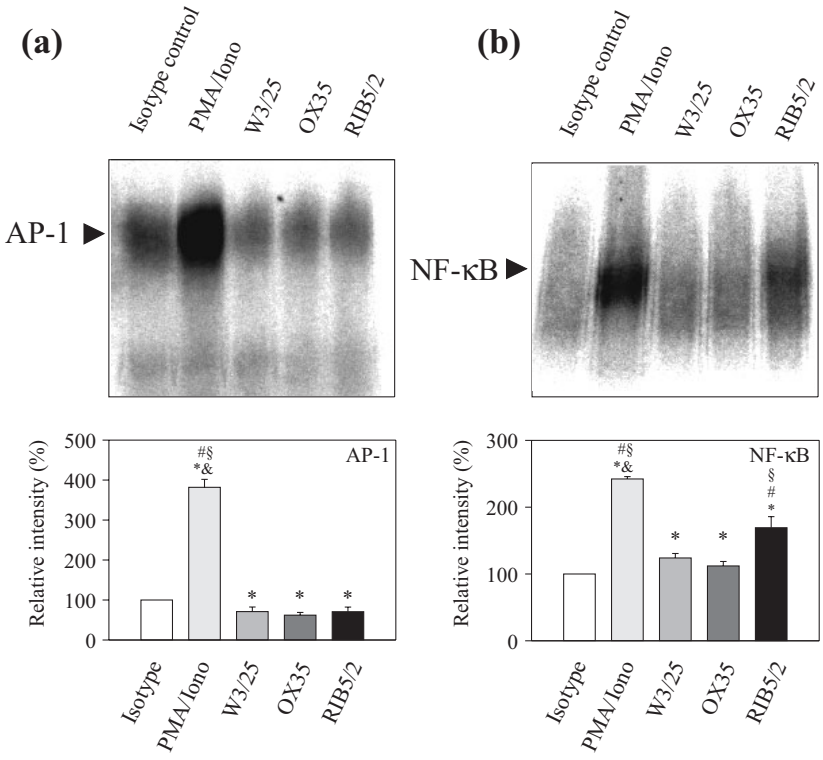
Production of TNF-α by spleen $CD4^+$ T cells after anti-CD4 preincubation, as measured by bioassay (means ± SEM of three experiments; pooled T cells from three normal rats each). The accelerating mAb, RIB5/2, induced a significantly stronger increase of secreted TNF-α than the other mAbs. Specificity was ensured by adding a neutralizing anti-TNF-α mAb to supernatants from RIB5/2-treated cultures. $^*P \leq 0.05$ in comparison with the isotype control, $^{\#}P \leq 0.05$ in comparison with W3/25, $^{\S}P \leq 0.05$ in comparison with OX35.

The binding activities of the transcription factors activator protein-1 (AP-1) and NF-κB were determined in nuclear extracts of spleen $CD4^+$ T cells preincubated with anti-CD4 mAbs. The activity of AP-1 was slightly but significantly decreased by pretreatment with all three anti-CD4 mAbs, to a similar degree (Fig. 3). Interestingly, preincubation with the accelerating anti-CD4 mAb, RIB5/2, induced a significantly higher binding activity of NF-κB than did preincubation with the ameliorating W3/25 and OX35, which only moderately increased the NF-κB binding activity (see Fig. 3).

Discussion

The present study shows differential clinical efficacy of three anti-CD4 mAbs in the preventive treatment of AA. The lack of differential clinical efficacy of the same mAbs in established AA may be explained by a completely divergent immunological constellation in connection with the known differential effects of anti-CD4 mAbs on naive and memory T cells. Although successful treatment with anti-CD4 mAbs has been achieved in various arthritis models [12,16–18], there is no evidence for a differential efficacy of anti-CD4 mAbs in arthritis models to date. Thus, this finding may represent the experimental counterpart of the conflicting results observed thus far in studies of human RA [6,8,10].

Figure 3



Electromobility shift assay (EMSA) of nuclear extracts from CD4+ T cells (preincubated with anti-CD4 mAbs or stimulated with PMA/ionomycin) for AP-1 **(a)** and NF-κB **(b)**. One representative phosphoimage of three independent experiments is shown (upper panels). Quantification of the bands of all three experiments is shown below (means ± SEM). All three anti-CD4 mAbs slightly decreased the AP-1 activity, and RIB5/2 induced a profound, significant increase of NF-κB activity. $^{*}P \leq 0.05$ in comparison with the isotype control, $^{\#}P \leq 0.05$ in comparison with W3/25, $^{\S}P \leq 0.05$ in comparison with OX35, $^{\&}P \leq 0.05$ in comparison with RIB5/2.

Role of molecular features of the antibodies

Isotype, affinity, and epitope

In contrast to a reported study of experimental allergic encephalomyelitis [19], the present study did not confirm a role for the mAb isotype in AA, as the isotypes of the three anti-CD4 mAbs did not match their differential preventive effects in AA. The ameliorating mAb W3/25 had a very high affinity, as previously reported [20], whereas the affinity of the other ameliorating mAb, OX35, was comparable to that of the accelerating mAb, RIB5/2. On the other hand, a contribution of the strikingly higher k_{diss} of the mAb RIB5/2 to its differential clinical effects cannot be excluded, in analogy to high and low k_{diss} for agonistic and antagonistic TCR-peptides, respectively [21]. In spite of these considerations, recognition of different epitopes by the mAbs [14], with distinct functional consequences for the target cells, remains the most likely explanation for their differential clinical efficacy [22]. While the binding sites of W3/25 and OX35 are situated in the C′–C″ region of domain D1 and the B–C region of domain D2 of the CD4 molecule, respectively [23], we could roughly localize the epitope of RIB5/2 in the F–G region of domain D1, as demonstrated by effective competition with the mAb OX65 (data not shown), known to bind this region of the CD4 molecule [23]. Binding of anti-CD4 mAbs to separate epitopes of the CD4 molecule could result in differential effects on T-cell functions by inducing distinct conformational changes of the extracellular and the intracellular parts of the CD4, resulting in modified interaction with other signaling molecules, as has been discussed recently with regard to the aspartate receptor [24].

Influence on T-cell reactivity

In the present study, the ameliorating anti-CD4 mAbs W3/25 and OX35 (but not the accelerating mAb, RIB5/2) numerically/significantly increased the DTH to the arthritogen *M. tuberculosis*. In the total T-cell population, two of the three anti-CD4 mAbs (and in purified CD4+ T cells, all three anti-CD4 mAbs) increased the *in vitro* reactivity to ConA. In the case of CD4+ T cells, this increased reactivity was negatively correlated with the clinical score of individual animals. The successful mode of action of anti-CD4 mAbs in arthritides may therefore be based on increasing the reactivity of a subpopulation of regulatory T cells, an

interpretation that is supported by: i) the increase of T-cell reactivity in some patients successfully treated with anti-CD4 mAbs [6], ii) an increased DTH to antigens like tetanus toxoid in less active versus active rheumatoid arthritis [25], iii) the reduced responsiveness to poly-specific stimulation of spleen cells from rats with acute AA in comparison with healthy animals [26], and iv) the almost complete restoration of T-cell responsiveness upon disease resolution/transition into the chronic phase [27].

Production of TNF-α in vitro

The accelerating mAb RIB5/2 significantly increased the secretion of TNF-α, as observed in primary $CD4^+$ T cells both upon anti-CD4 preincubation and upon CD4 cross-linking after stimulation with TCR (see Fig. 2). In view of the known local and systemic importance of TNF-α in experimental AA [28–30] and human RA [31], this effect may contribute to the acceleration of the onset of AA after pretreatment with the mAb RIB5/2. This finding may be important for the understanding of pathogenesis in arthritis despite predominant production of TNF-α by macrophages/monocytes (reviewed in [32]), since TNF-α production by T cells has been demonstrated both in the synovial membrane [33] and in lymphoid organs (authors own unpublished observations).

Signal transduction in vitro

Since TNF-α production is known to be regulated predominantly by NF-κB [34], it was revealing that the accelerating mAb, RIB5/2, besides inducing TNF-α secretion, also strongly upregulated NF-κB binding activity. This was especially striking because binding of another major transcription factor involved in T-cell activation, AP-1 [35], was even slightly downregulated by the anti-CD4 mAbs, in line with recently published data in murine cells [36]. These findings in our study support, again, the notion that NF-κB-mediated upregulation of TNF-α secretion (or vice versa) may contribute to the acceleration of disease onset in AA upon preventive treatment with RIB5/2.

The differential clinical efficacy of anti-CD4 mAbs is not restricted to arthritis but is also observed in transplantation models [37]. However, while preventive treatment with the anti-CD4 mAb RIB5/2 leads to an acceleration of AA, it is a very effective anti-CD4 mAb for the induction of tolerance in transplantation models [14,37]. This indicates that clinical efficacy (and its time course) may depend on the actual immunological constellation and that a given anti-CD4 mAb may have beneficial effects only in particular pathologies and/or stages of disease. From the results of the present study, it is evident that the individual features and effects of a particular anti-CD4 mAb have to be assessed before treatment trials in order to predict its clinical efficacy *in vivo*. This may require several *in vivo*, *ex vivo*, or *in vitro* assays of T-cell function in order to reveal subtle differences between anti-CD4 mAbs. Experimental models such as transgenic mice expressing human but not mouse CD4 may make it possible to address such questions in the future [38]. Recently, preclinical testing has been exploited in murine CD4-knockout/human CD4-transgenic (huCD4-transgenic) systems to assess the immunological effects of a particular anti-human-CD4 mAb in various disease models [39,40]. Analogous preclinical testing may prove useful also for the comparison of anti-human-CD4 mAbs targeting distinct epitopes.

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Supplementary material

Supplementary methods

Animals and antibodies

Female Lewis rats (8–16 weeks old; body weight 150–220 g) were obtained from Charles River (Sulzfeld, Germany) or from the Medical Experimental Center of the University of Leipzig. Female inbred Wistar–Prob rats (12–16 weeks old) were bred in the Medical Experimental Center. The animals were kept under standard conditions, two per cage, with food and water *ad libitum* and a 12 h/12 h light/dark cycle.

The mouse anti-rat-CD4 mAbs W3/25, OX35, and RIB5/2 (IgG_1, IgG_{2a}, and IgG_{2a}, respectively) were used as ascites fluid for *in vivo* treatment or purified on Protein A–sepharose (for OX35 and RIB5/2) or Protein G–sepharose (for W3/25) columns (Pharmacia, Freiburg, Germany) for all *in vitro* experiments. The maximal endotoxin content of the ascites was 58 IU/ml (W3/25), 17 IU/ml (RIB5/2), and 5 IU/ml (OX35); the final endotoxin concentrations for *in vitro* experiments were less than 1 IU/ml. For flow cytometry, directly FITC-labeled W3/25 and OX35 (gifts from P Kühnlein, Institute of Virology, University of Würzburg), and the mAbs G4.18 (anti-CD3, Pharmingen, Hamburg, Germany), OX8 (anti-CD8, Serotec, Oxford; UK), OX81 (anti-IL4, Pharmingen), A5-4

(anti-IL-10, Pharmingen), and DB-1 (anti-IFN-γ, Serotec) were used. MAbs MOPC21 (IgG_1), UPC10 (IgG_{2a}, both from Sigma, Deisenhofen, Germany) and mouse anti-glucose-oxidase FITC/phycoerythrin (PE) (IgG_1, IgG_{2a}, Dako, Hamburg Germany) served as isotype-matched control antibodies. The anti-TCRα/β mAb R73 was a kind gift of T Hünig (Institute of Virology, University of Würzburg). For sandwich ELISA analysis of cytokine concentrations, the following antibodies were used: DB-1 (anti-IFN-γ), biotinylated rabbit anti-rat IFN-γ (both Biosource, Ratingen, Germany); rabbit anti-rat IL-2, biotinylated A38-3 (anti-IL-2, both Pharmingen), A5-7, biotinylated A5-6 (both anti-IL-10, Pharmingen); OX81 (anti-IL-4), biotinylated rabbit anti-rat IL-4 (Biosource).

Affinity

The mAbs were bound to a BIAcore surface via goat anti-mouse Ig-Fc and incubated with various concentrations of soluble rat CD4 (a gift of N Barclay, Oxford, UK). The data were analyzed in accordance with the method of Karlsson *et al.* [S1], and the association rate constant k_{ass}, the dissociation rate constant k_{diss}, and the affinity constant K_A (k_{ass}/k_{diss}) were calculated.

Adjuvant arthritis and anti-CD4 treatment

Lewis rats (8–12 weeks old) were given 0.5 mg heat-inactivated *M. tuberculosis* (Difco, Detroit, MI, USA) in 100 µl paraffin oil (Riedel de Haën, Seelze, Germany), which was injected intradermally into the base of the tail. For preventive treatment, the rats ($n = 6$ in each group) received 3 mg of RIB5/2, 3 mg of W3/25, or 2 mg of OX35 intraperitoneally (corresponding to 17.1 and 11.4 mg/kg, respectively, on the basis of a mean body weight of 175 g) on days –1, 0, 3, and 6, i.e. 1 day before AA induction, on the day of induction (day 0), and thereafter. The lower dose of OX35 (2 mg) was chosen on the basis of previous experiments demonstrating its high clinical efficacy in AA [S2]. The control group received PBS [S3]. In addition, preventive treatment with an isotype-matched mAb from birth had shown no effect on rat AA in a previous study [S3].

For determination of the arthritis score, each paw was graded according to the extent of erythema and edema of the periarticular tissue, on a scale of 0–4, where 0 = no inflammation, 1 = unequivocal inflammation of one paw joint, 2 = unequivocal inflammation of at least two paw joints or moderate inflammation of one paw joint, 3 = severe inflammation of one or more paw joints, and 4 = maximum inflammation of one or more paw joints [S4,S5]. The scores were then added to obtain the total score (maximal possible score of 16 for each animal).

Flow cytometry

For determination of cell depletion and modulation of surface molecules, blood samples (100 µl) were taken via retro-orbital puncture on day 8 or day 13 after induction of AA. Whole-blood cells were stained with saturating amounts of directly FITC-labeled W3/25, OX35, G4.18, OX8, or respective isotype, followed by erythrocyte lysis. Ten thousand events were analyzed using an Epics XL flow cytometer (Coulter, Krefeld, Germany) and the results displayed as histograms.

Delayed-type hypersensitivity

To assess the DTH, either 10 µg of the arthritogen *M. tuberculosis* in 50 µl PBS, or 50 µl PBS only, were injected intradermally into the pinna of the left or the right ear, respectively, on day 13 after induction of AA. One day after injection, the swelling of the ears was determined with a gauge (Hahn & Kolb, Stuttgart, Germany). Swelling was expressed as the difference (mm) between the thickness of the left and the right ear.

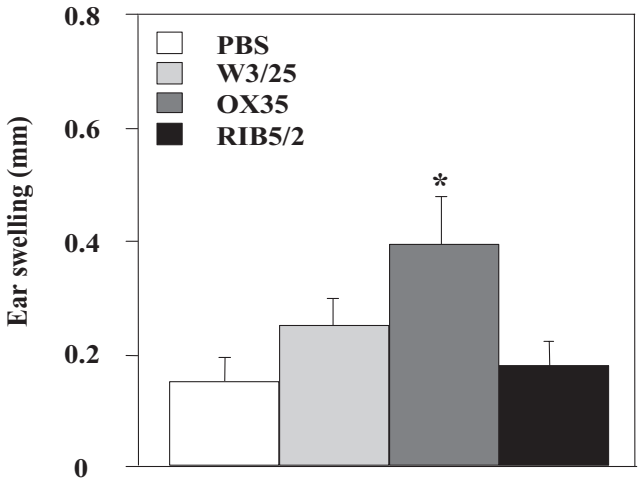
Supplementary Table 1

Affinity data of the anti-CD4 mAb

Anti-CD4 mAb	k_{ass} ($M\ s^{-1}$)	k_{diss} (s^{-1})	K_A (M^{-1})
W3/25	4.7×10^5	1.0×10^{-4}	4.9×10^9
OX35	0.8×10^5	0.8×10^{-3}	1.0×10^8
RIB5/2	2.7×10^5	3.7×10^{-3}	0.7×10^8

k_{ass} = association rate constant; k_{diss} = dissociation rate constant; K_A = affinity constant (k_{ass}/k_{diss}).

Supplementary Figure 1



In vivo delayed-type hypersensitivity to *Mycobacterium tuberculosis* on day 13 after preventive treatment of AA, i.e. when the clinical differences between the accelerating and ameliorating anti-CD4 mAbs were maximal. The data are expressed as means ± SEM ($n = 6$ for all groups, one representative of three *in vivo* experiments) of the difference between the swelling of the left (mycobacterium-treated) and the right (PBS-treated) ears. *$P \leq 0.05$ in comparison with PBS-treated rats.

T-cell reactivity *in vitro*

T cells were purified from spleens as described elsewhere [S6]. Briefly, the spleens were passed through a stainless-steel sieve and the resulting suspension was centrifuged through Lymphoprep (Pharmacia). Cells contained in the interphase were then washed twice and loaded onto a 10-ml syringe with 1.2 g nylon wool (Polyscience, Eppelheim, Germany). After incubation for 1 hour at 37°C, 5% CO_2, the cells were eluted with RPMI 1640/GlutaMaxI containing 10% FCS, 15 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (thereafter called R10F, all from GIBCO-BRL, Eggenstein, Germany). The resulting total T-cell suspension was ≥95% $CD3^+$, as evaluated by flow cytometry. Either purified total T cells were directly used for proliferation assays, or $CD4^+$ T cells were first negatively purified by adding 5 μg/1 × 10^7 cells OX8 mAb for 30 min on ice and, after washing, 75 μl Dynabeads-M450 coupled to goat anti-mouse IgG (Dynal, Hamburg, Germany). The suspension was again incubated for 30 min on ice and then separated by a magnetic particle concentrator (MPC®, Dynal). The purity of $CD4^+$ T-cell populations was always ≥95%, as assessed by flow cytometry. For enrichment of dendritic cells (DC), suspensions of spleen cells from healthy rats were subjected to an overnight adhesion step on Petri dishes (Falcon®, Becton Dickinson, Heidelberg, Germany) and then centrifuged through 2 ml of a 14.5% metrizamide solution (Sigma) in R10F. DC were then irradiated with 15 Gy to prevent their proliferation in subsequent assays. For proliferation assays, 1 × 10^5 purified total or $CD4^+$ spleen T cells per well of 96-well round-bottom plates were incubated with 1 × 10^4 DC per well and 1 μg/ml ConA (Sigma) for 72 hours. Then 1 μCi/well ^{3}H-thymidine (Amersham-Buchler, Braunschweig, Germany) was added. After an additional incubation for 16 hours, cells were harvested onto fiber filters and cell-bound radioactivity was measured by β-scintillation counting (Matrix96®, Canberra Packard, Dreieich, Germany).

Primary allogeneic mixed lymphocyte culture

Total T cells or purified $CD4^+$ T cells from spleens of healthy Lewis rats, and allogeneic DC from spleens of Wistar–Prob rats, were prepared as described above. Total or $CD4^+$ T cells were seeded at 4 × 10^5 cells per well in 96-well flat-bottom plates, together with 1, 2, or 4 × 10^4 DC per well and 1, 5, or 10 μg/ml of the respective purified anti-CD4 mAb (or isotype control mAbs). After incubation for 72 hours (37°C, 5% CO_2), bromodeoxyuridine (BrdU) was added and incubation was continued for an additional 16 hours. The proliferation was determined by a BrdU cell-proliferation ELISA (Boehringer Mannheim, Mannheim, Germany) in accordance with the supplier's recommendations.

Cell stimulation

For anti-CD4 preincubation, spleen $CD4^+$ T cells were incubated with the anti-CD4 mAbs or isotype control mAbs (10 μg per 1 × 10^7 cells) for 30 min at 4°C and washed once. The bound mAbs were then cross-linked with goat anti-mouse IgG (Jackson Lab, 20 μg/1 × 10^7 cells) for 1 hour at 37°C. After washing, the cells (1 × 10^6 per ml and per well) were seeded in 24-well plates previously coated with R73 and harvested after 24 and 48 hours.

Cytokine assays

Anti-CD4-preincubated cells were washed, fixed with 4% paraformaldehyde in PBS, permeabilized (0.5% saponin in PBS, 1% FCS, 0.01% NaN_3; for this step, supplemented with 10% rat serum), and incubated with 1 μg anti-IFN-γ FITC, anti-IL-4 PE, anti-IL-10 PE, or directly labeled isotype-control mAbs for 30 min at 4°C. FACS analysis was performed using a FACScan® flow cytometer (Becton Dickinson).

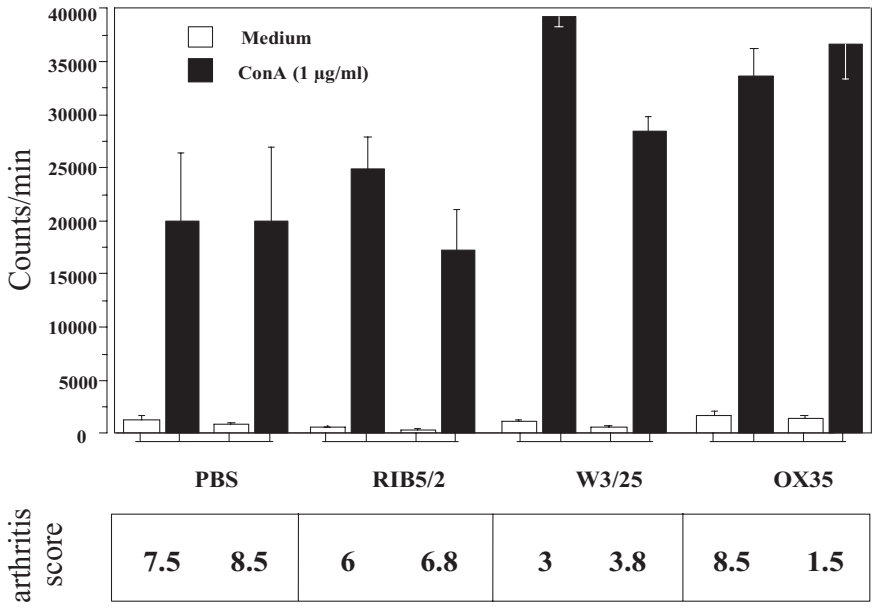
Detection of TNF-α was performed using a bioassay based on the lysis of the WEHI164/13 cell line after exposure to TNF-α. Assay specificity was ensured by adding a neutralizing mAb to rat TNF-α (100 μg/ml; clone 45418.111; R&D Systems, Wiesbaden, Germany).

Culture supernatants were analyzed for IFN-γ, IL-2, IL-4, and IL-10 by sandwich ELISA with the above-mentioned capture and detection antibodies. Recombinant cytokine standards were as follows: IFN-γ (Laboserv), IL-2, IL-10, and IL-4 (Pharmingen); range for IFN-γ, IL-2, and IL-10 was 39–5000 pg/ml; range for IL-4 was 9.85–1250 pg/ml. Cytokine concentrations in the culture supernatants were calculated from a standard curve using the software EasyFit (SLT, Crailsheim, Germany).

Electrophoretic mobility shift assays

Purified spleen $CD4^+$ T cells were preincubated with the anti-CD4 mAbs as above and then either stimulated on anti-CD3 mAb-precoated 6-well plates (1 × 10^7 cells per well) or with a combination of phorbol myristoyl acetate (PMA; 10 ng/ml) and ionomycin (250 ng/ml) for 4 hours at 37°C. After stimulation, the cells were washed once with PBS and microcentrifuged (10,000 × ***g***) at 4°C for 1 min. The pellet was resuspended in 400 μl ice-cold buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1 mM dithiothreitol, and 1 mM Na_3VO_4) and placed on ice for 15 min. Subsequently, 25 μl of a 10% Nonidet P-40 solution (Boehringer Mannheim) were added to the sample and the cells were homogenized by vortexing for 30 s followed by microcentrifugation for 1 min. The nuclear pellets were resuspended in 50 μl of buffer B (20 mM HEPES, 400 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM PMSF, 1 mM dithiothreitol, and 1 mM Na_3VO_4) and shaken for 15 min at 4°C. After microcentrifugation for 5 min at 4°C, the supernatants were aliquoted and stored at −70°C until further use. The nuclear extracts (10 μg total protein) were than

Supplementary Figure 2



Concanavalin A (ConA) reactivity of total spleen T cells (day 13 of adjuvant arthritis) from rats preventively treated with anti-CD4 mAbs. Proliferation data from two individual animals of each treatment group are expressed as means ± SEM of triplicate cultures from one *in vivo* experiment. The corresponding arthritis score for the animals is shown below the graph.

incubated with 2×10^8 cpm of ^{32}P-labeled, double-stranded oligonucleotide probe (sense strand only; AP-1: 5′-CGC TTG ATG AGT CAG CCG GAA-3′; NF-κB: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; both from Promega, Mannheim, Germany; NF-AT: 5′-CGC CCA AAG AGG AAA ATT TGT TTC ATA-3′; Santa Cruz, Heidelberg, Germany) in 25 µl binding buffer (1 M Tris, 1 M boric acid, 0.02 M EDTA, 5% glycerol) supplemented with poly[dI-dC] (0.16 mg/ml Pharmacia) and 2 mM dithiothreitol. The samples were separated on a 4% polyacrylamide gel at 200 V. After scanning in a phosphor imager (BAS-1000, Fuij Photo Film Co. Ltd, Japan), the bands were quantified using the PCBAS 2.09g software (Fuij Photo Film Co. Ltd).

Statistics

Differences between experimental groups were evaluated with the two-tailed nonparametric Mann–Whitney (*U*) test. The Spearman rank correlation test was used to verify whether the *ex vivo* ^{3}H-thymidine uptake of CD4$^+$ T cells correlated with the arthritis score of individual rats. Statistical significance was accepted at $P \leq 0.05$.

Supplementary results

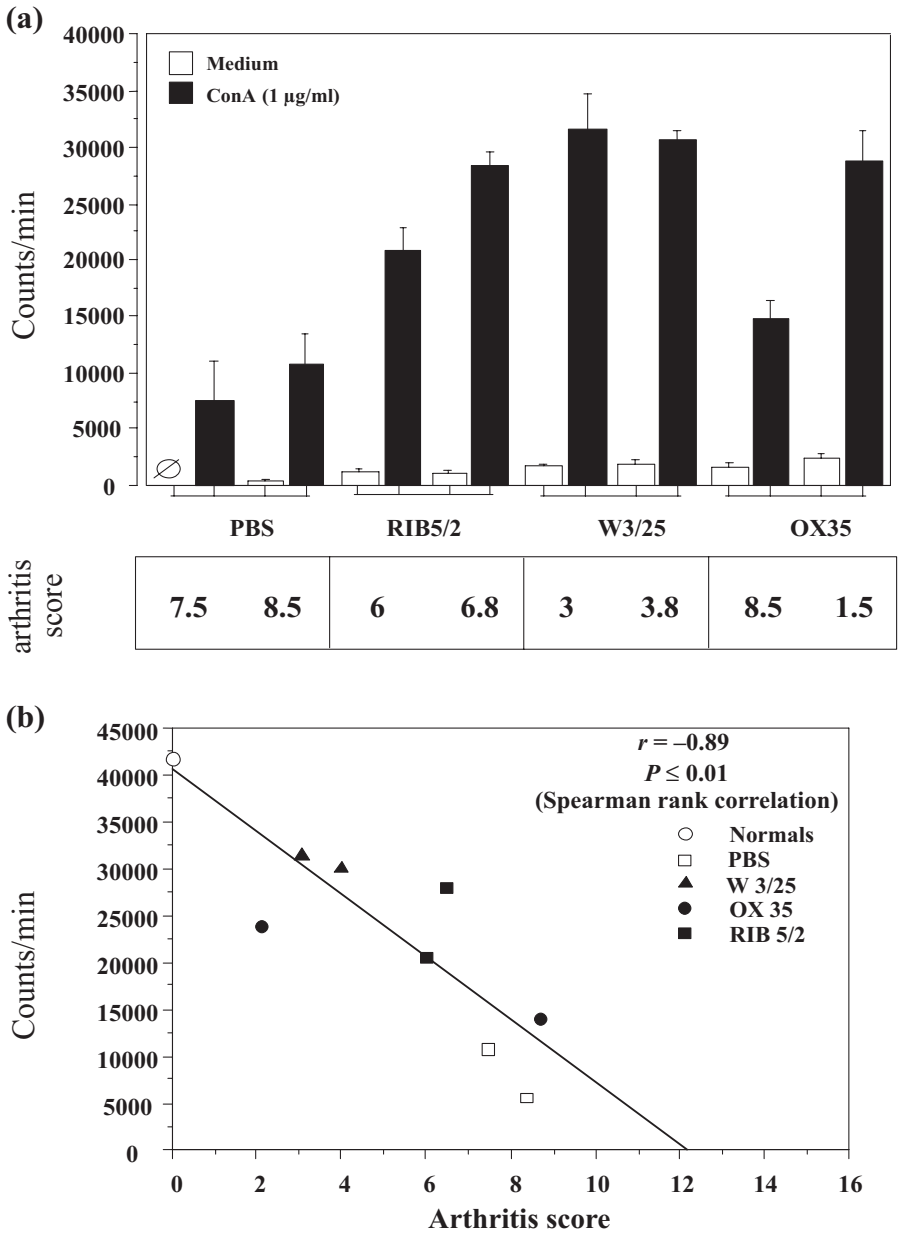
T-cell reactivity *in vitro*

Total T cells and CD4$^+$ T cells from spleens of individual rats (day 13 of AA) were stimulated *in vitro* with ConA. The *in vitro* proliferation rates of total T cells from OX35- and W3/25-treated animals in response to polyspecific stimulation with ConA were higher than those of PBS-treated rats, while T cells of the RIB5/2-treated rats showed proliferation rates similar to those of T cells from PBS-treated animals (Supplementary Fig. 2). ConA stimulation of CD4$^+$ T cells resulted in generally lower proliferation rates than those of total T cells in PBS-treated rats. However, the values of CD4$^+$ T cells from individual, anti-CD4-treated animals were always higher than those of the PBS-treated control group (Supplementary Fig. 3a). There was a highly significant negative correlation between the degree of the ConA-induced proliferation of CD4$^+$ spleen T cells and the severity of the arthritis score, i.e. the lower the arthritis score, the higher the proliferation rate (Supplementary Fig. 3b).

Mixed lymphocyte culture

In these experiments, the inhibitory potency of the anti-CD4 mAbs on the mixed lymphocyte culture, an equivalent of a transplant rejection, was investigated as a model for MHC-dependent T-cell activation. The anti-CD4 mAbs moderately inhibited the proliferation of total spleen T cells, depending on the stimulator cell concentration (Supplementary Fig. 4a). The accelerating mAb RIB5/2 led to significant inhibition at all concentrations of mAb (1, 5, and 10 µg/ml; only 1 µg/ml is shown in Supplementary Fig. 4a) and DC, whereas the other two anti-CD4 mAbs significantly inhibited the proliferation only in some cases. Furthermore, RIB5/2 inhibited the proliferation to a statistically significant degree at

Supplementary Figure 3

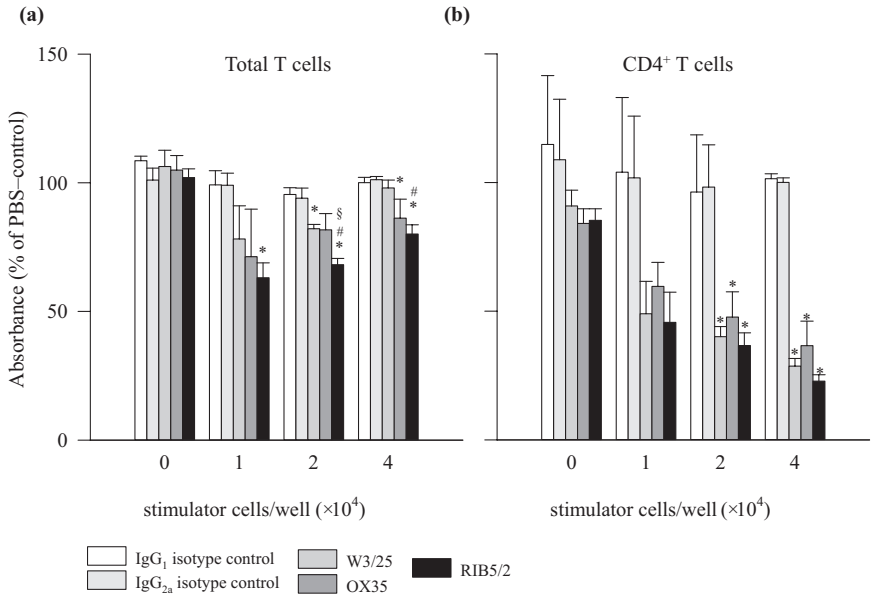


Concanavalin A (ConA) reactivity **(a)** of $CD4^+$ spleen T cells (day 13 of adjuvant arthritis) from preventively treated rats with anti-CD4 mAbs. Proliferation data from two individual animals of each group are expressed as means ± SEM of triplicate cultures. The corresponding arthritis score for the animals is shown in the middle. The correlation between reactivity to ConA and the severity of arthritis is shown in **(b)**. Normals = normal, untreated rats.

1 µg/ml and 2×10^4 DC/well more strongly than both W3/25 and OX35. Thus, these differential results (inversely) match the differential clinical efficacy seen with the three mAbs.

The possibility that $CD8^+$ T cells would mask the inhibitory potency of the anti-CD4 mAbs on the proliferation of $CD4^+$ T cells in a total T-cell population was excluded by using purified $CD4^+$ T cells. They showed a clearer inhibition of their proliferation rates (50 to 80% in comparison with the isotype) when used as responder cells. However, in contrast to the total T-cell population, there was no significant difference in the inhibitory potency among the three anti-CD4 mAbs (Supplementary Fig. 4b).

Supplementary Figure 4



Mixed lymphocyte culture of total **(a)** and CD4+ **(b)** spleen T cells from normal Lewis rats with different densities of allogeneic (Wistar–Prob) stimulator cells, supplemented with isotype controls (IgG_1, IgG_{2a}), or the anti-CD4 mAbs W3/25, OX35, and RIB5/2 at 1 μg/ml. Data are expressed as means ± SEM of the relative absorbance from three independent experiments (triplicates in each experiment), normalized to the absorbance of PBS-treated cultures (= 100%). $^{*}P \leq 0.05$ in comparison with the respective isotype control, $^{\#}P \leq 0.05$ in comparison with W3/25, $^{\S}P \leq 0.05$ in comparison with OX35.

Depletion and modulation in peripheral blood *in vivo*

In order to characterize the depleting and modulating capacity of the three mAbs, FACS of peripheral blood mononuclear cells was performed on days 8 and 13 of AA (Supplementary Table 2A,B). Two different anti-CD4 mAbs (W3/25 and OX35, which do not compete for CD4 binding) were employed to differentiate coating with therapeutic mAb from depletion of CD4+ T cells/CD4-modulation.

Treatment with W3/25

After treatment with W3/25, the percentage and/or MFI (mean fluorescence intensity) of CD3+ cells in the peripheral blood on days 8 and 13 was numerically lower than in the PBS-treated control group, resulting in a significantly lower MFI on day 13 (note: the fact that on day 8 only two animals of the W3/25-treated group were investigated likely prevented statistical significance). Furthermore, a decrease of CD4+ cells was observed when the cells were stained with OX35 (more prominent on day 13; see Supplementary Table 2B). After *ex vivo* staining with W3/25 – the mAb also used for treatment – only 7% (day 8) and 28% (day 13) of the peripheral blood mononuclear cells were CD4+. This fact, and the positive staining with an anti-mouse IgG on days 8 and 13 (18% and 9%, respectively), showed that the therapeutic anti-CD4 mAb was present on the cells as long as 7 days after the last injection of the mAb. After treatment with W3/25, the percentage of CD8+ cells did not show any significant changes on days 8 or 13; however, the MFI for CD8 on day 13 was significantly decreased (see Supplementary Table 2B).

Therefore, the anti-CD4 mAb W3/25 induced only weak depletion of CD4+ cells, as indicated by unchanged percentages of CD8+ cells. In addition, there was modulation of the CD4 and co-modulation of the CD3 surface molecules and indirect modulation of the CD8 molecule (see Supplementary Table 2B). The anti-CD4 mAb W3/25 therefore represented a weakly depleting, but modulating antibody.

Treatment with OX35

In the peripheral blood of OX35-treated rats, the percentage of CD3+ cells was significantly lower than in PBS-treated animals (see Supplementary Table 2A,B). The percentage of CD4+ cells in the OX35-treated group was also significantly lower than that of the PBS control, both upon staining with W3/25 and OX35 (see Supplementary Table 2A,B). The therapeutically applied OX35 was no longer present on the cells at this stage, as demonstrated by negative staining for mouse IgG. The percentage of CD8+ cells of OX35-treated rats was either significantly or numerically increased compared to the PBS-treated control on day 8 or 13, respectively, whereas the MFI of the CD8 molecule was significantly decreased on both days.

Supplementary Table 2

Cytofluorometric analyses of peripheral blood mononuclear cells on day 8 (A) and day 13 (B) of anti-CD4 treatment (data from one representative of three *in vivo* experiments).

(A) Day 8

	Treatment							
	PBS ($n = 4$)		W3/25 ($n = 2$)		OX35 ($n = 4$)		RIB5/2 ($n = 3$)	
Antigen (FACS)	%	MFI	%	MFI	%	MFI	%	MFI
Isotype control	0 ± 0.0	107 ± 9.0	4 ± 0.5	98 ± 2.0	1 ± 0.3	82 ± 3.3	0 ± 0.0	86 ± 7.7
CD3	70 ± 5.5	122 ± 1.7	47 ± 5.0	99 ± 1.5	31 ± 1.9*	93 ± 1.5*	28 ± 7.2*	95 ± 0.7*
CD8	21 ± 1.0	162 ± 1.3	27 ± 3.0	110 ± 1.5	33 ± 2.0*	109 ± 5.0*	39 ± 3.2*	111 ± 0.3*
CD4 (W3/25)	55 ± 3.2	116 ± 0.8	7 ± 2.5	98 ± 1.5	23 ± 1.7*	97 ± 1.2*	8 ± 1.5*	95 ± 0.7*
CD4 (OX35)	52 ± 7.5	150 ± 0.9	45 ± 8.0	93 ± 3.0	26 ± 4.1	122 ± 2.4	8 ± 1.2*	121 ± 1.0*
Mouse IgG	1 ± 0.3	105 ± 1.9	18 ± 2.0	101 ± 0.5	2 ± 0.3	79 ± 2.4*	1 ± 0.0	85 ± 3.2*

(B) Day 13

	Treatment							
	PBS ($n = 3$)		W3/25 ($n = 4$)		OX35 ($n = 4$)		RIB5/2 ($n = 3$)	
Antigen (FACS)	%	MFI	%	MFI	%	MFI	%	MFI
Isotype control	1 ± 0.6	78 ± 1.3	2 ± 1.7	87 ± 4.1	1 ± 0.3	81 ± 3.7	1 ± 0.3	71 ± 1.7
CD3	53 ± 2.3	142 ± 0.6	44 ± 2.3	116 ± 3.0*	39 ± 2.5*	94 ± 1.0*	38 ± 1.5*	96 ± 2.0*
CD8	20 ± 3.8	144 ± 1.5	23 ± 1.7	128 ± 3.2*	30 ± 7.4	108 ± 1.6*	41 ± 2.3*	113 ± 0.9*
CD4 (W3/25)	46 ± 1.0	110 ± 1.9	28 ± 2.0*	109 ± 1.9	25 ± 2.9*	104 ± 1.6	20 ± 0.9*	106 ± 0.7
CD4 (OX35)	46 ± 0.7	154 ± 0.7	29 ± 3.8*	144 ± 5.9	23 ± 5.3*	102 ± 2.6*	17 ± 2.5*	116 ± 6.4*
Mouse IgG	4 ± 2.5	77 ± 2.5	9 ± 2.0	91 ± 3.5*	1 ± 0.2	82 ± 2.7	2 ± 0.0	76 ± 1.2

Results of surface staining are expressed as the mean ± SEM of the percentage of positive cells and the mean fluorescence intensity (MFI). FACS = flow cytometry. *$P \leq 0.05$ in comparison with PBS control.

In comparison with W3/25, the mAb OX35 induced a clearer depletion of $CD4^+$ cells of the peripheral blood, as indicated by a significant increase in the percentage of $CD8^+$ cells. In addition, the mAb OX35 induced CD4-modulation and CD3-co-modulation, as shown by a profound decrease of $CD4^+$ cells and a decrease of the MFI for CD4. Thus, OX35 represented a depleting and modulating antibody.

Treatment with RIB5/2

RIB5/2 also induced a significant reduction of $CD3^+$ cells in comparison with the PBS group (see Supplementary Table 2A,B). The percentage of $CD4^+$ cells was also significantly decreased on days 8 and 13. In contrast, there was a significant increase of $CD8^+$ cells in the periphery of RIB5/2-treated rats in comparison with the PBS-treated control (see Supplementary Table 2A,B). Similarly to the findings for mAb OX35, mAb RIB5/2 induced T-cell depletion and CD4 modulation; however, the degree of peripheral T-cell depletion was stronger than in the case of OX35.

Thus, since both ameliorating and accelerating mAbs (OX35 and RIB5/2) depleted $CD4^+$ T cells from peripheral blood, and since all three mAbs were modulating, there was no apparent match of these parameters with the clinical efficacy.

Cytokine production after anti-CD4-preincubation

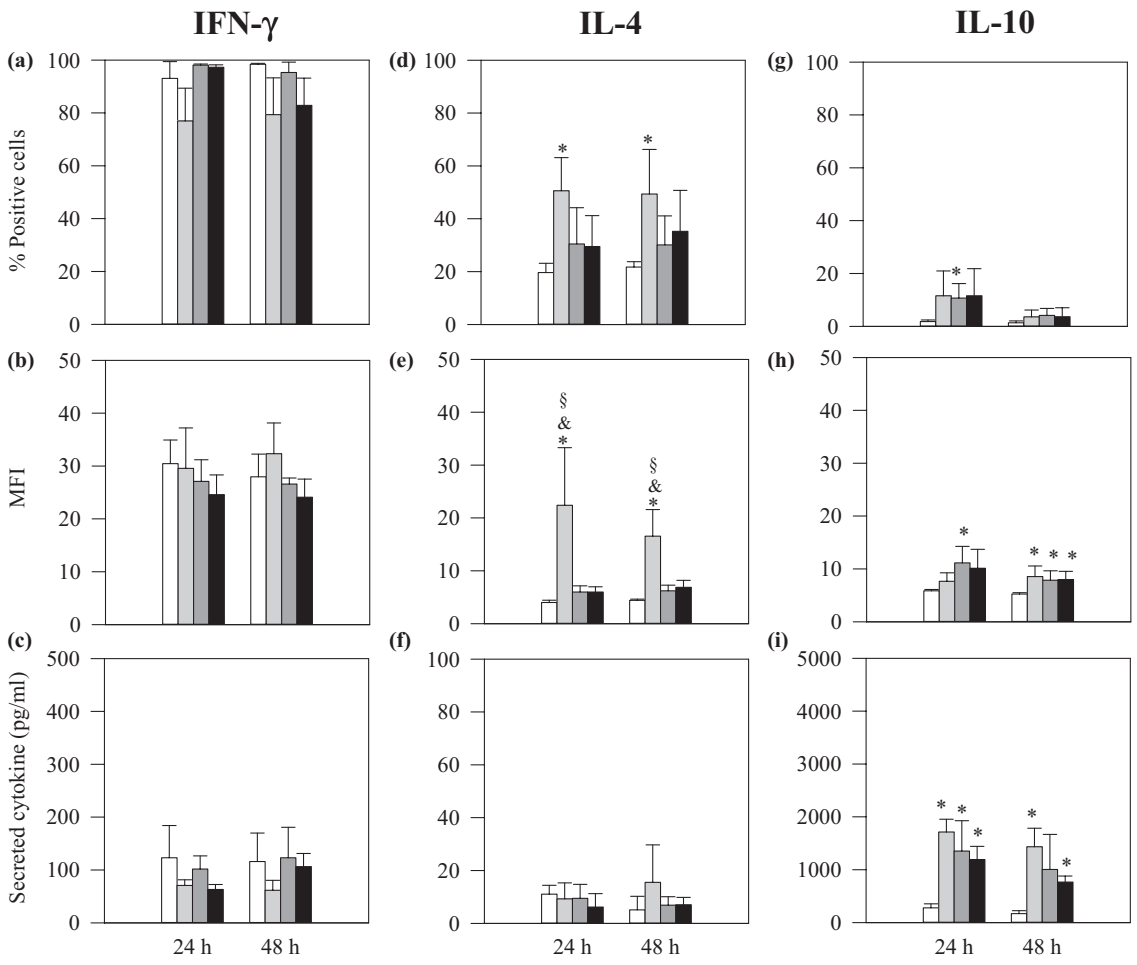
IFN-γ

Intracellular staining of cells preincubated with anti-CD4 mAbs or isotype controls showed a uniformly high expression for IFN-γ (Supplementary Fig. 5a,b; between 80% and 100% positive cells; MFI approximately 30). There were no differences between the anti-CD4 mAb-treated or isotype-treated cultures for the concentration of IFN-γ in the supernatant (Supplementary Fig. 5c).

IL-4

The anti-CD4 mAb W3/25 induced a significantly higher percentage of IL-4-positive cells at 24 and 48 hours than did the isotype control (Supplementary Fig. 5d); the MFI

Supplementary Figure 5



Production of IFN-γ **(a, b, c)**, IL-4 **(d, e, f)**, and IL-10 **(g, h, i)** by spleen CD4+ T cells after anti-CD4-preincubation. Percentage of cytokine-positive cells (upper panels), their mean fluorescence intensity (MFI) after intracellular staining (middle panels), and secretion of the cytokine into cell culture supernatant as measured by ELISA (lower panels), shown as means ± SEM of three independent experiments (triplicate determinations in pooled cells from three normal rats in each experiment). $^{*}P \leq 0.05$ in comparison with isotype control, $^{\#}P \leq 0.05$ in comparison with W3/25, $^{\S}P \leq 0.05$ in comparison with OX35, $^{\&}P \leq 0.05$ in comparison with RIB5/2. For key to bars, see Supplementary Figure 4.

was also significantly higher in comparison with OX35 and RIB5/2 (Supplementary Fig. 5e). In contrast, there were no significant differences of the IL-4 concentration in the supernatant of the cultures (Supplementary Fig. 5f). This observation may be attributable to a delayed secretion of IL-4 or consumption of the cytokine during culture.

IL-10

At 24 h, a numerical increase of the percentage of IL-10-positive cells could be detected after preincubation with the ameliorating mAb W3/25 and the accelerating mAb RIB5/2, whereas the other ameliorating mAb, OX35, induced a significant increase (Supplementary Fig. 5g). This increase was no longer observed at 48 h (Supplementary Fig. 5g). In contrast, the MFI for IL-10 was increased both 24 h and 48 h after pretreatment with anti-CD4 mAbs. At 24 h, only the increase induced by the mAb OX35 was significant, but at 48 h all three mAbs induced a significant increase of the MFI (Supplementary Fig. 5h). Preincubation with all anti-CD4 mAbs induced a significantly higher secretion of IL-10 than did the isotype control, not only after 24 h, but also after 48 hours (the latter increase did not reach statistical significance in the case of OX35). None of the parameters, however, showed significant differences among the three different anti-CD4 mAbs (Supplementary Fig. 5i).

Supplementary discussion

Depleting/modulating capacity

The use of nondepleting anti-CD4 mAbs for treatment of human rheumatoid arthritis is being discussed at present; such treatment would be intended to influence the reactiv-

ity of $CD4^+$ T cells, rather than remove them completely (particularly since depletion may induce transient immunodeficiency). *In vivo,* the depleting and modulating capacities of the three anti-CD4 mAbs were different. Whereas W3/25 was a weakly depleting, but clearly modulating, mAb, RIB5/2 and OX35 were moderately or strongly depleting and modulating mAbs, respectively; this pattern did not match the treatment effects of the three mAbs. In the present study, $CD4^+$ T-cell depletion was assessed only in the peripheral blood, the compartment most easily accessible also in humans. Because depletion/modulation in peripheral blood may differ from that in other compartments (e.g. lymphoid organs), the use of the terms 'depleting' or 'nondepleting' has to be restricted to the blood. In the case of the mAbs W3/25 and OX35, however, studies with marked, antibody-coated cells indicate a redistribution of the cells from lymphoid organs to the liver, possibly including phagocytosis and depletion of the opsonized 'cells' by liver macrophages (unpublished observations). Contrasting earlier findings concerning the depleting capacity of the mAb RIB5/2 [S7,S8] may be attributable to different time points of investigation, different amounts of injected mAb or number of therapeutic injections, and different experimental models (personal communication, Dr M Lehmann, University of Rostock).

T-cell reactivity *in vitro*

Differential effects of the three anti-CD4 mAbs on the mixed lymphocyte culture were noted only occasionally, especially with total T cells as responders. The accelerating mAb RIB5/2 was again more effective. The observation of differential effects only with total T cells, but not in $CD4^+$ T cells, indicates a differential contribution of the $CD4^+$ and $CD8^+$ T-cell subpopulation and suggests that interactions between $CD4^+$ and $CD8^+$ T cells [S9] are critical for T-cell activation and the effects of anti-CD4 mAbs on this process.

Cytokine secretion *in vitro*

Whereas IFN-γ secretion was completely unaffected by anti-CD4 preincubation and IL-4 was only increased by the mAb W3/25 (as previously reported; [S10]), IL-10 secretion was strikingly induced by all three anti-CD4 mAbs. At least in the case of the ameliorating mAbs W3/25 and OX35, this effect is compatible with the anti-inflammatory role of IL-10 in arthritis [S11,S12] and has been reported also after anti-CD4 therapy in transplantation [S13]. The fact that the total number of IFN-γ-positive and IL-4-/IL-10-positive T cells exceeds 100% indicates the presence of T_H0 cells in this particular experimental system.

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