

Aerosolized Anti-T-Cell-Receptor Antibodies Are Effective against Airway Inflammation and Hyperreactivity

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Key Words

Aerosolized monoclonal antibodies · Airway hyperreactivity · Inflammation, airways · Ovalbumin · T cell receptor

Abstract

Aerosolized monoclonal antibodies (mAbs) specific for T-cell receptors (TCR) were used to manipulate T-cell function in airways of ovalbumin (OVA)-sensitized and -challenged mice with airway hyperresponsiveness (AHR). The inhaled mAbs were found to be effective at low doses, had little or no systemic effect and specifically abrogated both effector and regulatory functions of the targeted T cells. Specific mAbs targeting $\alpha\beta$ T cells suppressed and those targeting $\gamma\delta$ T cells enhanced AHR. Moreover, specific mAbs directed against subsets of $\gamma\delta$ T cells varied in their effect on AHR. Using this approach of targeting either $\alpha\beta$ or $\gamma\delta$ T cells reduced airway eosinophilia, although the effect of mAbs specific for $\alpha\beta$ T cells was stronger. The use of aerosolized anti-TCR mAbs may offer an effective approach for the treatment of airway inflammation and AHR.

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Introduction

T lymphocytes are key players in the development and regulation of allergic airway hyperreactivity (AHR) and asthma. To a large extent, their role has been elucidated in animal models of allergic inflammation, such as ovalbumin (OVA)-sensitized and -challenged mice [1]. In these mice, an absolute requirement for $\alpha\beta$ T cells was confirmed using genetically $\alpha\beta$ -T-cell-deficient [T cell receptor (TCR)- $\beta^{-/-}$] mutants, and a regulatory role for $\gamma\delta$ T cells was discovered using either genetically $\gamma\delta$ -T-cell-deficient (TCR- $\delta^{-/-}$) mice or mice rendered transiently deficient in functional $\gamma\delta$ T cells by treatment with anti-TCR- δ monoclonal antibodies (mAbs) [2]. Modifying a previously developed protocol of targeting $\alpha\beta$ T cells with mAb against TCR- β [3], mAbs against $\alpha\beta$ and $\gamma\delta$ T cells were first administered systemically [2, 4], but we recently found that aerosolized mAbs against TCRs can also effectively inactivate pulmonary T cells and alter airway responsiveness [5]. Aerosolized anti-TCR mAbs were initially investigated in a model where mice are exposed to three consecutive OVA challenges alone without previous OVA sensitization (3N regimen). Under these conditions, $\gamma\delta$ T cells exerted a negative regulatory effect on airway responsiveness [2] and aerosolized anti-TCR mAbs, ei-

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ther specific for all $\gamma\delta$ T cells or for the $V\gamma 4+$ T cell subset, abrogated this negative regulatory effect at doses far lower than those used for systemic mAb injections [5]. This observation indicated that aerosolized mAbs could be used to specifically target TCR-defined subpopulations of pulmonary T cells and effectively neutralize their biological functions.

It seemed reasonable that treatment with aerosolized mAbs specific for the $\alpha\beta$ TCR might similarly neutralize $\alpha\beta$ -T-cell functions and thereby prevent AHR. Therefore, the effect of aerosolized anti-TCR mAbs was determined in a model of $\alpha\beta$ -T-cell-dependent AHR involving two systemic injections of OVA followed by three airway challenges (2ip3N). Treatment with aerosolized anti-TCR mAbs was effective in abrogating $\alpha\beta$ -T-cell functions and associated AHR. This locally targeted and highly specific approach provides an alternative to nonspecific immunosuppressive drugs or systemic applications of reagents directed to block T-cell function, such as anti-CD4 mAbs [6].

Methods

Animals

C57BL/6 mice were purchased from the Jackson Laboratories and cared for in the animal facility at the National Jewish Medical and Research Center, Denver, Colo., USA. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee.

Airway Challenge with OVA with and without Aerosolized Anti-TCR mAbs

C57BL/6 mice were either OVA-sensitized and -challenged (2ip3N protocol) or only OVA-challenged (3N protocol), as previously described [2]. The OVA challenge consisted of an aerosolized OVA exposure (1% in saline, total volume 10 ml/exposure) using ultrasonic nebulization for 20 min on 3 consecutive days. During the third and last aerosol exposure, mAbs against TCR (anti-TCR- β , anti-TCR- δ , anti-TCR- $V\gamma 1$, or anti-TCR- $V\gamma 4$) were added to the OVA solution at doses described in the Results. For comparison purposes, intravenous injection of mAbs was performed 1 week before OVA challenge, as previously described [2]. Airway responsiveness was assessed 48 h following the last OVA challenge in all treated mice. For each of these treatments, groups of at least 4 mice of each type were analyzed in every independent experiment, as detailed in the text and figure legends.

Determination of Airway Responsiveness

Airway responsiveness was assessed as changes in airway resistance and dynamic compliance after challenge with aerosolized methacholine (MCh) via the airways. Anesthetized, tracheostomized mice were mechanically ventilated, and lung function was assessed as a modification to previously described procedures [2, 7, 8]. A four-way connector was attached to the tracheostomy tube (18-gauge stainless-steel cannula), with two ports connected to the inspiratory

and expiratory sides of two ventilators. Ventilation was achieved at 160 breaths/min and a tidal volume of 0.15 ml with a positive end-expiratory pressure of 2–4 cm H_2O (Harvard Apparatus, South Natick, Mass., USA). Aerosolized MCh was administered for 10 breaths at a rate of 60 breaths/min in increasing concentrations (6.25, 12.5, 25, 50, 100 mg/ml MCh) with a tidal volume of 0.5 ml by the second ventilator (Shinano Manufacturing, Tokyo, Japan). The Plexiglas chamber containing the mouse was connected with a 1.0-liter glass bottle filled with copper gauze to stabilize the volume signal for thermal drift. Transpulmonary pressure was detected by a pressure transducer with one side connected to the fourth port of a four-way connector and the other side connected to a second port on the plethysmograph. Changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer and then referenced to a second copper-gauze-filled 1.0-liter glass bottle. Flow was measured by digital differentiation of the volume signal. Lung resistance (R_L) and dynamic compliance (C_{dyn}) were continuously computed (Labview, National Instruments, Tex., USA) by fitting flow, volume and pressure to an equation of motion. After each aerosol MCh challenge, the data were continuously collected for 1–5 min, and maximum values of R_L and minimum values of C_{dyn} were taken to express changes in airway function. Baseline and saline controls were all similar among all examined mice and results were reported as percentages of saline control.

Staining Reagents

For cytofluorographic analysis, mAbs were conjugated with N-hydroxysuccinimido-biotin (Sigma) and/or fluorescein isothiocyanate isomer I on Celite (Sigma). $0.5\text{--}1 \times 10^6$ cells/ml in 96-well plates (Falcon-Becton-Dickinson, Franklin Lakes, N.J., USA) were stained by using one-, two- or three-color techniques and analyzed cytofluorographically on XL2 (Coulter, Miami, Fla., USA) counting a minimum of 150,000 events per gated region. The following mAbs against murine TCR structures, all generated in Armenian hamsters, were grown as previously described [9]: TCR- β (clone H57-597 [10]), TCR- δ (clone GL3 [11]; clone 403A10 [12], a gift from Dr. S. Tonegawa), TCR- $V\gamma 1$ (clone 2.11 [13], a gift from Drs. P. Pereira and R. Sciammas), and TCR- $V\gamma 4$ (clone UC3-10A6 [14], a gift from Dr. J. Bluestone). FITC-conjugated anti-murine TCR- $V\gamma 4$ mAbs (clone UC3-10A6) were purchased from Pharmingen (San Diego, Calif., USA).

Targeting T Cells with Anti-TCR mAb

During OVA challenge on the 3rd and last day of OVA exposure, mAbs against TCR- β , TCR- δ , TCR- $V\gamma 1$, or TCR- $V\gamma 4$ were added to the OVA solution and airway responsiveness assessed 48 h later. Presence of T-cell subsets was assessed after lung digest using a previously described procedure [5].

Bronchoalveolar Lavage Fluid

Immediately after assessment of AHR, lungs were lavaged via the tracheal tube with 1 ml of Hank's balanced solution. Bronchoalveolar lavage fluid (BALF) cells were stained with Leukostat (Fischer Diagnostics, Pittsburgh, Pa., USA) on cytospin slides and differentiated in a blinded fashion by counting at least 200 cells under a light microscope [2].

Statistical Analysis

All results are expressed as means $\pm$ SD. Analysis of variance was used to determine the levels of difference between all groups. Pairs of groups were compared by unpaired two-tailed Student's *t* test. The *p* values for significance were set to 0.05.

Results

Because of our earlier observations that inhaled aerosolized and intravenously injected antibodies specific for TCR- δ had essentially the same effects on pulmonary $\gamma\delta$ -T-cell populations, namely functional inactivation and partial depletion [5], we first determined minimal antibody requirements in the inhalation protocol and whether this protocol could be extended to $\alpha\beta$ T cells as well. To define effective concentrations of aerosolized mAbs, we used a previously established model of AHR in which mice are exposed to aerosolized OVA on 3 consecutive days, without prior sensitization (3N regimen). Although the 3N regimen is not sufficient to establish OVA-specific hyperreactivity, these mice do develop AHR to MCh in an $\alpha\beta$ -T-cell-independent manner when deficient in $\gamma\delta$ T cells [2]. Mice treated with this 3N regimen received increasing doses of the hamster anti-mouse TCR- δ mAb mixture (1:1) of GL3 and 403A10 mAbs added directly to the OVA solution used in the last of the three aerosol exposures (fig. 1). To test for possible nonspecific effects of the antibody treatment, we included nonspecific hamster immunoglobulin at a concentration equivalent to the highest concentration of the specific antibody. Responsiveness to MCh was determined 48 h later. We found maximal effects of the aerosolized anti-TCR- δ mAbs at 10 μ g/ml, and still an effect at 1 μ g/ml of the specific antibody that exceeded the nonspecific effect of the hamster IgG at a dose ten times higher (fig. 1). These data established a concentration-dependent effect for the aerosolized anti-TCR- δ mAb treatment. We did not further escalate doses to avoid systemic depletion as determined by $\gamma\delta$ -T-cell counts in the spleen (data not shown).

In order to assess the effects of anti-TCR- β mAb, a model of $\alpha\beta$ -T-cell-dependent AHR is required. We therefore tested aerosolized hamster anti-mouse TCR- β mAb (H57-597) in mice that were systemically sensitized with OVA followed by airway challenge with OVA on 3 consecutive days (2ip3N regimen). The mAb was again added to the OVA solution used during the last of three aerosol challenges, at a concentration of 10 μ g/ml, and responsiveness to MCh was determined 48 h after the last aerosol exposure. As shown in figure 2A and 2B, the aerosolized anti-TCR- β mAb reduced AHR in OVA-sensitized and -chal-

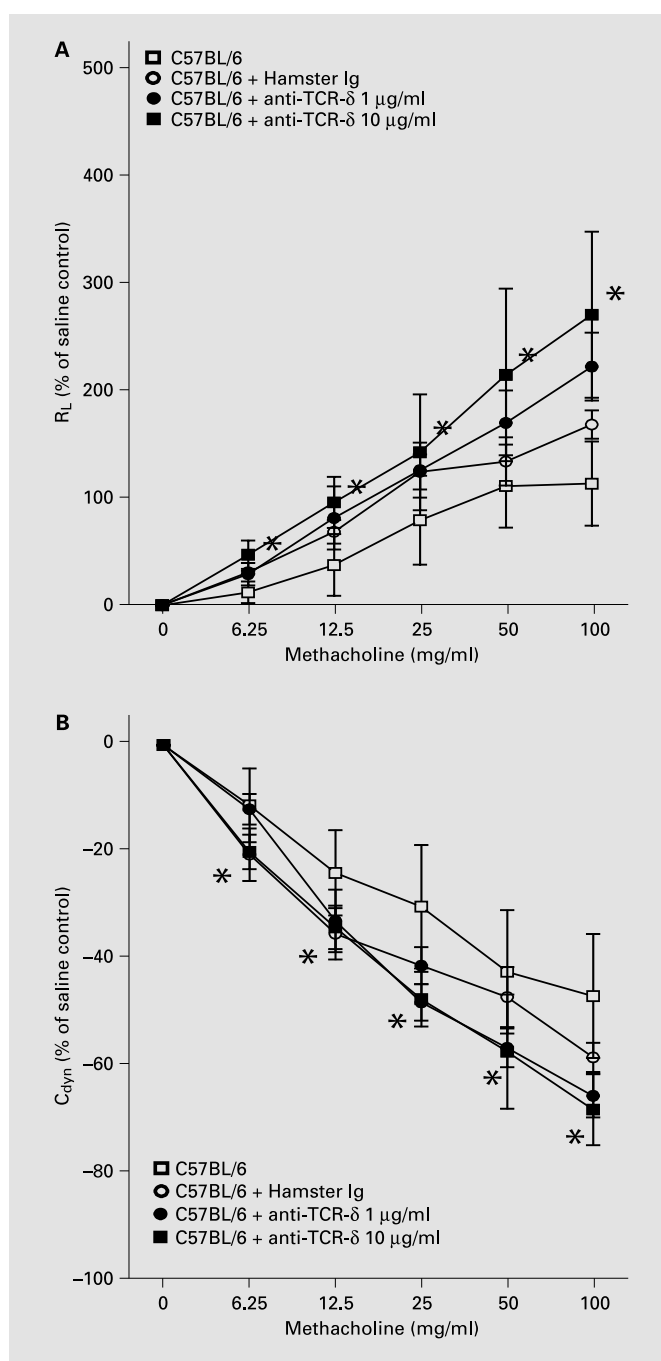


Fig. 1. Minimal effective concentrations of aerosolized mAbs against TCR- δ regulate airway responsiveness: after 3N treatment, changes in R_L (A) and C_{dyn} (B) were compared in C57BL/6 mice that received aerosolized anti-TCR- δ mAbs during their third and last ovalbumin (OVA) aerosol exposure. □ = Aerosolized OVA alone; ○ = aerosolized OVA with hamster IgG at 10 μ g/ml; ● = aerosolized OVA with anti-TCR- δ mAbs at 1.0 μ g/ml; ■ = aerosolized OVA with anti-TCR- δ mAbs at 10 μ g/ml. No significant differences in baseline responses to saline were observed in any of these groups. Each curve represents data from at least three independent experiments using a total of 9–12 mice. **p* < 0.05; error bars indicate SD.

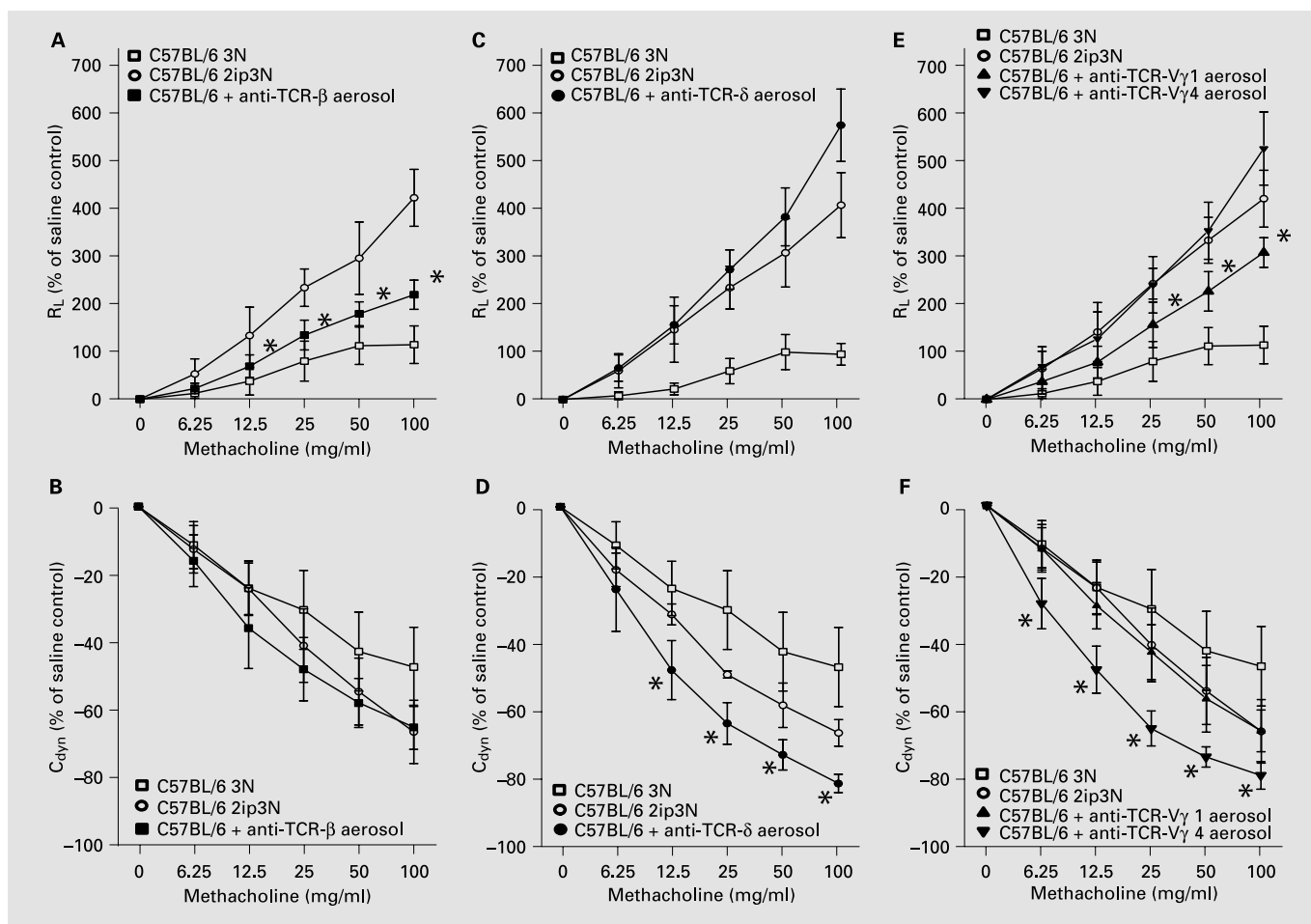


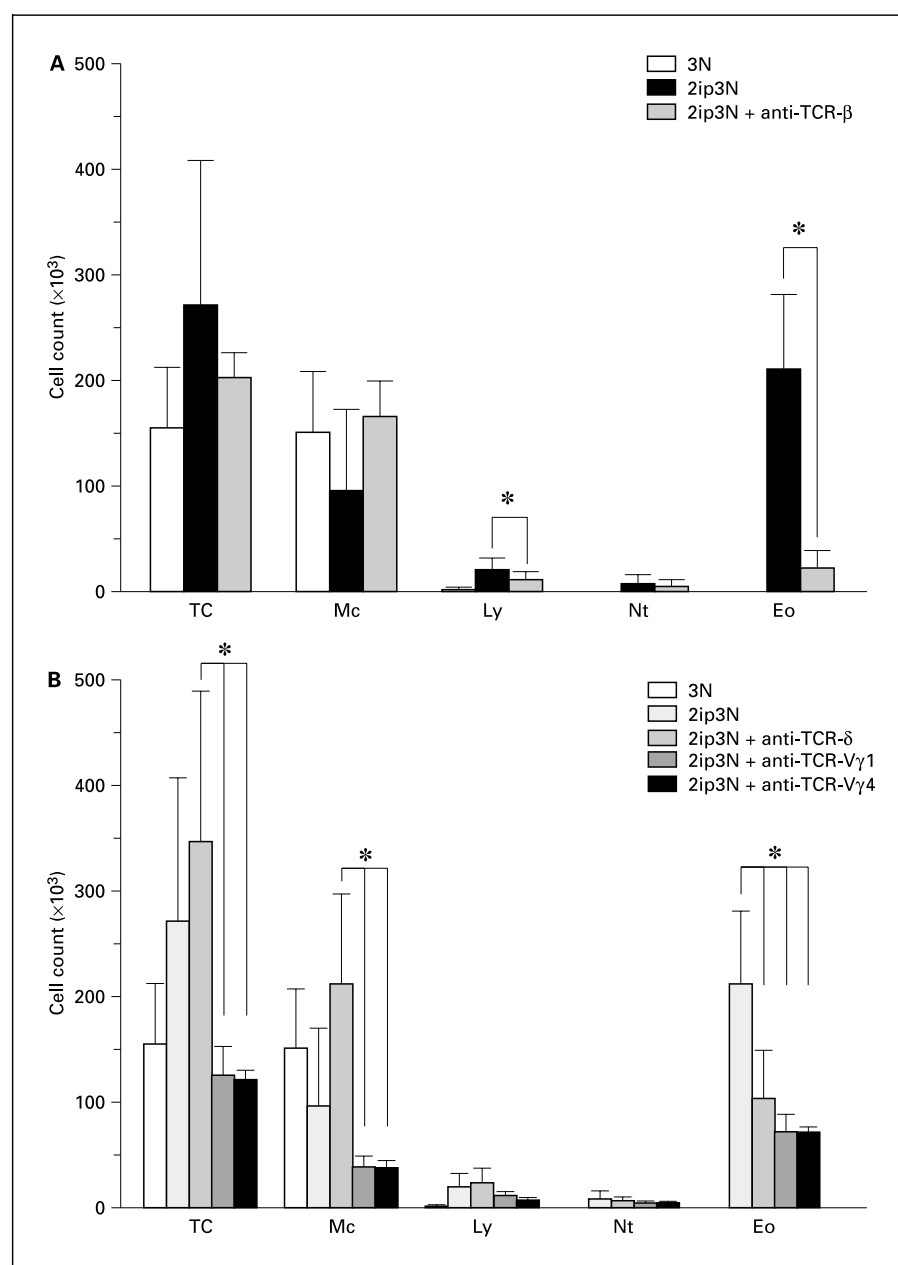
Fig. 2. Aerosolized anti-TCR mAbs specifically modulate AHR (A–F). R_L (A, C, E) and C_{dyn} (B, D, F) were measured in C57BL/6 mice that received the following aerosol treatments: □ = OVA challenge alone (3N); ○ = OVA sensitization and challenge (2ip3N); ■ = 2ip3N with anti-TCR- β mAb; ● = 2ip3N with anti-TCR- δ mAb; ▲ = 2ip3N with mAb anti-TCR-V γ 1; ▼ = 2ip3N with mAb anti-TCR-V γ 4. No significant differences in baseline responses to saline were observed in any of these groups. Each curve represents data from at least three independent experiments using a total of 9–12 mice. * $p < 0.05$ (for those experimental groups where aerosolized mAbs showed a marked and novel change in AHR).

lenged mice. Similar to our published findings in the 3N model [5], the mAb treatment resulted in partial depletion of pulmonary T cells, as determined by flow cytometry of nylon-wool-purified lung cells (data not shown). Taken together, these data confirm our earlier study demonstrating the absence of AHR in TCR- $\beta^{-/-}$ mice [2] and show that treatment with aerosolized anti-TCR- β mAbs has essentially the same effect on lung resistance as systemic treatment with anti-TCR- β mAbs or genetic TCR- β deficiency [2]. In contrast to earlier intravenous depletion studies [2], the aerosolized anti-TCR- β mAbs resulted in little change for C_{dyn} , which may indicate that $\alpha\beta$ T cells cannot be sufficiently inactivated in small airways [15, 16].

Next, we examined the effects of aerosolized anti-TCR- δ mAbs in 2ip3N-treated mice (fig. 2C, D). Anti-TCR- δ mAbs increased AHR. However, unlike 3N-treated mice [2, 5], the effect on R_L in 2ip3N-treated mice was small, but aerosolized anti-TCR- δ mAbs clearly reduced C_{dyn} . Thus, aerosolized anti-TCR- β and anti-TCR- δ mAbs have opposite and specific effects on AHR.

For further comparison, we examined the effects of two aerosolized $\gamma\delta$ -T-cell-subset-specific mAbs on AHR (fig. 2E, F), using the same exposure protocol (2ip3N). A mAb targeting V γ 1+ T cells (hamster anti-mouse mAb clone 2.11) had effects similar to the anti-TCR- β mAb, both with regard to R_L and C_{dyn} . By contrast, a mAb tar-

Fig. 3. Cellular BALF of 2ip3N-treated C57BL/6 mice after treatment with aerosolized mAbs directed against TCR- β (**A**), TCR- δ (**B**), TCR-V γ 1 (**B**) and TCR-V γ 4 (**B**). **A** Effect of aerosolized treatment with mAb against TCR- β . The open bars represent C57BL/6 mice treated with 3N alone, solid black bars are 2ip3N-treated mice and gray solid bars indicate mice treated with aerosol anti-TCR- β mAb. **B** Effect of aerosol treatment with mAbs against TCR- δ , TCR-V γ 1 and TCR-V γ 4. Open bars represent 3N-treated C57BL/6 mice, solid black bars 2ip3N-treated C57BL/6 mice, horizontal-hatched bars aerosol anti-TCR- δ -treated mice, left-hatched bars aerosol-treated anti-TCR-V γ 1 and right-hatched bars aerosol-treated anti-TCR-V γ 4 treated mice. **A, B** The BALF cell composition includes total cell counts (TC), macrophages (Mc), lymphocytes (Ly), neutrophils (Nt) and eosinophils (Eo). * $p < 0.05$.



getting V γ 4+ T cells (hamster anti-mouse mAb clone UC3) had effects similar to the anti-TCR- δ mAb. These data further emphasize the specificity of the treatment with aerosolized anti-TCR mAbs [2, 5].

We also examined effects of the antibody treatments on airway inflammation as detected by cellular infiltration in the BALF following OVA sensitization and challenge (fig. 3). Notably, aerosolized anti-TCR- β mAbs suppressed the eosinophilic response (fig. 3A). Small effects on lymphocyte counts were also seen. Anti-TCR- δ mAbs

reduced the eosinophilic response albeit not as effectively as anti-TCR- β mAb (fig. 3B). Finally, both anti-TCR-V γ 1 and anti-TCR-V γ 4 mAbs inhibited eosinophilia, again different to their opposite effects on AHR, and both antibodies also reduced BAL macrophage counts while a pan-specific anti-TCR- δ mAb did not. Taken together, these data document an array of specific effects of the aerosolized anti-TCR antibodies on the allergic response and the airways.

Discussion

In this study, we have explored the use of aerosolized inhaled mAbs specific for the TCR as a means for targeting T-cell populations involved in the development of AHR. Our data confirm $\alpha\beta$ T cells as effectors and $\gamma\delta$ T cells as regulators of AHR [2, 5]. We also demonstrate that $\alpha\beta$ T cells can be specifically targeted with aerosolized anti-TCR mAbs. Since $\alpha\beta$ T cells are effectors of AHR in models of asthma, these observations have implications for the treatment of diseases associated with AHR, including asthma. It now would appear feasible to selectively inactivate intrapulmonary allergen-specific $\alpha\beta$ T cells with clonotypic anti-TCR mAbs. Due to the low antibody doses required in the aerosol treatments, systemic impairment in the immune defenses may be avoided. As demonstrated by our earlier work, aerosolized mAbs against anti-TCR led to a reduction, but not to a depletion in pulmonary or systemic T cells [5]. In this study, the specificity of the aerosolized mAbs was demonstrated indirectly, by targeting T-cell populations with different functions. As would be predicted for specific antibody targeting, the effect of the antibody treatment on AHR depended on the function of the targeted subset. Moreover, if one assumes that R_L and C_{dyn} reflect AHR in different compartments of the lung [15, 16], differences in the $V\gamma$ -specific antibodies in their relative effects on R_L and C_{dyn} may reflect different distributions within the lung of the targeted T-cell subsets. The alternative that the effect of the aerosolized antibody treatment depends on intrinsic properties of the antibodies other than their ligand specificity is contradicted by our previous studies using systemic antibody treatment [2, 5]. In addition, coadministration of OVA with mAbs may lead to interference of antigen uptake or increased scavenging activity of mAbs by activated macrophages. While this cannot be excluded, allergen-specific IgG is usually implicated in interfering with antigen uptake [17]. In the present study, all anti-TCR mAbs used are of related structure (hamster IgG), but are not allergen specific. This structural relatedness strongly suggests that the differential activity of the mAbs reflects their specificity.

It also seems important that very small doses of the aerosolized anti-TCR mAbs are sufficient to modulate AHR, both to decrease it in the case of anti-TCR- β and anti-TCR- $V\gamma 1$ mAbs and to increase it as was shown with anti-TCR- δ and anti-TCR- $V\gamma 4$ mAbs. The reason for this high efficiency is probably a result of aerosolized antibodies targeting T cells localized in the airways and directly involved in the development of AHR. In our earlier study examining the role of pulmonary $\gamma\delta$ T cells in

non-sensitized mice [5], we documented an absence of systemic effects of the aerosolized anti-TCR mAbs in the time frame of the experiment (i.e. within 48 h after the last OVA aerosol treatment). The same protocol of antibody treatment was used in the present study. We assume that individual mice did not receive more than 10 μ g of antibody based on a single aerosol treatment of total 100 μ g mAb delivered to 10 mice per exposure box [18]. However, it is known from studies with other aerosolized proteins and similar exposure protocols in mice that only small fractions are actually deposited in the lung [19–21] and, therefore, the actual antibody dose is likely to be orders of magnitude lower. Obviously, the low dose, the high efficiency and the ease of inhalation make treatment with the aerosolized mAbs against TCR an alternative to other less specific and more systemic regimens.

In addition, our data suggest that the $\gamma\delta$ -T-cell subsets play different roles in the regulation of AHR, because aerosols with anti-TCR- $V\gamma 4$ and anti-TCR- $V\gamma 1$ mAbs had different effects on both C_{dyn} and L_R . In fact, our data reveal a previously unappreciated complexity of T-cell effects on the airways of OVA-sensitized and -challenged mice. Thus, while it is well known that cytokines produced by $\alpha\beta$ T cells promote eosinophil responses, our data suggest that $\gamma\delta$ T cells may also contribute to eosinophilia, despite their regulatory effect on AHR. Not only aerosolized anti-TCR- β but also anti-TCR- δ mAbs substantially reduced eosinophil numbers in BALF of 2ip3N-treated mice. This finding is reminiscent of other studies in which $\gamma\delta$ T cells were found to be instrumental in the development of lung eosinophilia [2, 22]. We have reported that TCR-defined subsets of $\gamma\delta$ T cells differ in their functions [23], and that $V\gamma 4$ + T cells but not $V\gamma 1$ + T cells function as negative regulators of AHR [5]. It thus seems possible that some $\gamma\delta$ T cells regulate AHR while others promote eosinophil responses [24, 25]. Moreover, AHR and eosinophilia are not always correlated and are probably determined by different underlying mechanisms [26]. We have not yet investigated in detail effects of $\gamma\delta$ T cells on eosinophil responses in the airways but it seems possible that $V\gamma 1$ + T cells and perhaps also $V\gamma 6$ + T cells would promote eosinophilia. This influence of $\gamma\delta$ T cells on eosinophils may reflect a more general role of $\gamma\delta$ T cells in regulating granulocyte recruitment during inflammation [27]. Finally, the here-described application of aerosolized mAbs against TCR- δ does not lead to a reduction in cellularity in the BALF, while those mAbs against the subsets $V\gamma 1$ and $V\gamma 4$ do. This is consistent with recent studies suggesting that $V\gamma 1$ + and $V\gamma 4$ + T cells have differential effects on inflammation of the lung [4, 28].

In summary, we have shown that aerosolized inhaled mAbs specific for the TCR can be used effectively to manipulate T-cell functions in the airways, including effector functions of $\alpha\beta$ T cells and regulatory functions of $\gamma\delta$ T cells, and that such treatment can be used to ameliorate AHR and allergic inflammation in mice.

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