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Fc receptor binding of anti-CD3 monoclonal antibodies is not essential for immunosuppression, but triggers cytokine-related side effects

A major drawback to the use of OKT3, a mouse anti-CD3 monoclonal antibody (mAb), as an immunosuppressive agent is the associated cytokine release syndrome. We used a mouse model to elucidate the properties of anti-CD3 mAb responsible for these cytokine-related side effects. We have previously demonstrated that the hamster anti-CD3 mAb 145-2C11 induced strong cytokine release and morbidity *in vivo*, whereas two rat anti-CD3 mAb 17A2 and KT3 did not. In the current study, we show that the mitogenic capacity of soluble anti-CD3 mAb *in vitro* correlates with their induction of side effects *in vivo*. Mitogenesis *in vitro* and tumor necrosis factor-α (TNF-α) release *in vivo* induced by anti-CD3 mAb could be inhibited by the anti-FcγR mAb 2.4G2, indicating that FcγR binding of anti-CD3 mAb is responsible for their mitogenic properties and for their induction of side effects. Importantly, the two non-mitogenic rat anti-CD3 mAb were equally capable of suppressing skin allograft rejection as the mitogenic hamster anti-CD3 mAb, suggesting FcγR binding of anti-CD3 mAb is not essential for their immunosuppressive properties. This suggestion is reinforced by our demonstration that administration of 2.4G2 *in vivo* did not interfere with immunosuppression of skin allograft rejection by 145-2C11. These findings suggest that clinical use of non-mitogenic anti-CD3 mAb will result in effective immunosuppression without cytokine-related side effects.

1 Introduction

The murine anti-CD3 mAb OKT3 is widely used for prevention and treatment of clinical organ allograft rejection because of its known immunosuppressive properties [1, 2]. However, treatment with this mAb is accompanied by severe clinical symptoms, such as fever, chills, nausea, vomiting, headache, and diarrhea. These side effects have been related to an early systemic release of several cytokines, such as IL-2, TNF-α, IFN-γ and IL-6 [3, 4], probably produced by activated T lymphocytes [5]. This so-called cytokine release syndrome precludes the use of anti-CD3 mAb in the treatment of autoimmune diseases.

We developed a mouse model to elucidate the properties of anti-CD3 mAb responsible for these undesirable side effects. The strength of this model is that it is based on three anti-CD3 mAb with similar immunosuppressive capacity in a mouse skin transplantation model [6]. Furthermore, all three mAb induced T cell depletion and TcR/CD3 complex modulation [6]. One of these mAb, the hamster mAb 145-2C11 [7], triggers strong cytokine release, accompanied by severe physical reactions consisting of piloerection, hypothermia, hypomotility and diarrhea [8, 9]. Interestingly, this was not observed with the anti-CD3 mAb 17A2 (rat IgG2b) [10] and KT3 (rat IgG2a) [6, 11].

In the present study, the role of FcγR was assessed by using an FcγR-blocking mAb, 2.4G2 [12] both *in vitro* and *in vivo*. Our results demonstrate that FcγR binding *in vivo* is responsible for cytokine release and side effects following anti-CD3 mAb treatment. Most importantly, this study provides direct evidence that FcγR binding of anti-CD3 mAb *in vivo* is not necessary for immunosuppression. Therefore, these data may have important clinical implications.

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2 Materials and methods

2.1 Monoclonal antibodies

We used the anti-mouse CD3 mAb 17A2, rat IgG2b [10], KT3, rat IgG2a [11] and 145-2C11, a hamster mAb [7]. 2.4G2 [12], a rat IgG2b mAb directed against mouse FcγRII/III [13], that also binds FcγRI via its Fc-portion [12], and $F(ab')_2$ fragments of 2.4G2 (kindly provided by Dr. M. Daëron, Institut Curie, Paris, France [14]) were used to block FcγR binding of anti-CD3 mAb. As isotype control mAb, we used PH2-4a (rat IgG2b), PH2-104 (rat IgG2a), both directed against *Escherichia coli* β-galactosidase (kindly provided by Dr. J. van Denderen, Department of Immunology, Rotterdam, The Netherlands), and anti-TcRγδ mAb GL3 (hamster Ig) [15]. We used P1.17, a mouse IgG2a Ab of unknown specificity [16], to compete for FcγRI binding.

mAb were purified from hybridoma culture supernatants by protein G (Pierce, Oud-Beijerland, The Netherlands) affinity chromatography, as described [17].

2.2 Mice

For all experiments *in vitro* and as skin graft recipients, we used C57BL/Ka mice (H-2^b). B6.C-H-2^{bm12} (H-2^{bm12}) mice

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were used as skin graft donors. All mice were bred at the Department of Immunology of the Erasmus University, Rotterdam. Mice were kept in light-cycled rooms and had access to acidified water and pelleted food *ad libitum*.

2.3 Proliferation assays

Spleen cells (2×10^5/well) were cultured in 200 µl RPMI 1640 medium (Gibco, Paisley, GB) supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 5×10^{-5} M β-mercaptoethanol, 100 IU/ml penicillin and 50 µg/ml streptomycin. Cells were incubated with different concentrations of anti-CD3 mAb in round-bottom tissue culture plates (Falcon). Alternatively, cells were cultured on anti-CD3 mAb coated flat-bottom tissue culture plates (Falcon). After 48 h of culture at 37 °C in 5% CO_2 in air, 0.5 µCi [^{3}H]thymidine was added to each well, and 18 h later, cells were harvested and [^{3}H]thymidine incorporation was measured in a liquid scintillation counter. The role of FcγR in anti-CD3 induced mitogenesis was examined by adding 10 µg/ml 2.4G2 mAb or an equivalent molar amount (6.7 µg/ml) 2.4G2 F(ab')$_2$ fragments to culture wells. In other wells, 10 µg/ml P1.17 (mouse IgG2a) [16] was added to compete for high-affinity FcγRI binding. In control wells, cells were cultured with isotype-control mAb or Con A (5 µg/ml).

2.4 Blocking of FcγR *in vivo*

The role of FcγR binding in TNF-α release and immunosuppression induced by 145-2C11 was examined by blockade of FcγR *in vivo*. C57BL/Ka mice were injected i.p. with 250 µg of 2.4G2 mAb 12 to 18 h before injection of 10 µg 145-2C11. Our primary goal was to achieve short-term blocking of FcγR, since the effects of 10 µg of 145-2C11 can be detected early after injection [6, 18]. This dose of 2.4G2 has previously been shown to inhibit FcγR-mediated sequestration of immune complexes for at least 24 h [19].

2.5 Detection of serum TNF-α

For induction of TNF-α release, 10 µg 145-2C11 mAb were injected i.v. The role of FcγR in TNF-α release was studied by 2.4G2 administration, 12 to 18 h before 145-2C11 mAb injection. Control mice were injected with 2.4G2 alone or 0.5 ml BSS. At 1, 2, 4, 8, and 24 h after 145-2C11 mAb injection, 3 or 5 mice per group were killed using carbon dioxide asphyxiation. Blood was obtained via heart puncture in sterile tubes and left to clot overnight at 4 °C. After centrifugation, serum samples were aliquotted and stored at -70 °C.

Serum TNF-α levels were determined by a cytotoxicity assay on WEHI-164 clone 13 cells [20]. Briefly, trypsinized WEHI-164 cells were seeded in flat-bottom tissue culture plates (Falcon) at 1×10^4 cells/100 µl complete medium. After overnight cell adherence at 37 °C, serum samples and actinomycin D (1 µg/ml) were added and incubated for 24 h at 37 °C. Cytotoxic activity was measured with the MTT assay [21].

2.6 Skin grafting

The immunosuppressive effect of 145-2C11 mAb on MHC class II-disparate skin allograft rejection was studied by administration of 10 µg mAb the day before grafting. FcγR-blockade was achieved by 2.4G2 mAb injection 12 to 18 h before 145-2C11 treatment. Control mice received 2.4G2 mAb alone or 0.5 ml BSS.

Tail skin of B6.C-H-2^{bm12} donors was grafted to the dorsal thorax of C57BL/Ka recipients using a modification of the method of Billingham and Medawar [22]. Grafts were considered rejected when no viable donor skin was detectable.

2.7 Statistical analyses

The induction of T cell proliferation and TNF-α release by anti-CD3 mAb was compared by analysis of variance (ANOVA). If ANOVA revealed significant differences, the groups were compared using Student's *t*-test. Graft survival of groups was compared by Mann Whitney tests. Values of $p < 0.05$ were considered significant.

3 Results

3.1 T lymphocyte proliferation induced by immobilized and soluble anti-CD3 mAb

To induce T cell proliferation, anti-CD3 mAb had to be cross-linked either by FcγR$^+$ cells or by solid phase immobilized mAb. The use of immobilized anti-CD3 mAb enabled the study of their mitogenic properties independent of their FcγR binding capacity [23]. Figure 1 A shows that immobilized anti-CD3 mAb, rat IgG2a and hamster mAb induced comparable T cell proliferation. The proliferation curve of rat IgG2b anti-CD3 mAb was significantly different from that of the two other anti-CD3 mAb. At higher concentrations, 17A2 (rat IgG2b) was significantly

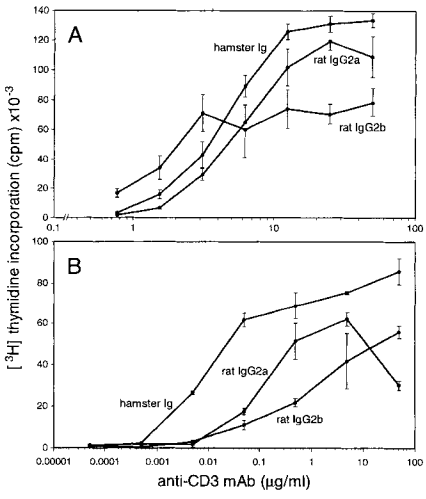

Figure 1. Proliferation induced by anti-CD3 mAb. C57BL/Ka spleen cells were stimulated with different concentrations of immobilized (A) or soluble (B) rat IgG2b, rat IgG2a or hamster anti-CD3 mAb. The data represent mean cpm of triplicate cultures ± SD. Experiments were repeated three times, yielding essentially identical results.

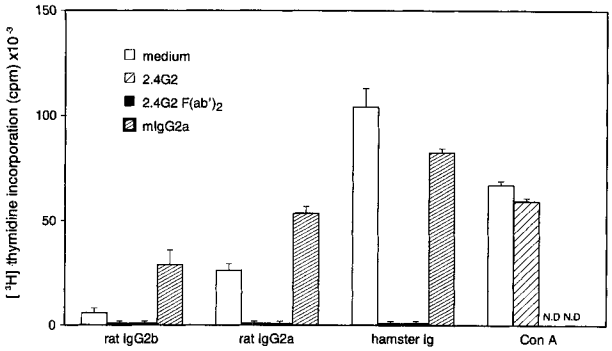

Figure 2. Effect of FcγR-blocking on proliferation induced by soluble anti-CD3 mAb or Con A. C57BL/Ka spleen cells were cultured in medium alone or medium supplemented with 2.4G2 mAb, F(ab')$_2$ fragments of 2.4G2 or mIgG2a. T cell mitogenesis was induced by addition of 10 μg/ml rat IgG2b, rat IgG2a or hamster anti-CD3 mAb. Data represent mean cpm of triplicate cultures ± SD. N.D. = not determined. Similar results were obtained in a second experiment (not shown).

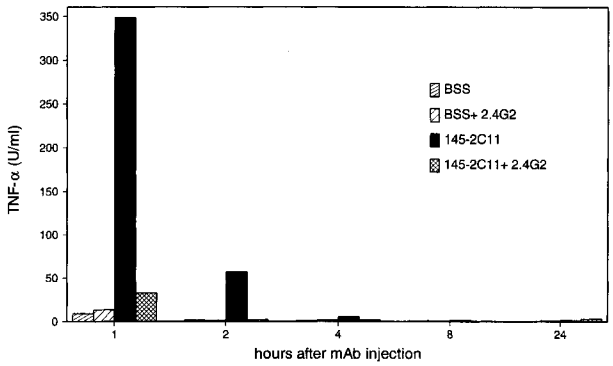

Figure 3. Effect of anti-FcγR mAb *in vivo* on TNF-α release triggered by 145-2C11 mAb. C57BL/Ka mice received either 10 μg 145-2C11 mAb i.v. (t = 0 h), 250 μg 2.4G2 mAb (t = - 12 to - 18 h) or a combination of 145-2C11 and 2.4G2 mAb. Control mice received 0.5 ml BSS. At 1, 2, 4, 8 and 24 h after treatment, 3 (2.4G2 and BSS group) or 5 (other groups) mice per group were killed for detection of serum TNF-α. This experiment was performed twice. Results represent the mean serum TNF-α levels (SEM ≤ 15 %) from one representative experiment.

less mitogenic than the other mAb, whereas at lower concentrations this mAb was significantly more mitogenic.

The capacity of soluble anti-CD3 mAb to induce T cell proliferation has been shown to correlate with the extent of their interaction with FcγR [24]. Soluble rat IgG2b and rat IgG2a anti-CD3 mAb induced comparable T cell proliferations (Fig. 1 B). To induce T cell proliferation, high concentrations (consistently 50–100 times higher than 145-2C11 mAb) of rat anti-CD3 mAb were needed. Neither of the isotype control mAb (PH2-4a, PH2-104 and GL3) induced proliferation (data not shown).

Addition of FcγR-blocking mAb (10 μg/ml 2.4G2) completely inhibited proliferation induced by all three soluble anti-CD3 mAb (Fig. 2). This inhibition was specific, since addition of 10 μg/ml of an isotype-control mAb (PH2-4a) had no effect on anti-CD3 mAb-induced proliferation (data not shown), and Con A induced proliferation was not affected by 2.4G2 mAb (Fig. 2). Highly purified F(ab')$_2$ fragments of 2.4G2 that only block FcγRII/III binding had a similar inhibitory effect to that of intact 2.4G2 (Fig. 2). In addition, competition for FcγRI binding by adding 10 μg/ml P1.17 (mouse IgG2a) did not inhibit anti-CD3 mAb-induced T cell proliferation (Fig. 2). Thus, mitogenesis caused by all three anti-CD3 mAb involved FcγRII/III molecules. The ability of low doses of hamster mAb to induce significant T cell proliferation suggests that this mAb has a high affinity for either FcγRII or FcγRIII.

3.2 Effect of FcγR-blocking mAb on TNF-α release triggered by 145-2C11 *in vivo*

Of the three anti-CD3 mAb, only 145-2C11 mAb induced cytokine-related side effects [6]. TNF-α has been shown to play a crucial role in these side effects [6, 9]. Administration of 10 μg hamster anti-CD3 mAb 145-2C11 *in vivo* induced a strong increase in serum TNF-α levels (Fig. 3). The highest TNF-α serum levels were measured 1 h after 145-2C11 injection. To determine the role of FcγR in this TNF-α release, we temporarily blocked FcγR by giving 250 μg 2.4G2 mAb 12 to 18 h before 145-2C11 injection. Treatment with 2.4G2 mAb completely abrogated the rise of serum TNF-α levels. Anti-FcγR mAb itself did not trigger TNF-α release. These results show that binding of 145-2C11 mAb to FcγR bearing cells is necessary for TNF-α release *in vivo*.

3.3 Effect of FcγR blocking *in vivo* on 145-2C11-induced immunosuppression of skin allograft rejection

Since FcγR blockade significantly inhibited the cytokine release syndrome caused by 145-2C11 mAb, it was essential to determine whether this treatment interferes with the immunosuppressive effect of this anti-CD3 mAb. Therefore, we studied the effect of the different treatment schedules on skin allograft rejection. The day after 145-2C11 injection, C57BL/Ka mice received an MHC class II-disparate B6.C-H-2^{bm12} skin graft. As shown in Fig. 4, a single dose of 10 μg 145-2C11 significantly prolonged skin allograft survival compared to the untreated control group. The administration of 250 μg 2.4G2 mAb had no

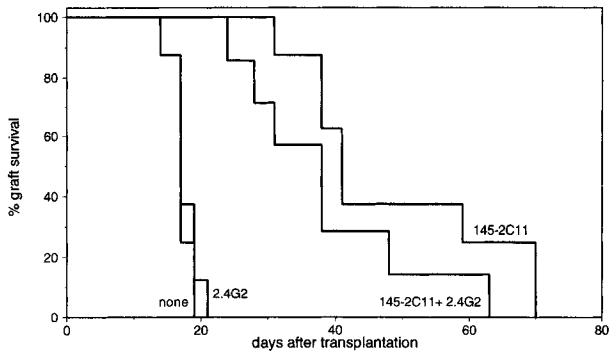

Figure 4. Effect of FcγR-blockade *in vivo* on 145-2C11-induced immunosuppression of skin allograft rejection. C57BL/Ka mice received an MHC class II-disparate B6.C-H-2^{bm12} skin graft. On the day before grafting, the mice were treated with 10 μg 145-2C11 (n = 8). One group received 250 μg 2.4G2 12 to 18 h before 145-2C11 administration (n = 7) and another received 2.4G2 mAb alone (n = 8). Control mice (n = 8) received no treatment.

effect on the immunosuppression induced by 145-2C11. Furthermore, the group that received 2.4G2 mAb alone showed the same graft survival as the untreated control group. These results show that FcγR-mediated binding of anti-CD3 mAb is not essential for immunosuppression.

4 Discussion

Treatment and prevention of organ allograft rejection using OKT3 is very successful [1, 2]. However, this treatment is complicated by the OKT3-induced cytokine release syndrome. In this study, we used a mouse model with three anti-CD3 mAb to characterize the properties of anti-CD3 mAb that are responsible for induction of cytokine-related side effects. Since all three mAb have been shown to be equally immunosuppressive [6], this model allows the study of T cell activating capacities of anti-CD3 mAb independent of their immunosuppressive properties.

Differences in T cell activation by anti-CD3 mAb might be caused by differences in epitope recognition or affinity of the mAb [24, 25]. Earlier studies [10, 11] showed cross-competition between 17A2, KT3 and 145-2C11 mAb, suggesting that the three anti-CD3 mAb recognize similar or closely related epitopes. The hamster mAb 145-2C11 has been shown to be specific for the CD3 epsilon chain [7]. Furthermore, the anti-CD3 mAb bound with similar affinity (K_d of 2.5×10^{-9}–3×10^{-9} M) to lymph node cells (data not shown).

Mitogenesis and T cell activation *in vitro* caused by anti-CD3 mAb is dependent on interaction of these mAb with FcγR [26]. The finding that 145-2C11 $F(ab')_2$ fragments induce less cytokine release and morbidity in mice than intact 145-2C11 mAb suggests that FcγR binding is also involved in T cell activation *in vivo* [9, 27], though interpretations may be influenced by the short half-life of $F(ab')_2$ fragments. Our results demonstrate in a mouse model that intact non-mitogenic anti-CD3 mAb induced less cytokine-related side effects than mitogenic mAb [6]. This difference in mitogenesis is due to differences in FcR binding capacities of the anti-CD3 mAb, since in immobilized form, all three mAb induced T cell proliferation. The mitogenic anti-CD3 mAb 145-2C11 displayed a higher affinity for FcγRII or FcγRIII than the non-mitogenic mAb 17A2 and KT3. The first direct evidence for the role of FcγR binding in the cytokine-related side effects of anti-CD3 mAb is provided by our finding that blocking of FcγR binding *in vivo* resulted in complete inhibition of TNF-α release by 145-2C11 mAb.

Our previous study showed that the non-mitogenic rat anti-CD3 mAb 17A2 and KT3 are equally effective as the mitogenic 145-2C11 mAb in suppressing skin allograft rejection [6]. That FcγR binding of anti-CD3 mAb is not essential for their immunosuppressive properties is further shown by our finding that blocking of FcγR binding *in vivo* has no effect on the suppression of skin allograft rejection by 145-2C11 mAb.

Together, our data suggest that the use of non-mitogenic anti-CD3 mAb would cause the induction of fewer cytokine-related side effects, while retaining effective immunosuppression. Though $F(ab')_2$ fragments have been proposed to be useful in the clinical situation, a major drawback of these fragments is their extremely short half-life and thus the necessity of frequent administration. In addition, the production of these fragments requires great care, since even minimal contamination with intact mAb may induce significant T cell activation [26]. The use of whole non-mitogenic anti-CD3 mAb would therefore be much more preferable. An IgA switch variant of a murine anti-CD3 mAb, unable to interact with human FcγR, induces significantly less cytokine release and side effects in chimpanzees [28]. To date, the number of patients treated with these mAb are insufficient to allow conclusions on their immunosuppressive efficacy [29]. Alegre et al. [30] showed that mutations in the Fc portion of a humanized OKT3 resulted in reduced FcγR binding. *In vitro,* this mutated mAb also induced significantly less T cell activation than the parental mAb, but displayed the same immunosuppressive properties. Recently, this mutated mAb has been shown to be equally effective in suppressing human skin graft rejection as the parental anti-CD3 mAb and OKT3 in SCID mice reconstituted with human splenocytes [31].

In summary, interaction of the hamster anti-CD3 mAb 145-2C11 with FcγRII/III is responsible for its cytokine-related side effects. From a comparison of this mAb with two other anti-CD3 mAb that do not induce any morbidity, we conclude that mitogenesis *in vitro* correlates with cytokine release and morbidity *in vivo*. This finding is relevant for the development of new anti-CD3 mAb. Furthermore, FcγR binding is not essential for immunosuppression by anti-CD3 mAb. Together these data suggest that non-mitogenic anti-CD3 mAb are promising immunosuppressive agents in clinical tissue and organ transplantation and that they may also be useful for treatment of autoimmune diseases.

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