CTLA-4 Limits Anti-CD20–Mediated Tumor Regression

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Abstract

Purpose: The inhibition of tumor growth by anti-CD20 antibody (Ab) treatment is mediated by Ab- and complement-dependent cytotoxicity in xenograft tumor models. In addition, anti-CD20 therapy for B-cell lymphoma can result in intrinsic and extrinsic tumor resistance to further Ab treatment. However, adaptive immune response–related resistance has not been well studied in anti-CD20–mediated tumor control, and adaptive immunity has long been underestimated. The purpose of this study was to explore whether T cells are involved in mediating the effects of anti-CD20 therapy and what factors contribute to adaptive immune response–related resistance.

Experimental Design: Using a syngeneic mouse B-cell lymphoma model, we investigated the role of CD8+ T cells in anti-CD20–mediated tumor regression. Furthermore, we revealed how the tumor-specific T-cell response was initiated by anti-CD20. Finally, we studied adaptive immune response–related resistance in advanced B-cell lymphoma.

Results: CD8+ T cells played an essential role in anti-CD20–mediated tumor regression. Mechanistically, anti-CD20 therapy promoted dendritic cell (DC)–mediated cross-presentation. Importantly, macrophages were also necessary for the increase in the tumor-specific CTL response after anti-CD20 treatment, via the production of type 1 IFN to activate DC function. Furthermore, adaptive resistance is gradually developed through the CTLA-4 pathway in Treg cells in larger lymphomas. Further blockade of CTLA-4 can synergize with anti-CD20 treatment in antitumor activities.

Conclusions: The therapeutic function of anti-CD20 depends on tumor-specific CD8+ T-cell responses initiated by anti-CD20 through macrophages and DCs. CTLA-4 blockade can synergize with anti-CD20 to overcome adaptive immune response–related resistance in advanced B-cell lymphoma. Clin Cancer Res; 23(1); 193–203. ©2016 AACR.

Introduction

Rituximab, the first mAb used for cancer, was approved by the FDA for treating non-Hodgkin B-cell lymphoma almost 20 years ago. Rituximab is a murine–human chimeric IgG1 Ab that recognizes the human CD20 antigen on B cells, with a primary response rate from 40% to 70% (1–4). Rituximab can induce the death of tumor cells through Ab–dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and direct induction of apoptosis in vitro and in animal models (5). However, the exact contribution of each mechanism to the observed clinical activity of anti-CD20 Ab remains unclear. Using the receptor for the Fc region of immunoglobulin G (FcR)–deficient mice, the essential role of ADCC has been confirmed in the therapeutic function of anti-CD20 (6). Clinically, it has been observed that the FCGR3A polymorphism limits NK-cell–mediated cytotoxicity in rituximab treatment (7). An enhanced therapeutic function was observed by enhancing the binding affinity of anti-CD20 for CD16 (8). Further study showed that anti-CD20–induced lymphoma depletion is mediated by macrophage FcγRI, FcγRII, and FcγRIII (9, 10), and FcγRIIB inhibits its therapeutic function (11). These studies collectively showed that ADCC plays an important role in anti-CD20 therapy. Effective control of B-cell lymphoma by anti-CD20 in xenograft models further suggests direct killing or innate-mediated killing may be sufficient for the control of this type of tumor, whereas the role of the adaptive immune system has not been defined.

The role of adaptive immunity in anti-CD20 therapy had been long ignored until recently. Using the huCD20-EL4 tumor model, a murine T-cell lymphoma transplanted with the human CD20 molecule, one group reported that the induction of cellular immune responses might contribute to long-lasting protection by anti-human CD20 treatment. Intriguingly, only CD4+ T cells, not CD8+ T cells, are required for the control of the tumor (12). Using the same model, another group showed that anti-CD20 treatment could generate protective memory T-cell responses through different FcγRs, but the role of T cells in the primary treatment was not clear (13). Recent studies have shown that CD8+ T cells are essential for the anti-neu therapy of TUBO, a solid tumor model for breast cancer (14, 15). T-cell lymphoma might have abnormally high levels of cytokine expression, and the expression of human CD20 in mouse T-cell lymphoma has...
created many different antigenic epitopes and cytokine milieu after treatment in immunocompetent mouse models. The above two conditions raise the possibility that human CD20-transfected EL4 could induce stronger immunity for tumor control, whereas the natural B-cell lymphoma might not induce CD4⁺ T-cell–dependent tumor control.

Anti-CD20 therapy for B-cell lymphoma can result in intrinsic and extrinsic tumor resistance to further Ab treatment (16, 17). Extrinsic resistance was observed to be related to a defective natural immune response (17). Recent studies in blocking the coinhibitory signaling (CTLA-4 and PD-1/PD-L1) of T cells demonstrated that reversing T-cell suppression is important for effective cancer immunotherapy against solid tumors (18–24). However, adaptive immune response–related resistance has not been well studied in anti-CD20–mediated tumor control, and adaptive immunity has long been underestimated. We explored whether and what type of host response is essential for the therapeutic function of anti-CD20 against actual B-cell lymphoma. Intriguingly, we observed that CD8⁺ T cells, but not CD4⁺ T cells, were essential for the anti-mouse CD20 Ab therapy against A20, a B-cell lymphoma in a syngeneic mouse model. We have further observed an essential role of CTLA-4 in Tregs within advanced B-cell lymphoma in limiting anti-CD20–mediated tumor regression. Thus, anti-CTLA-4 and anti-CD20 combined treatment is a possible new strategy in overcoming adaptive resistance and preventing relapse in the clinical treatment of B-cell lymphoma.

**Materials and Methods**

**Mice**

Wild-type (WT) BALB/c and BALB/c-nude mice were purchased from the Vital River Laboratories. CL-4 mice were purchased from The Jackson Laboratory. CD11c-DTR mice were bred and housed at the Institute of Biophysics. Eight- to ten-week-old female mice were used in all experiments unless otherwise specified. All mice were maintained under specific pathogen-free conditions in the animal facility of the Institute of Biophysics. Animal care and use were in accordance with the Institutional Animal Care guidelines of the Institute of Biophysics, and all studies were approved by the Animal Care and Use Committee of the Institute of Biophysics.

**Production of anti-mouse CD20 Ab**

The V region of the heavy chain and light chain of anti-mouse CD20 Ab was linked with a GGGS-GGSGGSGGGS linker and was cloned into pEE12.4. The plasmid containing both the heavy chain and light chain was transfected into Chinese hamster ovary (CHO) cells, and stable clones were established according to the manual. The supernatant containing the anti-mouse CD20 Ab was purified using a Protein A column according to the manufacturer’s protocol.

**Tumor growth and treatments**

A20 cells (1 to 5 × 10⁶) were injected subcutaneously into the backs of 8- to 10-week-old mice. The tumor volumes were measured along three orthogonal axes (a, b, and c) and were calculated as follows: tumor volume = abc/2. The mice were treated with intraperitoneal or intratumoral injections of 100 μg of anti-mouse CD20 Ab. For CD8⁺ or CD4⁺ T-cell–depletion experiments, 200 μg of anti-CD8 Ab (TIB210) or 200 μg of anti-CD4 Ab (clone GK1.5) were injected intraperitoneally 1 day before anti-CD20 treatment. Finally, 100 μg of anti-Itifn1 Ab was used for type I IFN pathway blockade 1 day before anti-CD20 Ab treatment.

**Measurement of IFNγ-secreting T cells by ELISPOT assay**

Spleen or lymph node (LN) cells were resuspended in RPMI 1640 medium supplemented with 10% FBS, 2 mmol/L 1-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. A total of 3 × 10⁶ spleen or 2 × 10⁵ LN cells were used for the assay. A20 cells were irradiated with a single dose of 60 Gy (10 Gy/minutes for 6 minutes). The ratio of A20 and spleen or LN cells was 1:4. After 48 hours of incubation, the IFNγ production was determined with an IFNγ ELISPOT Assay Kit according to the manufacturer’s protocol (BD Biosciences). The visualized cytokine spots were enumerated with the ImmunoSpot Analyzer (CTL).

**Ex vivo DC cross-presentation assay**

A20-HA tumor-bearing BALB/c-nude mice were treated with 100 μg of anti-CD20 Ab or isotype control Ab (hlgG) on day 14. Three days later, the mice were sacrificed and draining lymph node (DLN) cells were digested with 1 mg/mL collagenase VIII.
(Sigma-Aldrich) and 200 μg/mL DNasel (Sigma-Aldrich) at 37°C for 30 minutes. DCs (CD11b^+^CD11c^+^B2^+^) were sorted out by FACS. Approximately 1 × 10^6^ DCs were mixed together with 1 × 10^6^ purified CL4 T cells. Two days later, the supernatants were collected and IFNγ, IL2, and TNFα were measured by cytokmetric bead array assay (BD Biosciences).

**Generation of bone marrow chimeras**

WT BALB/c mice were lethally irradiated with a single dose of 1,000 rad. The next day, the irradiated mice were adoptively transferred intravenously with 5 × 10^6^ CD11c^+^DTG donor bone marrow cells. The mice were maintained on sulfamethoxazole and trimethoprim (Bactrim) antibiotics diluted in drinking water for 4 weeks after reconstitution. The mice were injected with tumor cells approximately 12 weeks after reconstitution.

**In vitro culture and function assay of BMDCs and BMDMs**

Single-cell suspensions of bone marrow cells were obtained from BALB/c mice. The cells were plated in 10-cm Petri dishes and were cultured in RPMI 1640 medium containing 10% FBS, supplemented with 200 U/mL GM-CSF or 1,000 U/mL M-CSF. Fresh media with GM-CSF or M-CSF was added to the culture on day 3. BMDCs and BMDMs were harvested for stimulation assay on day 7. BMDCs or BMDMs were cocultured with A20-HA cells at the ratio of 1:1 in the presence of either 10 μg/mL anti-CD20 Ab or hlgG for 3 days. To determine the role of Fc and FcR interaction, FcR blocking Ab (2.4G2) was used at the same time. The expression of type I IFN was measured by ELISA. Next, the tumor cells were removed, and DCs or macrophages were incubated with isolated CD8^+^ T cells from naïve CL4 or BALB/c mice for 3 days. In one experiment, BMDMs were cocultured with Raji cells in the presence of 10 μg/mL rituximab or hlgG for 3 days. Next, the Raji cells were removed, and macrophages were incubated with isolated CD8^+^ T cells from BALB/c mice that were immunized three times with Raji cells.

**In vivo expression of type I IFN**

A20-bearing WT BALB/c mice were administered 100 μg of anti-mCD20 or hlgG on day 12. Three days later, DCs and macrophages from the tumor were sorted by FACS. RNA from sorted cells was extracted by RNAeasy Plus Mini Kit (Qiagen). RNA was digested with DNasel and reverse transcribed into cDNA for real-time PCR. The levels of gene expression were normalized to β-actin. The following primers were used: β-actin, forward primer: 5’-ACACCGGCCCAC-CAGTCCGG, reverse primer: 5’-ATGCCGATCCCTAGGTCAGG-GTCA GAATA IFNγ, forward primer: 5’-TCCCCTGACCAGGGAA-GATGCC, reverse primer: 5’-ATTGCCAGAGGAAGACAGGGCT.

**Flow cytometry**

Single-cell suspensions of cells were incubated with anti-CD16/32 (anti-FcγIII/II receptor, clone 2.4G2) for 30 minutes, then were stained with conjugated Abs. All fluorescently labeled Abs were purchased from BioLegend or ebioscience. Samples were analyzed on a FACSFortessa flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (TreeStar).

**Statistical analysis**

Data are shown as the mean ± SEM. Statistical analyses were performed using a two-tailed unpaired Student t test and Prism software (version 6.0, GraphPad Software). P < 0.05 was considered statistically significant.

**Results**

The therapeutic effect of anti-CD20 treatment is CD8^+^ T-cell dependent

A20 is a mouse B-cell lymphoma cell line derived from a spontaneous neoplasms found in an advanced-age BALB/c mouse (25). To investigate the role of anti-CD20 in mouse B-cell lymphoma, WT BALB/c mice were inoculated with A20 and were treated with anti-mouse CD20 Ab or isotype control Ab (hlgG). Similar to the clinical outcome of B-cell lymphoma treated with rituximab, anti-mouse CD20 Ab could control the tumor effectively (Fig. 1A). However, surprisingly, anti-CD20 Ab failed to control A20 in BALB/c-nude mice (Fig. 1B), suggesting that T cells play an essential role in anti-CD20 therapy. To further investigate which subset of T cells is needed for the anti-CD20-initiated elimination of lymphoma, we depleted either CD4^+^ or CD8^+^ T cells during anti-CD20 treatment. The depletion of CD4^+^ or CD8^+^ T cells was verified by FACS. CD4^+^ T cells were not necessary for the function of anti-CD20 (Supplementary Fig. S1). However, the therapeutic function of anti-CD20 was almost abolished after CD8^+^ T-cell depletion (Fig. 1C). Together, the results suggest that CD8^+^ T cells are essential for the function of anti-CD20 in controlling B-cell lymphoma.

To study the mechanism for the generation of the tumor-specific T-cell response, T cells from the DLN were isolated 11 days after Ab treatment to evaluate their responses against tumor antigens using the IFNγ ELISPOT assay. Irradiated A20 cells were used to stimulate the tumor-specific T-cell response, and irradiated 4T1 cells were used as a control. We observed that there was a 10-time increase in immunospots after anti-CD20 treatment (Fig. 1D). These data further support the induction of the anti-CD20-mediated tumor-specific T-cell response in the B-cell lymphoma. CD8^+^ T cells are the major cytotoxic T cells capable of lysing tumor cells. To investigate whether IFNγ is not only a biomarker for tumor-reactive T cells but also a key cytokine that can control tumors, we administrated a specific neutralizing Ab soon after anti-CD20 therapy. The antitumor activity of anti-CD20 decreased dramatically after IFNγ neutralization, and the tumor became resistant to anti-CD20 therapy (Fig. 1E). Collectively, these data demonstrate that the increased tumor-specific T-cell response after anti-CD20 treatment is necessary for its function in the control of B-cell lymphoma.

The increased cross-presentation function of DC is required for anti-CD20 treatment

Considering that CD8^+^ T cells are crucial for effective anti-CD20 therapy, we further investigated the mechanism by which anti-CD20 enhanced tumor-specific T-cell responses. Cross-priming is an essential pathway for the formation of the CD8^+^ T-cell response. To determine whether the cross-presentation function of APC is enhanced after anti-CD20 treatment, we transplanted an antigen epitope hemagglutinin (HA) into A20 cells. We sorted the DCs from the DLN after anti-CD20 treatment and incubated them with TCR-matched naïve T cells from CL4 Tg mice capable of recognizing HA. Two days after incubation, the supernatants were collected and measured using the cytokometric bead array (CBA) assay. There was more IFNγ, IL2, and TNFα secretion after anti-CD20 treatment (Fig. 2A), suggesting that the cross-presentation function of DC was enhanced after anti-CD20 treatment. To further study whether DCs are essential for effective anti-CD20 therapy, DCs were depleted by DT in CD11c^-^DTR mice. Complete
depletion was achieved, without affecting macrophages that much both in the tumor (Supplementary Fig. S2A and S2B) and DLN (Supplementary Fig. S2C and S2D). Depletion of DCs impaired the antitumor effect (Fig. 2B) and the T-cell response in anti-CD20 treatment (Fig. 2C). These data indicate that the increased cross-presentation function of APC is indeed essential for anti-CD20-mediated tumor regression.

Macrophages increase the cross-presentation function of DCs through type I IFN

To directly rule out the role of macrophages, we depleted macrophages using Clophosome. After macrophage depletion, we unexpectedly observed that the therapeutic function of anti-CD20 was also largely abolished (Fig. 3A). The Clophosome depletion experiment showed it could eliminate CD11c and F4/80 double-positive cells in the tumor (Supplementary Fig. S3A and S3B). Recently, the cellular and molecular origin of this subset of cells was reported (26). They proved it is a type of macrophage that differentiates from CCR2+ inflammatory monocytes (26). Because macrophages, DC, and tumor-specific T cells are all essential in anti-CD20 therapy, we hypothesized that macrophages may be the upstream stimulating cells of DCs or T effector cells. To check whether macrophages are necessary for the T-cell response, tumor-specific T cells were monitored in macrophage-depleted mice treated with anti-CD20. The tumor-specific T-cell response was significantly decreased after macrophage depletion (Fig. 3B). We also observed that the therapeutic function of anti-CD20 is NK-cell independent when using anti-asialo GM1 Ab for NK-cell depletion (Supplementary Fig. S4A). Furthermore, the depletion of NK cells did not affect the tumor-specific T-cell response (Supplementary Fig. S4B). Together, the data suggest that macrophages are essential for the tumor-specific T-cell response.

We have shown that both macrophages and the increased cross-presentation function of DCs through type I IFN are responsible for stimulating the tumor-specific T-cell response. To investigate if and how macrophages and DCs are cooperative in anti-CD20 treatment, we checked the cross-presentation function of DCs after macrophage depletion with anti-CD20 treatment. There was much less IFNγ expression using DCs from the DLN of mice with macrophage depletion (Fig. 3C), suggesting the cross-presentation function of DCs decreased dramatically in the absence of macrophages. In addition, NK cells are not responsible for increased DC function (Supplementary Fig. S4C). However, how macrophages activate the cross-presentation function of DC is unknown.

Figure 1.
The therapeutic effect of anti-CD20 treatment is CD8 T-cell dependent. A, WT BALB/c mice (n = 6/group) or BALB/c-nude mice (n = 6/group; B) were injected subcutaneously with 18 × 10⁶ A20 cells and were treated with 100 μg of anti-mCD20 or 100 μg of hIgG (Ctrl) by intraperitoneally on days 12 and 15 after A20 injection. This is the basic procedure for C–E. C, A20-bearing BALB/c mice (n = 6/group) were treated as in A. CD8+ T-cell-depleting Ab (TIB210, 200 μg/mouse) was administered twice a week starting on day 11. D, Eleven days after the final Ab treatment, DLN cells were collected, and the IFNγ ELISPOT assay was performed with medium or irradiated A20 cell restimulation. Irradiated 4T1 cells were used as the control. E, A20-bearing BALB/c mice (n = 6/group) were treated as in A. Mouse IFNγ Ab (R4-6A2, 200 μg/mouse) was administered twice a week starting on day 11. The mean ± SEM values are shown. Two-tailed Student t test was used for statistical analysis.
Type I IFN is known to be critical for the cross-priming of the tumor-specific T-cell response (27–29). Therefore, we tried to determine which cells are the major contributors for type I IFN, macrophages versus DCs. We observed that the macrophages expressed much higher levels of type I IFN than DCs after anti-CD20 treatment (Fig. 3D). In addition, type I IFN expression of macrophages induced by anti-CD20 was dependent on Fc and FcR interaction (Fig. 3D). The in vivo expression of type I IFN after anti-CD20 treatment was also checked by RT-PCR (Fig. 3E). The essential roles of type I IFNs in initiating antitumor T-cell responses by various antitumor therapies have been demonstrated in many studies (27–29). Therefore, we tried to elucidate the type of IFN expression in initiating antitumor T-cell responses by various antitumor therapies and tumor regression. To check the essential role of type I IFNs, the type I IFN pathway was blocked by anti-ifnar1 during anti-CD20 treatment. Indeed, blocking of the type I IFN pathway by antibodies resulted in an impaired cross-presentation function of DCs (Fig. 3F). Furthermore, the therapeutic function of anti-CD20 was largely abolished when the type I IFN pathway was blocked (Fig. 3G). Correspondingly, blockade of the type I IFN pathway diminished the tumor-specific T-cell response initiated by anti-CD20 (Fig. 3H). Collectively, these data indicate that anti-CD20 therapy can induce type I IFN, which could increase the cross-presentation function of DCs for potent T-cell responses against tumors. The increase in the APC cross-presentation function induced by anti-CD20 was also confirmed with human lymphoma cells in vitro. BMDCs presented an increased cross-priming function when cocultured with human B-cell lymphoma in the presence of anti-human CD20 Ab (rituximab; Fig. 3I), suggesting that rituximab might be capable of priming tumor-specific T cells in the patients.

Tumor-specific T cells are mainly primed in the DLN after anti-CD20 therapy

Several studies have indicated that anti-PD-1- or anti-CD47–mediated T-cell reactivation inside tumor tissues is essential for tumor regression (30, 31). Anti-CD20 can induce tumor cell apoptosis that triggers the cross-priming of tumor DCs to reanimate T cells. To investigate whether priming tumor-specific T cells in the tumor or DLN was essential for tumor regression, we utilized FITY720 treatment to block sphingosine 1-phosphate receptor-1, thereby prohibiting T cells from exiting the lymphoid organ (Supplementary Fig. S1). The therapeutic function of anti-CD20 was almost abolished after FITY720 administration (Fig. 4A), suggesting that the reactivation of T cells inside the tumor is not sufficient, and T cells in DLNs are essential for the therapeutic function. To monitor the tumor-specific T-cell changes after FITY720 administration, DLN cells and splenocytes were collected, and the IFNγ ELISPOT assay was performed. FITY720 had no influence on the activation of tumor-specific T cells in the DLN when treated with anti-CD20 (Fig. 4B). There were no detectable tumor-specific T cells in the spleen when treated with FITY720 (Fig. 4C), suggesting that tumor-specific T cells were not stimulated in the spleen. Taken together, tumor-specific T cells are largely primed in the DLN after treatment. Recirculation of those primed T cells to the tumor tissue is essential to control tumors.

CTLA-4 contributes to the adaptive resistance to anti-CD20 therapy

Anti-CD20 therapy was effective for controlling small tumors (Fig. 5A). However, when the tumors became more established, they became much more resistant to anti-CD20 treatment (Fig. 5B). Previous studies have shown that tumor-intrinsic resistance
Macrophages increase DC function through type I IFN. A, A20-bearing WT BALB/c mice (n = 5/group) were administered 100 μg of anti-mCD20 or 100 μg of hlgG on days 11 and 15. Macrophage-depleting reagent (Clophosome, 200 μg/mouse) was administered on day 10 and day 15. B, Eleven days after Ab treatment, DLN cells were collected, and the IFNγ ELISPOT assay was performed with medium or irradiated A20 cell restimulation. C, A20-HA-bearing BALB/c-nude mice (n = 5/group) were treated with anti-mCD20 and/or Clophosome on day 14. Three days later, DCs from DLN were sorted by FACS and then were cocultured with CL4 T cells. IFNγ production was measured 2 days later. D, Bone marrow–derived dendritic cells or macrophages were cocultured with A20-HA cells in the presence of anti-mCD20 and/or 2.4G2 for 3 days. IFNβ production was measured by ELISA. **P < 0.001. E, A20-bearing WT BALB/c mice (n = 5/group) were administered 100 μg of anti-mCD20 or hlgG on day 12. Three days later, DCs and macrophages from the tumor were sorted by FACS. Total RNA was extracted and used for the real-time PCR detection of IFNα. F, A20-HA-bearing BALB/c-nude mice (n = 5/group) were treated with anti-mCD20, or anti-mCD20 and anti-ifnar1, or hlgG on day 14. Three days later, DCs from the DLN were sorted by FACS and then were cocultured with CL4 T cells. IFNγ production was measured 2 days later. G, A20-bearing WT BALB/c mice (n = 5/group) were administered 100 μg of anti-mCD20 or hlgG on days 12 and 15. Mouse ifnar1-blocking Ab (anti-ifnar1, 100 μg/mouse) was administered twice a week starting on day 11. H, Eleven days after the Ab treatment, DLN cells were collected, and the IFNγ ELISPOT assay was performed with medium or A20 cell restimulation. I, Bone marrow–derived macrophages were co-cultured with Raji cells in the presence of either rituximab or hlgG for 3 days. Next, the tumor cells were washed out, and CD8+ T cells from Raji-immunized mice were added to the culture. IFNγ production was measured 3 days later. The mean ± SEM values are shown. Two-tailed Student t test was used for statistical analysis.
SEM values are shown. C, cells, we determined test was used for T cells are T-cell expansion. A20-bearing WT BALB/c mice and T cells were depleted by GK1.5 systemically. CD4+5/group) were administered 100 µg of anti-mCD20 or hlgG on day 11. FTY720 was administered every 2 days starting on day 10 through the end of the experiment. B and C, Eleven days after the Ab treatment, DLN cells (B) or splenocytes (C) were collected, and the IFN-γ ELISPOT assay was performed with medium or A20 cell restimulation. The mean ± SEM values are shown. Two-tailed Student t test was used for statistical analysis.

and innate immune response resistance account for the tumor nonresponsiveness to Ab treatment (16, 17). In addition, larger tumors might lead to an insufficient anti-CD20 molecule to contact each tumor cell. In light of the essential role of CD8+ T cells for initial tumor control in our current study, we wondered whether anti-CD20–initiated immunity was tempered by T-cell–mediated resistance. We investigated possible changes among T-cell subsets and observed that there were far fewer tumor-specific T cells after Ab treatment in the advanced tumor (data not shown). We propose that increased Treg cells might contribute to the reduction of CTL. Indeed, the percentage of Treg cells in CD4+ T cells in the tumor was increased in the large established B-cell lymphoma (Fig. 5C). In addition, Treg cells are the major immunosuppressive cells in the tumor compared with macrophages and myeloid derived suppressor cells (MDSCs) in large established tumors (Fig. 5D). Thus, Treg cells might contribute to the inhibition of CTL–mediated tumor regression (12). Anoth-

er recent study demonstrated that long-lasting antitumor protection and ADCC against human CD20-transfected EL4 could be triggered by anti-CD20 therapy, likely through differential Fc-receptor engagement (13). However, strong immunogenicity of human CD20, a lack of depletion of B cells, and strong cytokine production in T-cell lymphoma by anti-human CD20 could complicate the comprehensive understanding of T-cell involvement for anti-CD20–mediated tumor control and relapse. In our study, anti-mouse CD20 Ab was used for the treatment of a mouse

Discussion

Using xenograft models and in vitro assays, it has been well established that anti-CD20 therapy can increase CDC, ADCC, or apoptosis of lymphoma (5). Some clinical studies have reported that tumor-specific T-cell responses could also be detected after the use of rituximab in clinical treatment (40, 41). However, whether T cells are essential for anti-CD20–mediated primary B-cell lymphoma regression has been largely ignored. Using EL4-huCD20, a mouse T-cell lymphoma transduced with human CD20, a xenopeptide, it has been shown that CD4+ T cells are essential for anti-CD20–mediated tumor regression (12). Another recent study demonstrated that long-lasting antitumor protection and ADCC against human CD20-transfected EL4 could be triggered by anti-CD20 therapy, likely through differential Fc-receptor engagement (13). However, strong immunogenicity of human CD20, a lack of depletion of B cells, and strong cytokine production in T-cell lymphoma by anti-human CD20 could complicate the comprehensive understanding of T-cell involvement for anti-CD20–mediated tumor control and relapse. In our study, anti-mouse CD20 Ab was used for the treatment of a mouse
B-cell lymphoma (without transfection of human CD20) in the immunocompetent mouse model, to mimic the clinical use of rituximab in human B-cell lymphoma. We have observed that enhancing macrophage-dependent cross-priming for CTL induced by anti-CD20 is essential for antitumor therapy: (i) the anti-mouse CD20 Ab could control mouse B-cell lymphoma effectively in WT BALB/c mice but not in the BALB/c-nude mice, suggesting that T cells are required for anti-CD20 treatment; (ii) the essential role of T cells was also confirmed by the loss of a treatment effect after CD8 T-cell depletion and an enhanced tumor-specific T-cell response after anti-CD20 treatment; (iii) type I IFN-associated cross-priming is essential; (iv) macrophages increase the cross-presentation function of DCs through type I IFN; and (v) impressively, CTLA-4 from Tregs plays an essential role in adaptive resistance. These data reveal an essential role of the adaptive immune response in anti-CD20 therapy and resistance to this therapy.

Macrophages have been shown to induce ADCC in anti-CD20 therapy in a mouse model with T-cell lymphoma overexpressing human CD20 (9). Our data showed that macrophages also control mouse B-cell lymphoma treated with anti-mouse CD20. However, the function of macrophages depended on the induction of the tumor-specific T-cell response but not on ADCC alone. The function of macrophages is normal or increased in nude mice, but anti-CD20 failed to control B-cell lymphoma in the syngeneic model. In contrast to the xenograft model, innate cells against syngeneic tumors might not be as strong as xenograft tumors. In fact, T cells can suppress innate cell function (42). When macrophages were depleted, the therapeutic function of anti-CD20 was abolished in WT mice, and the tumor-specific T-cell response was also impaired. Our study suggests that tumor-specific T cells is the final effector cell in the control of tumors; in addition to killing some tumor cells by ADCC, macrophages play a critical role in inducing a tumor-specific T-cell response.

It is not easy to determine whether the tumor or lymphoid tissue is the major site essential for the generation of T-cell responses after Ab treatment because it might depend on the function of antibodies and/or type of tumor. Several studies have indicated that anti-PD-1 or anti-CD47–mediated T-cell reactivation inside tumor tissues is essential for tumor regression (30, 31). Blockade of T cells exiting from the lymph node during CD20 therapy could reduce the therapeutic effect, suggesting that the DLN might be the essential site for T-cell priming and expansion. Furthermore, increased activation of T cells against tumor cells can be detected in the DLN. The absence of A20 cells in the DLN suggests that APCs must carry antigens into the DLN to prime T cells, and cross-priming might be involved. Type I IFN is the best-known cytokine for the increased cross-priming of DCs to tumor-specific CD8\(^+\) T cells.
T cells are involved in this study whereas T cells in huCD20-EL4, a T-cell lymphoma, A20-bearing WT BALB/c mice were injected for about 60 days after 4/group) were administered for a rapid Th1 CD4 T-cell response.

It was shown that the therapeutic function of anti-CD20 depended on CD4 T cells expressing human CD20, a xenoprotein that triggers strong immune responses. Consistently, we observed that human CD20 is a high immunogenic antigen because human CD20-expressing A20 tumors were immunologically rejected in mice even when given 10-fold higher than the lethal dose (10^7 per mouse) at transplantation. Furthermore, the EL4-huCD20 model shows that the therapeutic function of anti-human CD20 depends on NK cells, which can strongly express IFNγ for a rapid Th1 CD4 T-cell response.

However, in our model, NK cells are not necessary, which is in accordance with another study also using mouse B-cell lymphoma treated with anti-mouse CD20 (9). Whether the xenoproteins or tumor antigens are the reasons for the different phenotypes of T-cell responses remains to be determined.

Ab-induced apoptosis and innate responses have been thought to be major mechanisms in Ab-initiated tumor regression (5). Therefore, studies on Ab resistance in tumor treatment have been mostly focused on the tumor-intrinsic resistance and innate immune response–related resistance (such as absent CD20 expression and FcgRIIIA polymorphism; refs. 16, 17). Our study has revealed that the adaptive immune response is essential for the initial control of tumors at the early phase, but tumor relapse could also be due to adaptive resistance in the adaptive immune system. CTLA-4 is one of the immune checkpoints of T cells after initial T-cell activation. CTLA-4 is important for the suppressive function of Tregs in tumor evasion (33–38). In our study, we observed a much higher percentage of Treg cells as the tumor progressed. Interestingly, these Tregs expressed a much higher level of CTLA-4 than other T cells. However, consistent with single anti-CTLA-4 treatment in the clinic (44), anti-CTLA-4 alone showed a minor effect for A20 regression in our mouse model. However, the function of anti-CTLA-4 was greatly enhanced after anti-CD20 treatment, suggesting that anti-CTLA-4 might release the brake of Tregs in anti-CD20 treatment. Anti-CTLA-4 could feasibly remove the suppression of Tregs. However, in anti-CTLA-4 single treatment, there are no functional T cells, so the tumor could not be controlled. Furthermore, in the anti-CD20 and anti-CTLA-4 combined treatment, it is possible that anti-CD20 initiates a tumor-specific T-cell response and anti-CTLA-4 removes Treg suppression to tumor-specific T cells, achieving a synergistic effect. Anti-CTLA-4 and anti-CD20 combined treatment could also generate a protective memory response against tumor relapse, which may also prevent tumor relapse. Our study raises the
In conclusion, we have characterized how anti-CD20 treatment initiated a potent tumor-specific T-cell response for tumor control. Ab could kill some tumor cells through ADCC by macrophages that produce type I IFN for cross-priming; DCs capture antigens; IFN binds IFNAR and activates DCs to better process tumor antigens for cross-priming T cells in the DLN; tumor-specific T cells travel to the tumor site for tumor control (Supplementary Fig S7). Importantly, this study reveals the unappreciated contribution of adaptive immune responses in the early elimination and late resistance of anti-CD20 therapy, raising the potential of using checkpoint blockade to overcome adaptive resistance in the future.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z. Ren, Y.-X. Fu
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