

**B cell-targeted therapy with anti-CD20 monoclonal antibody in a mouse model of Graves' hyperthyroidism**

Running title: B cell depletion in Graves' mouse model

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## Summary

Graves' disease is a B cell-mediated and T cell-dependent autoimmune disease of the thyroids which is characterized by overproduction of thyroid hormones and thyroid enlargement by agonistic anti-thyrotropin receptor (TSHR) autoantibody. In addition to antibody secretion, B cells have recently been recognized to function as antigen-presenting/immune-modulatory cells. The present study was designed to evaluate the efficacy of B cell depletion by anti-mouse (m) CD20 monoclonal antibody (mAb) on Graves' hyperthyroidism in a mouse model involving repeated injection of adenovirus expressing TSHR A-subunit (Ad-TSHR289). We observe that a single injection of 250  $\mu$ g/mouse anti-mCD20 mAb efficiently eliminated B cells from the periphery and spleen and to a lesser extent from the peritoneum for more than three weeks. B cell depletion before immunization suppressed an increase in serum IgG levels, TSHR-specific splenocyte secretion of IFN- $\gamma$ , anti-TSHR antibody production and development of hyperthyroidism. B cell depletion two weeks after the first immunization, a time point at which T cells were primed but antibody production was not observed, was still effective at inhibiting antibody production and disease development without inhibiting splenocyte secretion of IFN- $\gamma$ . By contrast, B cell depletion in hyperthyroid mice was therapeutically ineffective. Together, these data demonstrate that B cells are critical not only as antibody-producing cells but also as antigen presenting/immune-modulatory cells in the early phase of the induction of experimental Graves' hyperthyroidism and, although therapeutically less effective, B cell depletion is highly efficient for preventing disease development.

## Introduction

Organ-specific autoimmune diseases result from abnormal B and T cell recognition of self-autoantigen. Some of these diseases are largely mediated by humoral immune responses producing pathogenic autoantibodies, and others by cellular immune responses leading to destruction of target tissues by cytotoxic T cells. Graves' disease is a representative of the former, characterized by stimulatory autoantibodies against the thyrotropin receptor (TSHR) (thyroid stimulating antibody, TSAAb), which cause overproduction of thyroid hormones and thyroid hyperplasia [1]. As antibody producing cells, B cells are crucial immune cells in the pathogenesis of Graves' disease. In addition, other important aspects of B cell function in immune reactions have recently been clarified, including antigen-presentation, pro-inflammatory cytokine production, co-stimulatory molecule expression (CD80 and CD86), alterations in dendritic cell function, *etc.* [2]. Indeed, previous studies with mice genetically deficient for B cells (B cell KO mice) showed the requirement of B cells for development of autoimmune thyroiditis, type 1 diabetes and systemic lupus erythematosus (SLE) [3-5]. Impaired activation of TSHR-reactive T cells in B cell KO mice in a mouse Graves' model has also been demonstrated [6]. These data indicate the critical role of B cells not only for autoantibody production but also for CD4<sup>+</sup> T cell priming as professional antigen-presenting cells. B cells are therefore an ideal therapeutic target in terms of not only lowering activities of pathogenic antibodies but also dampening pathogenic autoimmune responses *per se* in autoimmune diseases.

However, B cell KO mice have a serious problem that these mice have major qualitative and quantitative abnormalities in the immune system [7,8]. By contrast, B cell depletion may be a feasible approach to study the function of B cells in autoimmune diseases. Indeed monoclonal antibodies to B cell-specific cell surface molecules such as CD19, CD20, CD79 and to a B cell-surviving factor (B cell lymphocyte stimulator, BLyS) have been successfully used to deplete B cells *in vivo* and to treat numerous autoimmune and malignant hematopoietic diseases

in humans and mice [2,9,10]. Transient depletion of B cells by these means can distinguish between the role of B cells during immune development and that during immune responses.

CD20 is a B cell-specific molecule that is expressed on the cell surface during the transition of pre-B to immature B cells but is lost upon plasma cell differentiation [11]. In human autoimmune diseases, rituximab, a chimeric anti-human CD20 monoclonal antibody, has proved to be effective for treatment of autoimmune diseases including rheumatoid arthritis, SLE, idiopathic thrombocytopenic purpura, hemolytic anemia and pemphigus vulgaris [12]. In addition, preliminary clinical studies have shown the therapeutic efficacy of rituximab in a small fraction of Graves' patients with mild hyperthyroidism [13-16]. In mice, anti-mouse CD20 monoclonal antibodies (anti-mCD20 mAbs) which efficiently eliminate mouse B cells *in vivo* have recently been isolated [11,17], and used to treat mouse models of autoimmune thyroiditis, systemic sclerosis, collagen- or proteoglycan-induced arthritis, Sjogren syndrome, SLE and type 1 diabetes [17-23]. Moreover, the soluble decoy receptor-Fc fusion proteins to block B cell surviving factors [BAFF, a BLyS/B cell-activating factor belonging to the TNF family; and APRIL, and a proliferation-inducing ligand] reduced TSAb activities and T<sub>4</sub> levels in a mouse model of Graves' disease [24].

In the present study, we evaluated the efficacy of anti-mCD20 mAb in a mouse model of Graves' disease we have previously established [24]. We found that this approach efficiently depleted B cells and that B cell depletion by this agent was effective for preventing Graves' hyperthyroidism. Our results indicate the requirement of antibody production and T cell activation by B cells in the early phase of disease initiation for the disease pathogenesis.

## **Materials and methods**

### **Mice**

Female BALB/c mice (6 weeks old) were purchased from Charles River Japan Laboratory Inc. (Tokyo, Japan) and were kept in a specific pathogen free facility. Animal care and all experimental procedures were performed in accordance with the Guideline for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee.

### **Experimental protocols**

Construction, amplification, purification of non-replicative recombinant human adenovirus expressing the human TSHR-A subunit (Ad-TSHR289) and determination of the viral particle concentration were described previously [24].

Mice were injected intramuscularly in the quadriceps with 100  $\mu$ l PBS containing  $10^{10}$  particles of Ad-TSHR289 on three occasions at three-week-intervals (the weeks 0, 3 and 6). Groups of mice were also treated by intraperitoneal (ip) injection of anti-mCD20 mAb (50 or 250  $\mu$ g/mouse, a single injection; 18B12, IgG2a) or control Ab (2B8, IgG2a) (gifts from R. Dunn and M. Kehry at Biogen Idec; refs. 17,18) at the indicated time points. Blood samples were obtained two weeks after the second immunization or four weeks after the third immunization.

### **Thyroxine (T<sub>4</sub>) and anti-TSHR Ab measurements**

Serum free T<sub>4</sub> concentrations were measured with a radioimmunoassay (RIA) kit (DPC free T<sub>4</sub> kit; Diagnostic Products, Los Angeles, CA). The normal range was defined as the mean  $\pm$  3 S.D. of control untreated mice.

Anti-TSHR antibodies in mouse sera were determined using two different methods, a

biological TSAb assay and a flow cytometric assay with Chinese hamster ovary (CHO) cells stably expressing the full-length human TSHR, as previously described [25]. The former measures the stimulating antibodies responsible for hyperthyroidism, and the latter the titers of anti-TSHR antibodies recognizing the native TSHR expressed on the cell surface irrespective of their function.

### **ELISA for measuring serum IgG concentrations**

ELISA wells were coated overnight with 100  $\mu$ l goat anti-mouse Ig (diluted 1:1,000, SothernBiotech, AL) and were then incubated with mouse sera (diluted 1:2,000). After incubation with horseradish peroxidase-conjugated anti-mouse IgG (diluted 1:3,000; A3673, Sigma-Aldrich Corp., St. Louis, MO), color was developed using orthophenylene diamine and H<sub>2</sub>O<sub>2</sub> as substrate, and optimal density (OD) was read at 492 nm.

### **Flow cytometry**

Splenocytes were stained with FITC or PE-conjugated anti-CD4 (H129.19), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-B220 (RA3-6B2), anti-IgM (II/41) and anti-FoxP3 (FJK-16s; Foxp3 staining kit) (PharMingen, San Diego, CA or eBioscience, San Diego, CA), and analyzed on a FACSCanto II flow cytometry using FACS Diva software (BD Biosciences, San Diego, CA).

### **Cytokine assays**

Splenocytes were cultured (triplicate aliquots) at  $5 \times 10^5$  cells/well in a 96-well round bottomed culture plate in the presence or absence of 10  $\mu$ g/ml TSHR289 protein as previously described (26). Four days later, the culture supernatants were collected. The concentrations of IFN- $\gamma$  were determined with Bio-Plex<sup>TM</sup> Suspension Array System (Bio-Rad, Tokyo, Japan). Cytokine production was expressed as ng or pg/ml using a standard curve of recombinant mouse

cytokines.

### **Statistical analysis**

Levels of T<sub>4</sub>, antibodies and cytokines, and incidences of hyperthyroidism were analyzed by t-test or by chi-square test, respectively. A 'p' value of less than 0.05 was considered statistically significant.

## Results

### B cell depletion by anti-mCD20 mAb

To determine the efficacy of anti-mCD20 mAb for B cell depletion, BALB/c mice were treated with a single ip injection of 50 or 250  $\mu\text{g}/\text{mouse}$  of either anti-mCD20 mAb or control mAb. Representative flow cytometric data on peripheral blood of naïve, anti-mCD20 mAb-treated and control mAb-treated mice are shown in Fig. 1A. Anti-mCD20 mAb reduced B220<sup>+</sup>IgM<sup>+</sup> B cell numbers in a dose-dependent manner, with 250  $\mu\text{g}/\text{mouse}$  mAb resulting in the depletion of B cells to less than five % of the baseline in the peripheral blood and spleen (Fig. 1B). The mAb was the least effective in the peritoneal cavity (Fig. 1B). This is thought to be due to inaccessibility of Fc receptor-bearing cells into the peritoneal cavity that mediate antibody-dependent cellular cytotoxicity [11,26]. The effect persisted for at least three weeks with an approximately 80 % recovery in six weeks (Fig. 1C). These data are essentially identical to those in the previous report that has studied the effect of anti-mCD20 mAb on different B cell subsets in BALB/c mice [22]. Despite the effective B cell depletion in the peripheral blood and spleen, serum basal IgG levels remained unchanged (see below).

Regarding T cell subsets, the percentages of CD4<sup>+</sup>CD44<sup>-</sup>CD62L<sup>+</sup> naïve, CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>+</sup> activated, CD4<sup>+</sup>CD44<sup>+</sup>CD62L<sup>-</sup> memory and CD4<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells remained unaltered two weeks after anti-mCD20 mAb injection (data not shown).

### Outcome of anti-mCD20 mAb treatment for Graves' hyperthyroidism and TSHR antibodies

The consequences of B cell depletion on Graves' hyperthyroidism were studied in a mouse model involving repeated injection of susceptible BALB/c mice with Ad-TSHR289 (24). Antibody treatment (250  $\mu\text{g}/\text{mouse}$ ) was performed at three different time points (Exps. 1, 2 and 3 in Fig. 2) and sera were analyzed at two time points, two weeks after the second immunization

(the week 5) and four weeks after the third immunization (the week 10).

In mice that received anti-mCD20 mAb five days before the first immunization (Exp. 1 in Fig. 2), development of hyperthyroidism was completely suppressed at the week 5 and markedly reduced at the week 10 (Fig. 3A). Likewise, the titers of anti-TSHR antibodies were also almost completely inhibited at the week 5 but began to increase at the week 10 (Fig. 3B), presumably because of recovery of B cell numbers (see Fig. 1C). However, pathogenic TSAb activities were still low in the anti-mCD20 mAb-treated mice at this time point (Fig. 3C), consistent with the lower incidence of hyperthyroidism (Fig. 3A). Thus the ability of B cell depletion to suppress development of TSAb and Graves' hyperthyroidism is relatively long lasting, even after circulating B cells recovered in the periphery. Thus B cell depletion by anti-mCD20 mAb is very effective at preventing the development of Graves' hyperthyroidism.

We next tested the outcome of injecting anti-mCD20 mAb 10 days after the first immunization (Exp. 2 in Fig. 2), a time point at which we previously found that T cells were already primed but anti-TSHR antibodies or hyperthyroidism were not induced [27]. Albeit slightly less effective than pre-treatment (Fig. 3), only 33% of immunized, anti-mCD20 mAb-treated mice became hyperthyroid compared with 73 % in immunized, untreated mice (Fig. 4A). Again, the levels of anti-TSHR antibodies were significantly lower in mice that received anti-mCD20 mAb (Fig. 4B).

In the third approach, anti-mCD20 mAb was administered to hyperthyroid mice (Exp. 3 in Fig. 2). This treatment was proved to be ineffective. Thus, the incidences of hyperthyroidism were decreased from 90 % in the immunized, untreated mice to 54 % in the immunized, anti-mCD20 mAb-treated mice (Fig. 5A), which were statistically insignificantly different. Moreover, the differences in levels of anti-TSHR antibodies and TSAb activities were also insignificant between two groups (Fig. 3B, C).

Of interest, immunization with Ad-TSHR289 significantly increased serum concentrations of IgG (Figs. 3D, 5D). However, anti-mCD20 mAb had no effect on the basal IgG levels (Fig. 3D).

### **Effect of B cell depletion on memory T cell responses**

TSHR antigen-specific splenocyte secretion of IFN- $\gamma$  *in vitro* was used as a measure of T cell activation because we have previously found that this cytokine is indispensable for the pathogenesis of Graves' disease [28]. In the first experiment, splenocytes were prepared two weeks after a single injection of AdTSHR289 from mice which received anti-mCD20 mAb five days before immunization (Exp. 1 in Fig. 2). Controls were splenocytes from immunized but not B cell-depleted mice, as well as splenocytes from unimmunized mice. In a T cell recall assay, splenocytes from Ad-TSHR289 immunized mice, but not from immunized and B cell-depleted mice, produced significantly increased amounts of IFN- $\gamma$  in response to TSHR antigen (Fig. 6A). Thus, anti-mCD20 mAb suppressed antigen-specific IFN- $\gamma$  synthesis by ~50 %. In the second experiment, T cell recall responses were studied in mice which received anti-mCD20 mAb 10 days after immunization with Ad-TSHR289 (Exp. 2 in Fig. 3). Splenocytes were prepared two weeks after immunization from these B cell depleted mice and from immunized but not B cell-depleted mice, as well as from unimmunized mice. In this case, splenocytes from both the immunized mice and the immunized and B cell depleted mice produced comparably increased amounts of IFN- $\gamma$  in response to TSHR antigen (Fig. 6B).

Overall, our findings indicate that B cells are important for disease initiation by stimulating T cell function and antibody production. However, B cell depletion prevents disease induction but is not efficacious once disease is clinically manifested.

## Discussion

This study was designed to evaluate the prophylactic and therapeutic potentials of B cell depletion on Graves' hyperthyroidism in a mouse model. Although clinical trials of B cell depletion by rituximab have previously been performed in a small numbers of Graves' patients [13-16], we believed that studies on animal models would have an important role because of substantial limitation of performing mechanistic studies on B cell contribution to Graves' disease in humans.

We first observed that anti-mCD20 mAb (18B12) efficiently depleted B cells in the periphery and spleen and to a less extent in the peritoneal cavity for a long time period, in agreement with previous findings [17]. Baseline serum IgG levels were unaffected, presumably because the majority of antibodies are produced from CD20<sup>-</sup> plasma cells [11]. However, the outcomes of anti-CD20 mAb-mediated B cell depletion on T cell subsets in the previous studies are controversial. Thus, a slight increase in percentages of naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells (CD44<sup>low</sup>CD62L<sup>high</sup>) and a decrease in memory T cells (CD4<sup>+</sup>CD44<sup>high</sup>CD62L<sup>low</sup>) were reported in one [17] but not in another study [8]. Furthermore, expansion of Treg was recently demonstrated in some [29, 30] but not another [23] studies in NOD mice. We found in this study no change in naïve/activated/memory T cell subsets and also in Treg subsets.

We then showed in the Graves' mouse model the excellent prophylactic effect of anti-mCD20 mAb for blocking induction of anti-TSHR antibodies and preventing hyperthyroidism. This outcome could be expected because anti-mCD20 mAb almost completely eliminated antibody-producing B cells before immunization. However, B cell depletion before immunization also significantly suppressed antigen-specific T cell activation in a T cell recall assay. Previously, suppression of *in vitro* T cell proliferation and/or proinflammatory cytokine (IFN- $\gamma$  and IL-17) secretion was reported [22,23], as well as *in vivo* proliferation of autoreactive T cells in response to endogenous autoantigens by B cell depletion

[8]. Thus, elimination of both antigen-presentation and antibody-production by B cells is possibly involved in this highly efficient prophylactic effect.

The effect of B cell depletion by anti-mCD20 mAb persisted even after the recovery of B cell number as previously reported in diabetes [23]. B cell depletion may be able to “reset” the immune system, by breaking the self-perpetuating vicious cycle of autoreactive B cell generation and T cell activation. However, in other cases, continuous B cell depletion was necessary [19]. It is therefore critical to clarify the reason(s) of these differences for optimizing treatment strategies.

B cell depletion after the first immunization, when T cells were primed, but anti-TSHR antibody production was not observed, was also effective at reducing hyperthyroidism, albeit to a lesser extent than when given before the first immunization. Because B cell depletion after immunization had no effect on already activated T cell function, the suppressive function of anti-mCD20 mAb at this time point is likely attributed to elimination of activated, autoreactive B cells. Although Bouraziz *et al.* [8] have elegantly demonstrated that the presence of both dendritic cells and B cells are necessary for full CD4<sup>+</sup> T cell activation, Yan *et al.* [31] have reported that B cells are the first subset of antigen-presenting cells for activating autoreactive T cells. Thus it is likely that requirement of antigen-presenting function of B cells is limited at the early step of autoantigen-presentation in induction of Graves’ hyperthyroidism. By contrast, therapeutic effect was not observed when mAb was given to hyperthyroid mice. In this case, autoreactive B cells might already have differentiated into CD20<sup>-</sup> plasma cells, and/or antigen-presenting ability of B cells may be no longer necessary once disease is manifested.

Preventive but not therapeutic effects of B cell depletion were reported in mouse models of systemic sclerosis, collagen-induced arthritis and Sjogren syndrome [19-21]. Also the efficacy of B cell depletion on ongoing immune responses/inflammation was reported when mAb were given prior to onset of clinically manifested diseases in spontaneous mouse models of SLE and type 1 diabetes [17,23] and a proteoglycan-induced arthritis model [22]. Thus, in these autoimmune diseases, as in Graves’ disease, B cells play a role in the early stages of

autoimmunity during autoreactive T cell activation/expansion and autoantibody production. By contrast, therapeutic efficacy was observed in experimental autoimmune thyroiditis [18], suggesting the necessity of B cells to maintain the disease activity. These different outcomes may arise because of differential requirements for B cells in initiating disease *vs.* in maintaining disease in different disease models.

In contrast to a lack of therapeutic effect in the majority of mouse studies, some degree of therapeutic effect of rituximab was observed in human autoimmune diseases [2]. Thus, in human trials, rituximab therapy reduced levels of IgG autoantibodies to citrullinated protein, cytoplasmic neutrophil antigen, C1q and TSHR (TSAAb), despite the lack of change in IgG levels [32-38]. It should be appreciated that most of the human studies that showed reduction in pathogenic antibodies and significant changes in some T cell subsets involved combination therapy of both rituximab and immunosuppressive drugs. However, autoantibody reduction does not always correlate with clinical efficacy [39,40], suggesting that the loss of other B cell functions contributes to suppression of autoimmune diseases. One reason for these differences between human and mouse studies may be that B cells augment T cell activation in response to continuous autoantigen challenge, and antibody-producing B cells/plasma cells are continuously generated in human diseases. For these reasons, it may be anticipated that B cell depletion therapy is more effective in humans than in mouse models.

In terms of antibody production, a drawback of anti-mCD20 mAb is its inability to deplete plasma cells, which do not express CD20. We considered that this problem could be overcome by the eventual demise of plasma cells, alone or in combination with B cell depletion. However, plasma cells have very long half-lives, measured in months or even years [11].

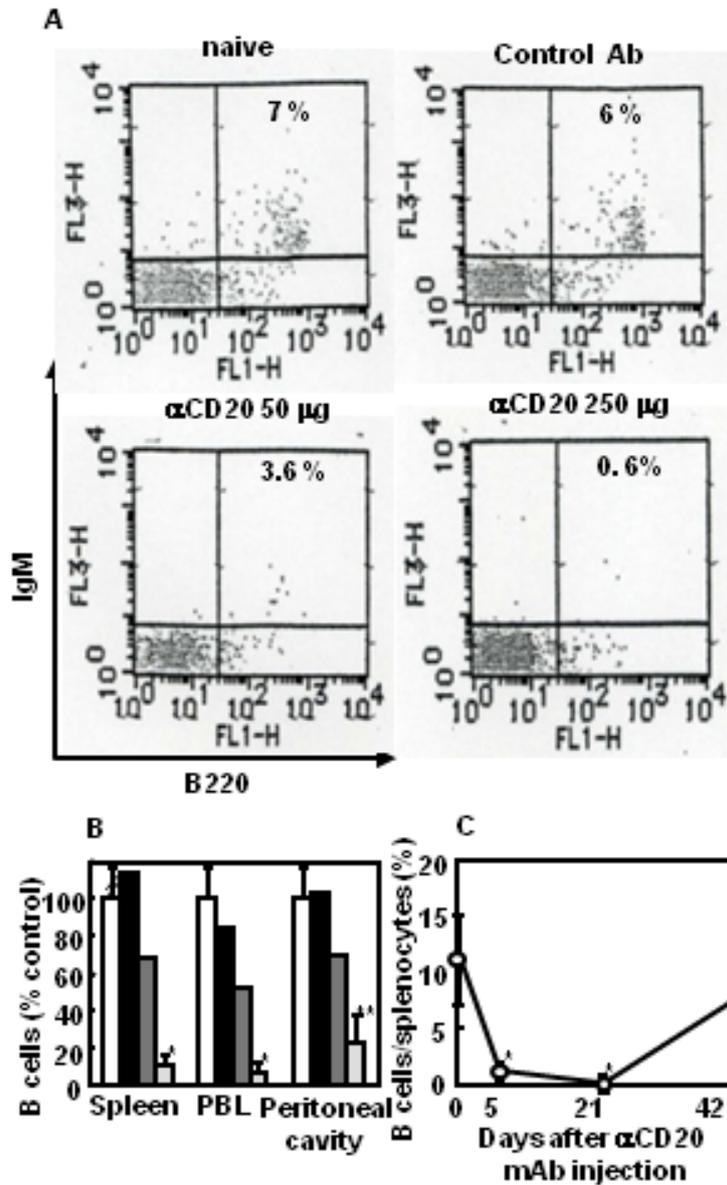
Finally, we here show that anti-mCD20 mAb efficiently depletes B cells and that, although therapeutically less effective, B cell depletion by this agent is highly efficient for preventing development of experimental Graves' hyperthyroidism. Our results indicate that B cells are critical not only as antibody-producing cells but also as antigen presenting/immune-modulatory cells in the early phase of the disease pathogenesis. Further studies are necessary to find

efficient means to therapeutically suppress the pathogenic autoantibody production as novel therapeutic modalities for Graves' disease and also other autoantibody-mediated autoimmune diseases.

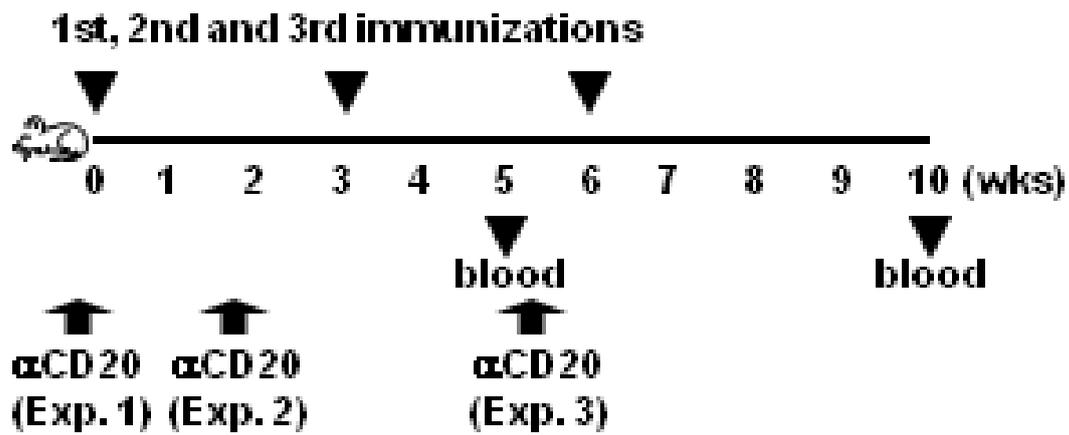
## **Acknowledgments**

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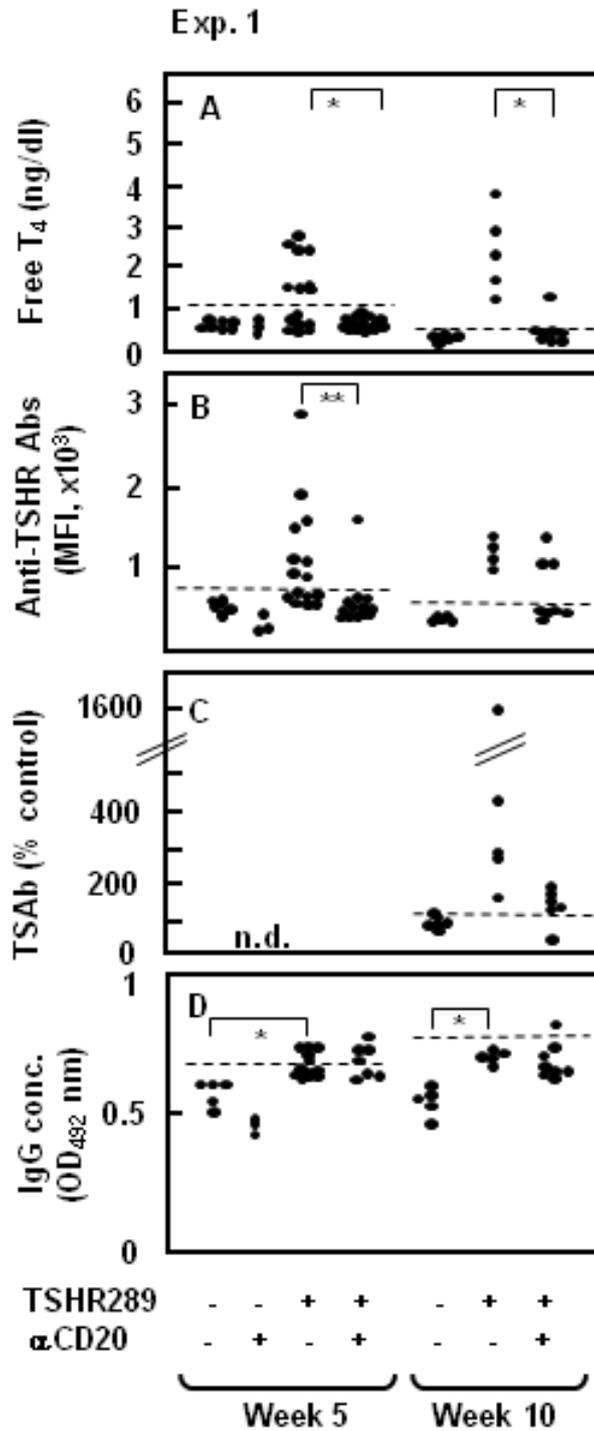
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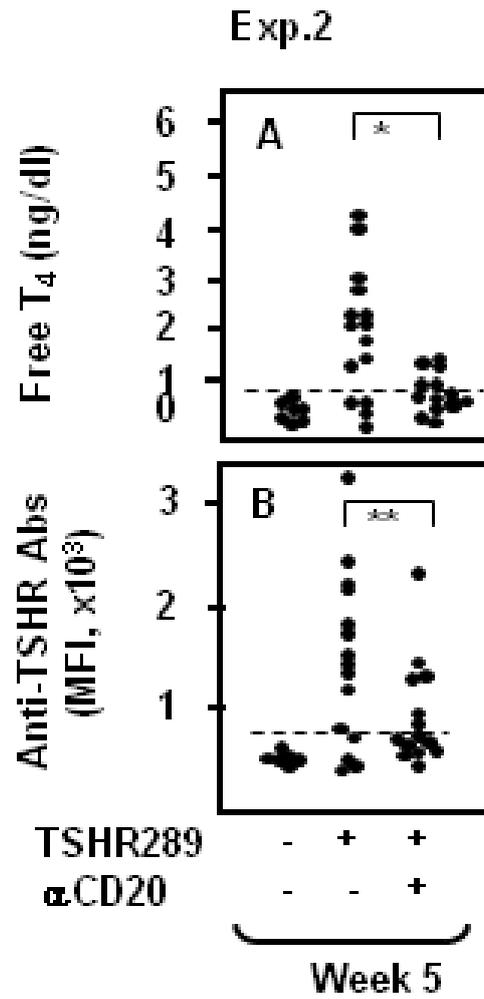
**Fig. 1.** Flow cytometric analysis of B220<sup>+</sup>IgM<sup>+</sup> B cells in mice. Mice were ip treated with PBS, 50 or 250  $\mu$ g anti-mCD20 mAb or 250  $\mu$ g control mAb, and B220 and IgM expression on splenocytes, the peripheral blood and the peritoneal cells was analyzed at indicated time points as described in the *Materials and methods*. (A) Representative flow cytometric data on peripheral blood from mice injected five days before. (B) Dose-dependent effects of anti-mCD20 mAb on B220<sup>+</sup>IgM<sup>+</sup> B cell numbers in spleen, the peripheral blood (PB) and the peritoneal cavity five days after injection of anti-mCD20 mAb. Data are mean  $\pm$  S.D (n = 4-6) or means of two mice. (C) Time course of the effect of anti-mCD20 mAb on B cell numbers in spleen. Data are mean  $\pm$  S.D. (n = 6-10). \* and \*\*, <0.01 and <0.05, respectively, as compared to the controls.



**Fig. 2.** Experimental designs for B cell-depletion study. Mice were immunized thrice (the weeks 0, 3 and 6) with Ad-TSHR289 as described in the *Materials and methods*. Anti-mCD20 mAb was given five days before the first immunization (Exp. 1), 10 days after the first immunization (Exp. 2) or two weeks and two days after the second immunization (a time when development of Graves' hyperthyroidism was confirmed). Blood was taken five and 10 weeks after the first immunization.

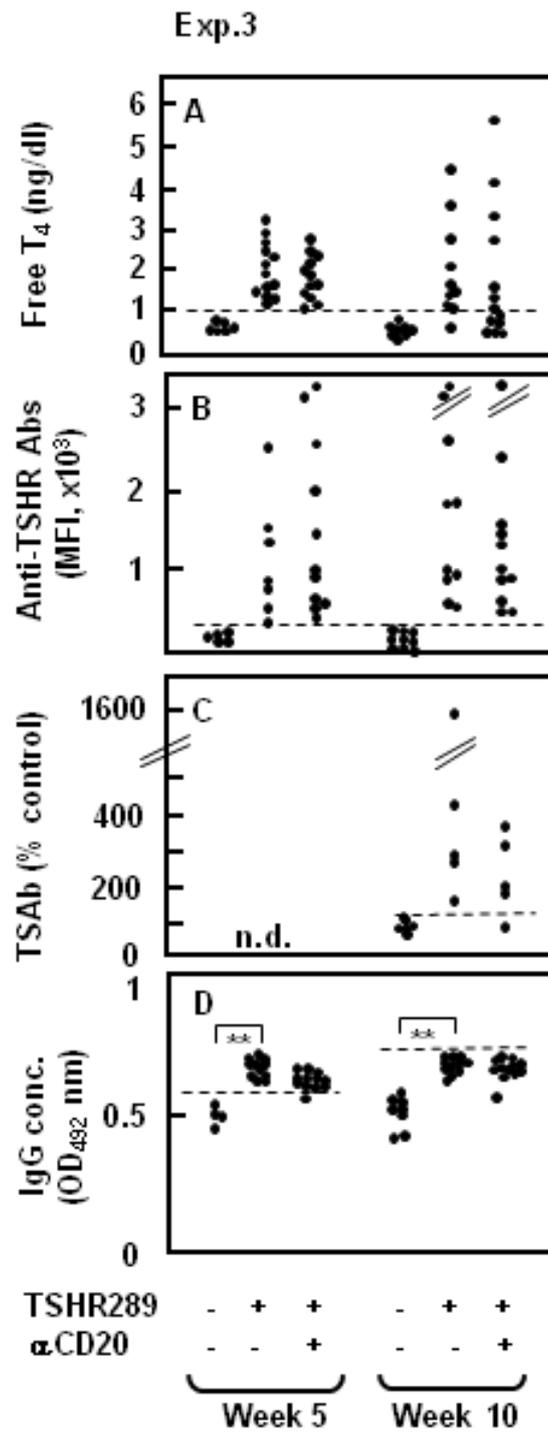


**Fig. 3.** T<sub>4</sub> concentrations, anti-TSHR antibody titers and IgG levels in mice in Exp. 1. Free T<sub>4</sub> were determined by RIA, anti-TSHR antibodies by flow cytometry and bioassay and serum IgG concentrations by ELISA in mice from Exp.1. Data are shown for individual mice. The horizontal broken lines designate the normal upper limits of free T<sub>4</sub>, anti-TSHR antibodies and IgG. \*, p < 0.01; \*\*, p < 0.05.

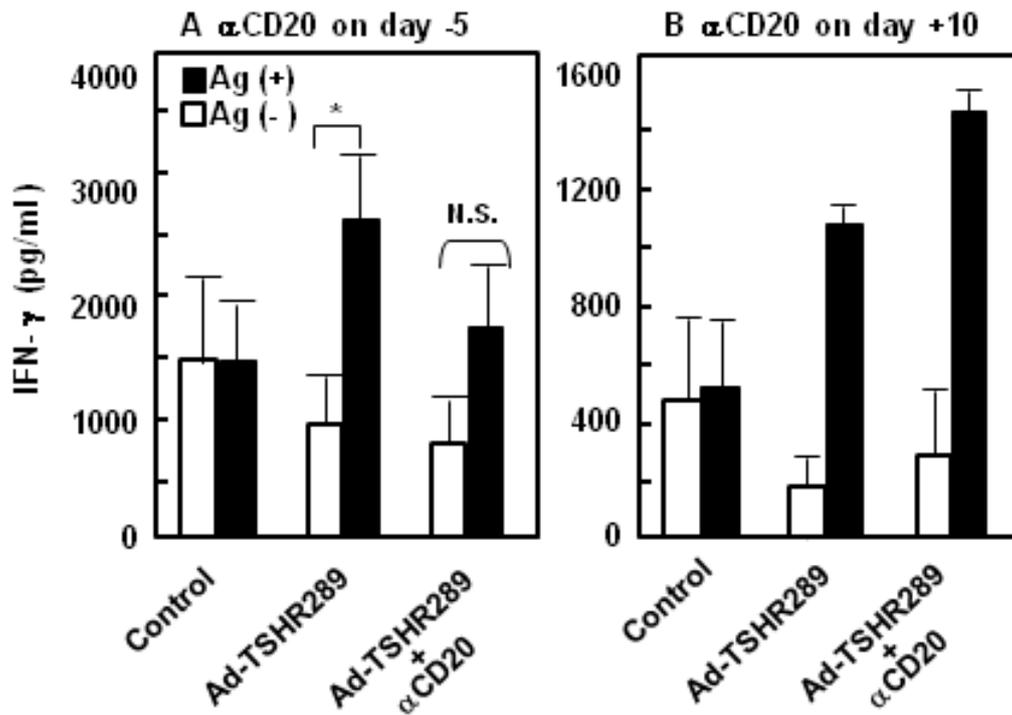


**Fig. 4.** T<sub>4</sub> concentrations and anti-TSHR antibody titers in mice in Exp. 2. Free T<sub>4</sub> and anti-TSHR antibodies were determined as in Fig. 3. Data are shown for individual mice. The horizontal broken lines designate the normal upper limits of free T<sub>4</sub> and anti-TSHR antibodies.

\*, p < 0.01; \*\*, p < 0.05.



**Fig. 5.** T<sub>4</sub> concentrations, anti-TSHR antibody titers and IgG levels in mice in Exp. 3. Hyperthyroid mice selected at the 5th week were divided into two groups of untreated and  $\alpha$ CD20-treated. The horizontal broken lines designate the normal upper limits of free T<sub>4</sub>, anti-TSHR antibodies and IgG. \*\*, p < 0.05.



**Fig. 6.** Antigen-specific splenocyte secretion of IFN- $\gamma$  in a T cell recall assay. (A) Mice injected with anti-mCD20 mAb five days before were immunized with Ad-TSHR289. Two weeks later, splenocytes were prepared and subjected to a T cell recall assay as described in the *Materials and methods*. Data are means  $\pm$  S.D. (n = 3 – 5). \*, p < 0.05. N.S., not significant. (B) Mice were injected with Ad-TSHR289 and 10 days later with anti-mCD20 mAb. Four days later splenocytes were prepared. Data are means  $\pm$  range (n = 2).

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