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Original Article

YB-1 immunization combined with regulatory T-cell depletion induces specific T-cell responses that protect against neuroblastoma in the early stage

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Neuroblastoma is the most common extracranial solid cancer in childhood and the most common cancer in infancy. Currently, no effective clinical treatments are available for advanced neuroblastoma. In a previous study, we screened Y Box protein 1 (YB-1) as a potential neuroblastoma-associated antigen from sera of AGN2a-immunized mice by serological analysis of recombinant cDNA expression libraries technique. The aim of this study is to explore if YB-1 immunization in the context of Treg depletion could induce protective immune response against the neuroblastoma in mice. YB-1 was expressed and purified by pET-15b prokaryotic expression system. It was demonstrated that anti-YB-1 $CD8^+$ T-cell responses could be induced by AGN2a immunization, and the strongest $CD8^+$ T-cell responses against AGN2a were induced by YB-1-immunized mice in the context of Treg depletion compared with YB-1 only immunization group and control group. Importantly, the survival rate of mice treated with YB-1 immunization combined with Treg depletion was 80% when challenged by 1×10^4 AGN2a cells, significantly higher than that of mice immunized with YB-1 alone ($P < 0.01$). Furthermore, T-cell adoptive therapy showed that the neuroblastoma growth was inhibited when T cells or splenic cells from YB-1-immunized mice with Treg depletion were transferred to AGN2a bearing mice. Both $CD4^+$ and $CD8^+$ T cells were involved in the anti-neuroblastoma responses induced by YB-1 immunization combined with Treg depletion. These results indicated that YB-1 immunization combined with Treg depletion could induce specific T-cell responses against neuroblastoma and could be a potential strategy for the prevention and treatment of neuroblastoma in the early stage.

Keywords neuroblastoma; YB-1; immunization; CD25

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Introduction

Tumors remain a leading cause of death in the developed countries and are slowly rising to the top in the developing countries. Surgery, radiotherapy, and chemotherapy can lead to a temporary remission for tumor growth, but are not effective in preventing cancer recurrence. The recently acquired knowledge in tumor biology yields potential new targets for more specific and effective target therapies. The prototype of a target therapy is immunotherapy. Sophisticated animal models and improved understanding of the various immune effectors mechanisms have revealed that the immune system can effectively control cancer growth [1].

Neuroblastoma is the most common extracranial solid tumor in children. For those >1 year of age with advanced disease, the 3-year progression-free survival is only 30% [2]. Currently, no effective clinical treatments are available for advanced neuroblastoma. Therefore, alternate therapies such as cancer antigen vaccines or cellular immunotherapy are urgently needed not only for relapsed patients but also as an adjuvant to prevent disease recurrence [3–6].

The serological analysis of recombinant cDNA expression libraries (SEREX) constructed from patient tumor was established by Sahin *et al.* [7,8] who demonstrated that this process could identify T-cell antigens as well as B-cell antigens. It is used in patient studies and has even been proven to identify intracellular antigens targeted by the immune system [9–11]. The identification of the NY-ESO-1 antigen in patients by SEREX demonstrated that both major histocompatibility complex (MHC) class II-restricted epitopes and MHC class I-restricted (HLA-A2) epitopes, targets of cytotoxic T-cell responses, could be identified with this technique [11].

In previous studies, we have screened the potential neuroblastoma-associated antigens from sera of co-stimulatory molecules engineered AGN2a-immunized mice or AGN2a-bearing mice by SEREX technique and identified Y

Box protein 1 (YB-1) as one of these antigens [12–14]. YB-1 is a 42-kDa multifunctional cellular protein that is expressed in various cancers [15]. In particular, YB-1 is localized in the nuclear compartment following cellular stress, such as radiation, drug treatment, hyperthermia, and viral infection, and is a potential target in cancer therapy [16]. Most importantly, anti-YB-1 $CD8^+$ T-cell responses could be induced by AGN2a immunization as demonstrated in previous studies [12,14].

T regulatory cells are known to suppress the immune response to self-antigens, including tumor self-antigens, and thwarting this tolerogenic role by their depletion has become a major focus in the development of new immunotherapeutic strategies for the treatment of human malignancy [17,18]. Golgher *et al*. [19] have demonstrated that $CD25^+$ T-cell depletion uncovers immune responses to the tumor cell type used as a vaccine, and importantly that this response broadens to include other syngeneic tumor cell types. According to the above reports, we supposed that treatment of experimental animals with tumor-associated antigen immunization in the context of anti-CD25 antibody treatment would induce a strong anti-neuroblastoma immune response.

To explore if YB-1 immunization combined with Treg depletion could induce stronger host immune responses against the neuroblastoma in mice, we identified that YB-1 could induce a T-cell response *in vivo*. Then A/J mice were immunized with YB-1 in the context of Treg depletion to detect the protective immune responses of tumor-associated antigens. This study may help to find a novel and potential way for the prevention and treatment of neuroblastoma in the early stage.

Materials and Methods

Mice and tumor cell lines

A/J mice, 6–8 weeks of age, were housed in the Medical College of Xi'an Jiaotong University (Xi'an, China). All animal care and experimental procedures in this study were approved by the Institutional Animal Care and Use Committee of the Xi'an Jiaotong University. An aggressive clone of Neuro2a (AGN2a) is the strain A-derived mouse neuroblastoma cell line and derived from successive *in vivo* passage.

Expression of YB-1 protein

The method to express YB-1 protein was described previously [12,14]. Briefly, full-length *YB-1* gene was cloned from AGN2a cDNA using the YB-1 upstream primer 5′-GGAATTCCATATGAGCAGCGAGGCCGAG-3′ and downstream primer 5′-CGGGATCCTTACTCAGCCCCGCCCTG-3′. Polymerase chain reaction fragments with *Nde*I and *Bam*HI restriction sites were inserted into pET-15b (Novagen, Madison, USA) and recombinant plasmid with inserted sequence was verified by DNA sequencing. Plasmid was transformed into *Escherichia coli* BL21 (DE3) and gene expression was induced with 0.8 mM isopropyl β-D-thiogalactoside. Then protein was purified using a Ni-NTA purification system (Invitrogen, Carlsbad, USA) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Purified proteins were resolved by SDS-PAGE (12%, NuPAGE gel system; Invitrogen), and transferred to PVDF membranes (0.45 μm; Invitrogen) using a NuPAGE electrophoresis system (Invitrogen). The membranes were probed with anti-human YB-1 (ARP, Belmont, USA) at a 1 : 1000 dilution, followed by AP-conjugated rabbit anti-mouse IgG (H + L) at a 1 : 2500 dilution. AP detection was performed using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP; picoBLUE Immunoscreening kit, Stratagene, La Jolla, USA).

Vaccination and immune assays

A/J mice were immunized by subcutaneous (s.c.) injection of 2×10^6 irradiated (5000 rad) AGN2a cells in the shaved right flank. Five days following the second of two weekly vaccinations, spleen cells were collected, and $CD8^+$ T cells were purified using the CD8a (Ly-2) Microbead kit (Miltenyi Biotech, Cologne, Germany) on a MACS device (Miltenyi Biotech). Enzyme-linked immunosorbent spot (ELISPOT) analysis to enumerate $CD8^+$ interferon-gamma (IFN-γ)-producing cells was carried out using the BD ELISPOT mouse IFN-γ set (Franklin Lakes, USA) and 96-well PVDF membrane plates (Millipore, Bedford, USA) according to the manufacturer's protocol. Briefly, peritoneal exudate cells (PECs) were used as antigen-presenting cells. About 1×10^5 PECs from naive A/J mice were placed in ELISPOT wells and loaded with 25 μg/ml of recombinant enhanced green fluorescent protein (EGFP) and YB-1 in 100 μl of media, respectively, for 4 h at 37°C. $CD8^+$ T cells (1×10^5 cells/100 μl) were added to each well for 18 h to test for antigen recognition. To identify whether antigen-specific $CD8^+$ T cells could directly recognise tumor cells, 1×10^4 neuroblastoma cells (AGN2a) were incubated with 5×10^4 $CD8^+$ T cells from mice immunized with EGFP and YB-1, respectively, at 37°C for 18 h. Spots were counted using an automated reader (Immunospot 3, C.T.L., Ltd, Cleveland, USA).

Flow cytometric analyses

Regulatory T cells, $CD8^+$, and $CD4^+$ T cells from mice peripheral blood were checked by flow cytometry 3 days after antibody treatment. FITC-anti-CD4, PE-anti-CD25, and PE-anti-CD8 antibodies (1 : 100) were from BD Biosciences Pharmingen. Blood cells were incubated with antibodies at 4°C for 15 min, and then the cells were

analyzed by a FACScan flow cytometer after the red blood cells were lysed.

Immunization and survival assay

A/J mice were divided into six groups with eight mice in each group. For depletion of Tregs, mice received 250 μg of bioreactor generated (Integra CL 1000, Chur, Switzerland) anti-CD25 monoclonal antibody (mAb), and clone PC61, by intraperitoneal (i.p.) injection 3 days prior to the first vaccination. For immunization groups, mice were immunized weekly for three times. The initial dose of proteins is 50 μg protein emulsified with CFA (complete Freund's adjuvant) per mice followed by 25 μg protein emulsified with IFA (incomplete Freund's adjuvant) per mice every week. Five days after the last immunization, mice were challenged with 1×10^4 or 1×10^5 AGN2a cells. Mice were examined daily until the tumors became palpable, after which the tumor volume was determined daily by measuring the diameter of the tumors using calipers. The tumor volume was calculated using the formula, $V = (ab^2)/2$, where a is the long axis, and b is the short axis [20]. Mice were sacrificed until the tumor size was $>300\ mm^3$ and the sera were collected for enzyme-linked immunosorbent assay (ELISA).

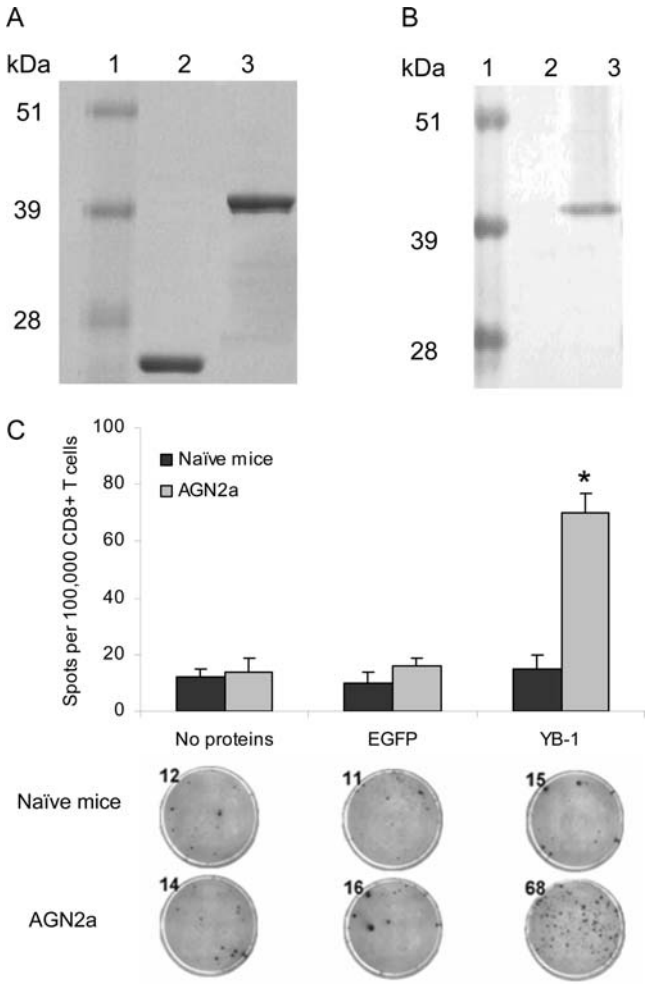

Figure 1 Anti-YB-1 CD8$^+$ T-cell responses were induced by AGN2a immunization SDS-PAGE (A) and western blot analysis (B) of YB-1 expression. Line 1, molecular weight marker; line 2, purified EGFP; line 3, purified YB-1. Proteins were probed with anti-YB-1 antibody. (C) About 1×10^5 syngeneic PECs loaded with buffer (no protein), recombinant EGFP, recombinant YB-1 were co-cultured with 1×10^5 CD8$^+$ T cells from the AGN2a-immunized mice and naïve mice, and then IFN-γ ELISPOT analysis of CD8$^+$ T cells were performed after 18 h. (B) About 1×10^4 neuroblastoma cells (AGN2a) were incubated with 5×10^4 CD8$^+$ T cells which from mice immunized with EGFP, YB-1, and YB-1 in the context with Treg depletion. Data are representative of three separate experiments. Each experiment consisted of T cells purified and pooled from five mice. Error bars represent the standard deviation calculated from triplicate ELISPOT wells. Values were shown as the mean ± SD ($n = 3$). *$P < 0.05$ vs. EGFP group. Representative ELISPOT images showed the spots for one well response.

Enzyme-linked immunosorbent assay

Antigen-specific IgG was detected by 96-well plates (EIA/RIA; Costar, Corning, USA) coated with bacterially expressed EGFP and YB-1 (1 μg per well) in carbonate buffer (45.3 mM $NaHCO_3$, 18.2 mM Na_2CO_3, pH 9.6). Diluted sera were added to the blocked wells and detected with rabbit anti-mouse IgG (H + L) labeled with alkaline phosphatase (Abcam, Cambridge, UK) and developed with NBT/BCIP.

Adoptive T-cell therapies

The donor A/J mice were immunized with YB-1 in the context of Treg depletion as described in the method of immunization and survival assay. Five days after last immunization, spleens were collected and processed into single-cell suspensions. The splenocytes were incubated with anti-Thy1.2-conjugated microbeads (Miltenyi Biotec), and the T cells were positively selected using a MACS device. The recipient A/J mice were inoculated with 1×10^4 AGN2a cells 8 days before T-cell adoptive treatment. The mice were also treated with 250 μg of anti-CD25 antibody by i.p. injection 3 days prior to the adoptive treatment. Mice were given a single intravenous injection of 5×10^6 Thy1.2-enriched T cells or 2×10^7 spleen cells, respectively. One day after T-cell adoptive treatment, mice were immunized with YB-1 weekly for three times as described in the method of immunization and survival assay. Mice were sacrificed until the tumor size was $>300\ mm^3$.

T-cell depletion assay

A/J mice were divided into three groups with eight mice in each group. For T-cell depletion group, mice were treated with either 250 μg anti-CD8 mAb or anti-CD4 mAb (BD Biosciences Pharmingen, San Diego, USA) on days 4, 7, 10, and 14 after the first immunization. Mice in all groups were immunized weekly for three times. The initial dose of proteins is 50 μg protein emulsified with CFA per mice

followed by 25 μg protein emulsified with IFA per mice every week. Five days after the last immunization, mice were challenged by 1×10^4 AGN2a cells. Mice were sacrificed until the tumor size was >300 mm^3.

Data analysis

Data were processed using the SPSS13.0 software package for Windows. All results were expressed as the mean ± SD (standard deviation). One-way analysis of variance was employed to determine the difference among groups. Results were considered statistically significant if $P < 0.05$.

Results

Anti-YB-1 CD8$^+$ T-cell responses induced by AGN2a immunization

Recombinant YB-1 was expressed in the pET-15b bacterial vector encoding an N-terminal His_6 sequence. Proteins containing the His_6 tag were purified from bacterial lysates by using a Ni-NTA column. SDS-PAGE data showed that the purified protein was 42 kDa and recognized by anti-YB-1 antibody [**Fig. 1(A,B)**].

CD8$^+$ T-cell response against YB-1 was detected in IFN-γ ELISPOT assays by using PECs as the antigen-presenting cells. CD8$^+$ T cells enriched from AGN2a-immunized mice by immune magnetic selection. Control cultures containing T cells plus non-protein or EGFP-loaded PECs had low numbers of IFN-γ-producing cells. In contrast, significant anti-YB-1 reactivity was found in T cells plus YB-1-loaded PECs [**Fig. 1(C)**]. These results demonstrated that anti-YB-1 CD8$^+$ T-cell responses were induced by AGN2a immunization.

YB-1 immunization in the context of Treg depletion induced strong cellular immune response against AGN2a cells *in vivo*

To identify if YB-1-specific CD8$^+$ T cells could directly recognize the tumor cells, AGN2a were incubated with CD8$^+$ T cells from mice immunized with EGFP and YB-1, respectively, with or without Treg depletion. As shown in **Fig. 2(A,B)**, Tregs were depleted totally 3 days after anti-CD25 antibody treatment. Significant anti-tumor reactivity was induced by CD8$^+$ T cells from mice immunized with YB-1, especially with YB-1 immunization in the context of Treg depletion [**Fig. 2(C)**]. This indicated that antigen-specific CD8$^+$ T cells did recognize the tumor cells effectively when mice were immunized with YB-1 in the context of Treg depletion.

Protective effect of YB-1 immunization in the context of Treg depletion

To identify the protective effect of YB-1 immunization in the context of Treg depletion, A/J mice were divided into six groups randomly and given different treatments [**Fig. 3(A)**]. Five days after the third immunization, YB-1-specific IgG was found in groups treated with YB-1 immunization [**Fig. 3(B)**]. For all the challenged groups, the tumor size was measured every 3 days. At the beginning, tumors grew slowly and no difference was observed among different groups. Tumors in naïve and EGFP-immunized mice grew faster than those in other groups 20 days later. Mice treated with anti-CD25 antibody alone group have no protective effect in their syngeneic neuroblastoma model. However, mice of YB-1-immunized group treated with anti-CD25 antibody were significantly protected from tumor challenge at both two challenge doses (80 and 40% survival of mice challenged with 1×10^4 and 1×10^5 AGN2a cells, respectively; $P < 0.05$ compared with non-vaccinated and EGFP-vaccinated mice) [**Fig. 3(C,D)**]. It was also found that 40% of mice from YB-1 alone immunized group were survived when challenged with 1×10^4 AGN2a cells but not 1×10^5 AGN2a cells. These data indicated that YB-1 immunization combined with Treg depletion could efficiently prevent tumor growth in the early stage.

Effect of adoptive T-cell therapy

T-cell adoptive therapy was used to further identify the tumor protective effect of YB-1 immunization combined with anti-CD25 antibody treatment. Tumor-bearing A/J mice were given with enriched T cells or spleen cells from YB-1-immunized mice in the context of Treg depletion [**Fig. 4(A)**]. Tumors in experiment groups grew slower than those without adoptive treatment (control). The survival rate of enriched T-cells treatment group and spleen cells treatment group were similar but obviously higher than that of the control group (50% and 60% survival of mice treatment with spleen cells and purified T cells, respectively; $P < 0.05$ as compared with the control group) [**Fig. 4(B)**].

CD4$^+$ and CD8$^+$ T cells were involved in the anti-neuroblastoma responses induced by YB-1 immunization in the context of Treg depletion

To determine which T cells are responsible for the protective anti-tumor immune responses induced by YB-1 immunization combined with anti-CD25 antibody treatment, mice were depleted of CD4$^+$ or CD8$^+$ T cells *in vivo* using mAbs [**Fig. 5(A)**]. More than 96% of CD8$^+$ and 95% of CD4$^+$ T cells were depleted by the mAb treatment [**Fig. 5(B)**]. Approximately 80% of non-antibody-treated mice (control group) survived 1×10^4 live AGN2a cells challenge. Depletion of either CD4$^+$ or CD8$^+$ T cells significantly reduced survival of the vaccinated mice [**Fig. 5(C)**, $P < 0.05$ compared with non-antibody-treated mice], indicating that both T-cell subsets are important for generating the anti-tumor responses.

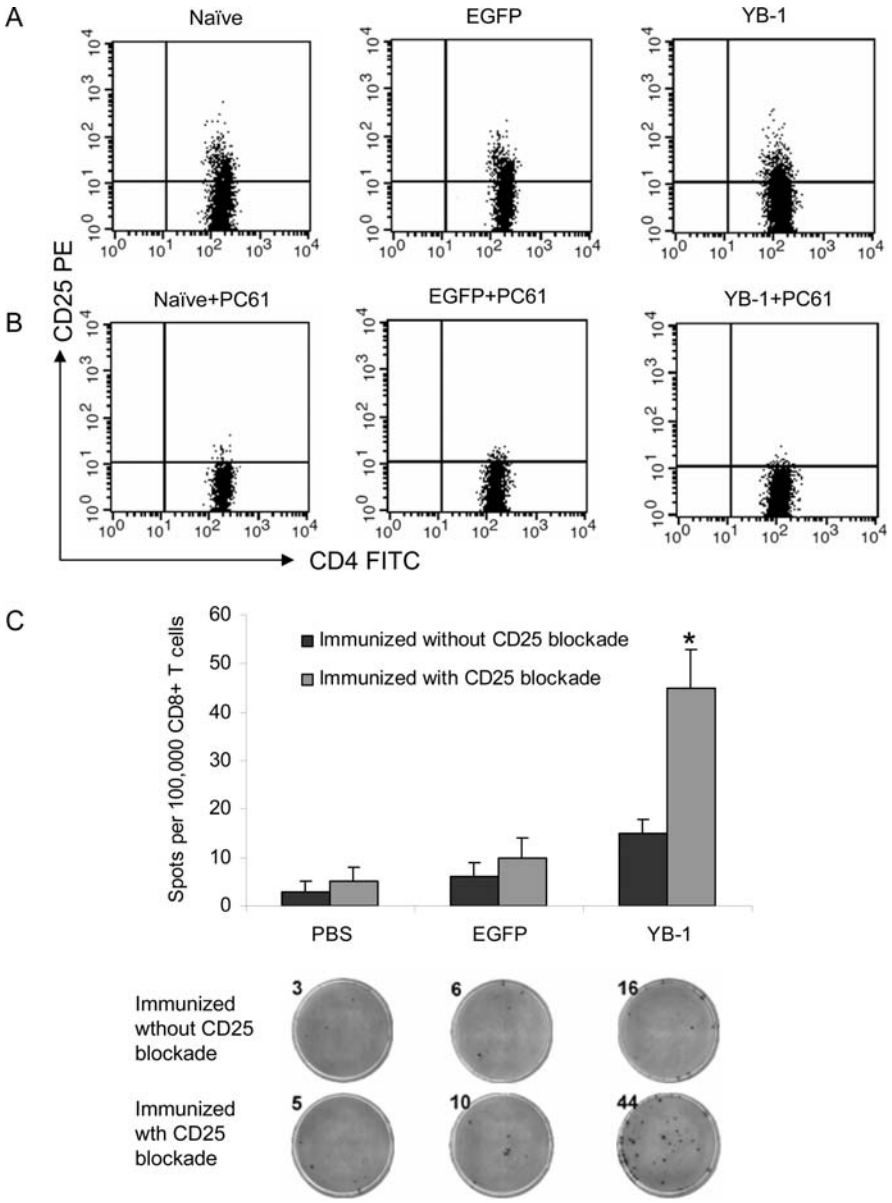

Figure 2 YB-1 immunization in the context of Treg depletion induced strong cellular immune response against AGN2a cells *in vivo* (A,B) Flow cytometric analysis of Tregs in peripheral blood. Tregs were analyzed by flow cytometry 3 days after anti-CD25 antibody treatment. (A) The regulatory T-cell ratio of groups untreated with anti-CD25 antibody. (B) The regulatory T-cell ratio of groups treated with anti-CD25 antibody. (C) About 1×10^4 neuroblastoma cells (AGN2a) were incubated with 5×10^4 $CD8^+$ T cells which from mice immunized with EGFP, YB-1 only, and YB-1 in the context with Treg depletion. Data are representative of three separate experiments. Each experiment consisted of T cells purified and pooled from five mice. Error bars represent the standard deviation calculated from triplicate ELISPOT wells. Values were shown as the mean ± SD ($n = 3$). *$P < 0.05$ vs. YB-1 only group. Representative ELISPOT images showed the spots for one well response.

Discussion

In this report, we tested whether vaccination with the neuroblastoma-associated antigen YB-1 combined with anti-CD25 antibody treatment could generate anti-neuroblastoma immunity. We discovered that potent anti-tumor immunity could be generated after tumor-associated antigen immunization. However, an adoptive transfer of T cells for the vaccination could also be effective to inhibit the tumor growth in the early stage. Both $CD4^+$ and $CD8^+$ T cells were involved in the anti-neuroblastoma responses induced by YB-1 immunization combined with anti-CD25 antibody treatment.

Tumor antigens, such as oncofetal antigens, oncogenes, overexpressed normal molecules, cancer-testis, and so on, are useful in identifying tumor cells and are potential candidates for use in cancer therapy [21–25]. Development of tumor vaccines has followed two major approaches: the use of tumor cells or cell lysates as immunogens or the use of well-characterized tumor antigens. YB-1 protein is a

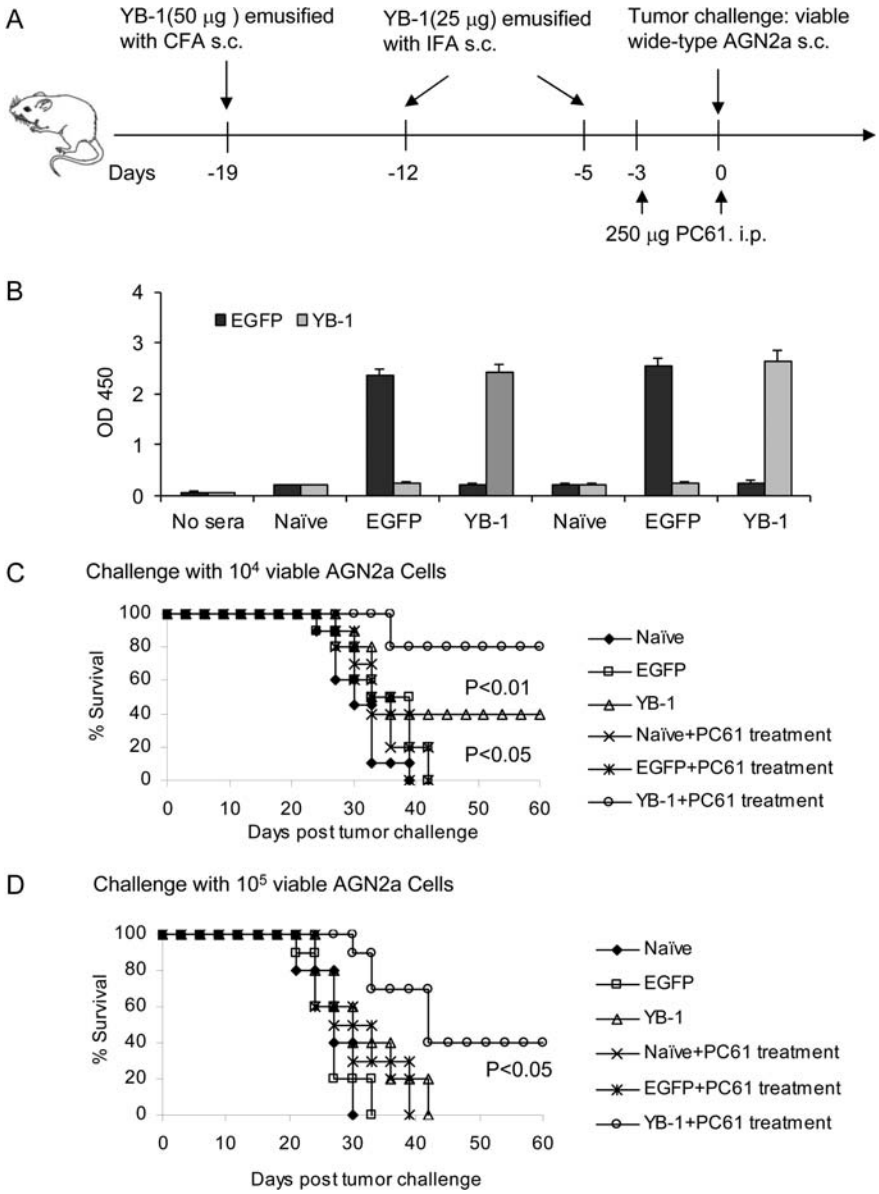

Figure 3 Protective effect of YB-1 immunization in the context of Treg depletion (A) Experiment design. (B) ELISA analysis of sera form immunized mice. Error bars show standard deviations for triplicate sample wells in the same assay. (C,D) YB-1 immunization combined with anti-CD25 antibody treatment significantly increased the survival rate of immunized mice challenged by 1×10^4 ($P < 0.01$) or 1×10^5 ($P < 0.05$) AGN2a cells compared with naïve and EGFP group.

multifunctional cellular protein that expressed in various cancers. YB-1 was found in 94.6% neuroblastoma cases and considered as a potential novel tumor marker for neuroblastoma [26]. YB-1 also triggers the expression of Her-2 and estrogen receptor alpha (ERalpha) in breast cancer [27]. In some cancers, such as nasopharyngeal cancer, YB-1 is a promising predictive marker of radioresistance and chemoradioresistance [28].

In this study, we first identified that anti-YB-1 $CD8^+$ T-cell responses could be induced by AGN2a immunization, and then the strongest $CD8^+$ T-cell responses against AGN2a were induced by YB-1-immunized mice in the context of Treg depletion. Furthermore, we found that YB-1 immunization in the context of Treg depletion could inhibit neuroblastoma (AGN2a) growth. The mechanism of these protective immune responses is based on YB-1 immunization and Treg depletion. YB-1 was one of the neuroblastoma-associated antigen from mice immunized with co-stimulatory molecules engineered AGN2a in the context of Treg depletion. Immune tolerance is a major barrier to effective immunization against tumor, and tumor establishment has been associated with tolerance to tumor antigens [29]. So breaking this barrier is very important for tumor treatment. It was reported that Treg depletion could uncover immune responses to the tumor cell type used as a vaccine, and importantly this response broadens to include

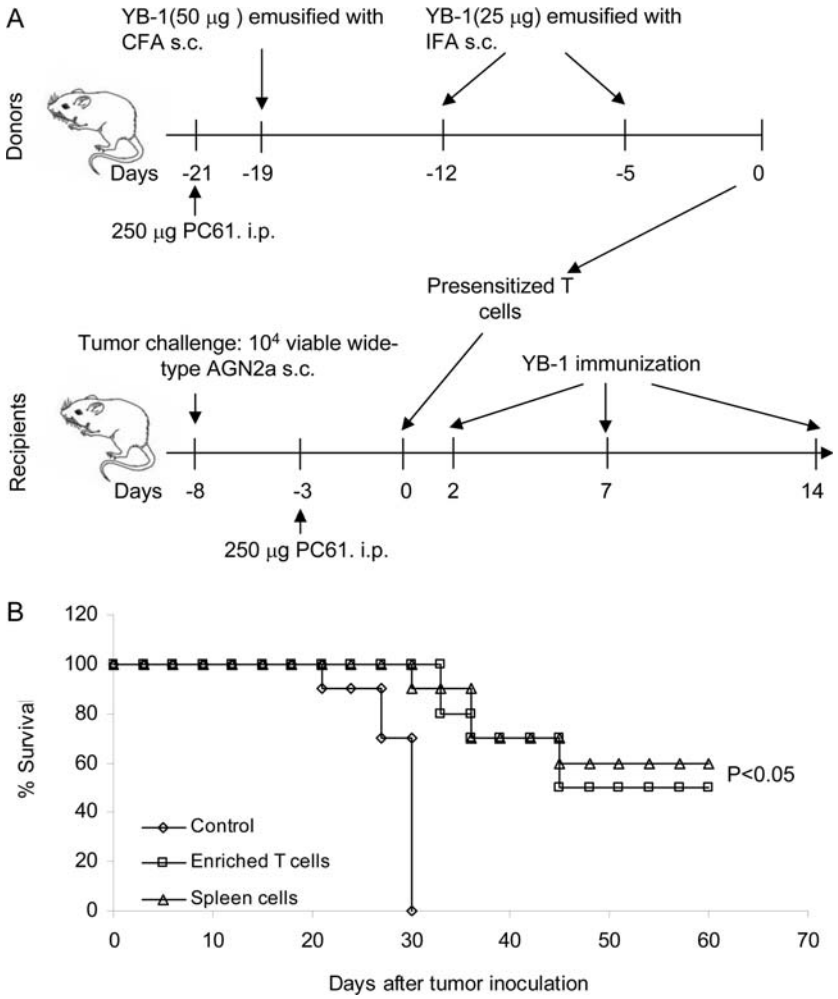

Figure 4 Effect of adoptive T-cell therapy (A) Experiment design. (B) Spleen cells and purified T cells adoptive treatment significantly increased the survival rate of immunized mice (50 and 60% survival of mice treatment with enriched T cells and spleen cells, respectively). $P < 0.05$ compared with control group.

other syngeneic tumor cell types [19]. Our results showed that 80% mice of YB-1 immunization combined anti-CD25 antibody treatment survived the tumor challenge [**Fig. 3(C)**]. These data indicated that YB-1 immunization combined with Treg depletion could prevent tumor growth.

Adoptive cellular therapy has the potential to boost the effectiveness of cancer vaccines [30]. In this report, we found both enriched T cells and spleen cells from YB-1 immunization and anti-CD25 antibody-treated mice could increase the survival rate of recipient mice [**Fig. 4(B)**, 50% and 60% survival of mice treatment with enriched T cells and spleen cells, respectively; $P < 0.05$ compared with control group]. These data further indicated that YB-1 immunization in the context of Treg depletion could inhibit tumor growth in the early stage.

Both $CD4^+$ and $CD8^+$ T cells were involved in the anti-neuroblastoma responses induced by YB-1 immunization combined with anti-CD25 antibody treatment [**Fig. 5(C)**]. This is surprising since the AGN2a tumor cells do not express MHC class II molecules. We speculate that the involvement of $CD4^+$ T cells in the effector response could be either by aiding in the prompt activation of $CD8^+$ cytotoxic effector cells and B cell to mature as antibody producing cell, or by directly serving as effector cells through tumoricidal cytokine production. Mechanisms that have been previously implicated in CD4 anti-tumor effector function include secretion of IFN-γ [31], or activation of macrophages capable of suppressing tumor growth [32].

This study is the primary study of immune therapy for neuroblastoma. Although the YB-1 immunization combined with Treg depletion was not enough to eliminate the tumor cells, it at least delayed the tumor growth. With an improved understanding of mechanisms underlying tumor-induced immune suppression, our vaccines will likely combine approaches designed to restore anti-tumor immune responses, eliminate tumor escape, and correct tumor-induced immune deviation to enable the host's immune system to more effectively deal with the tumor.

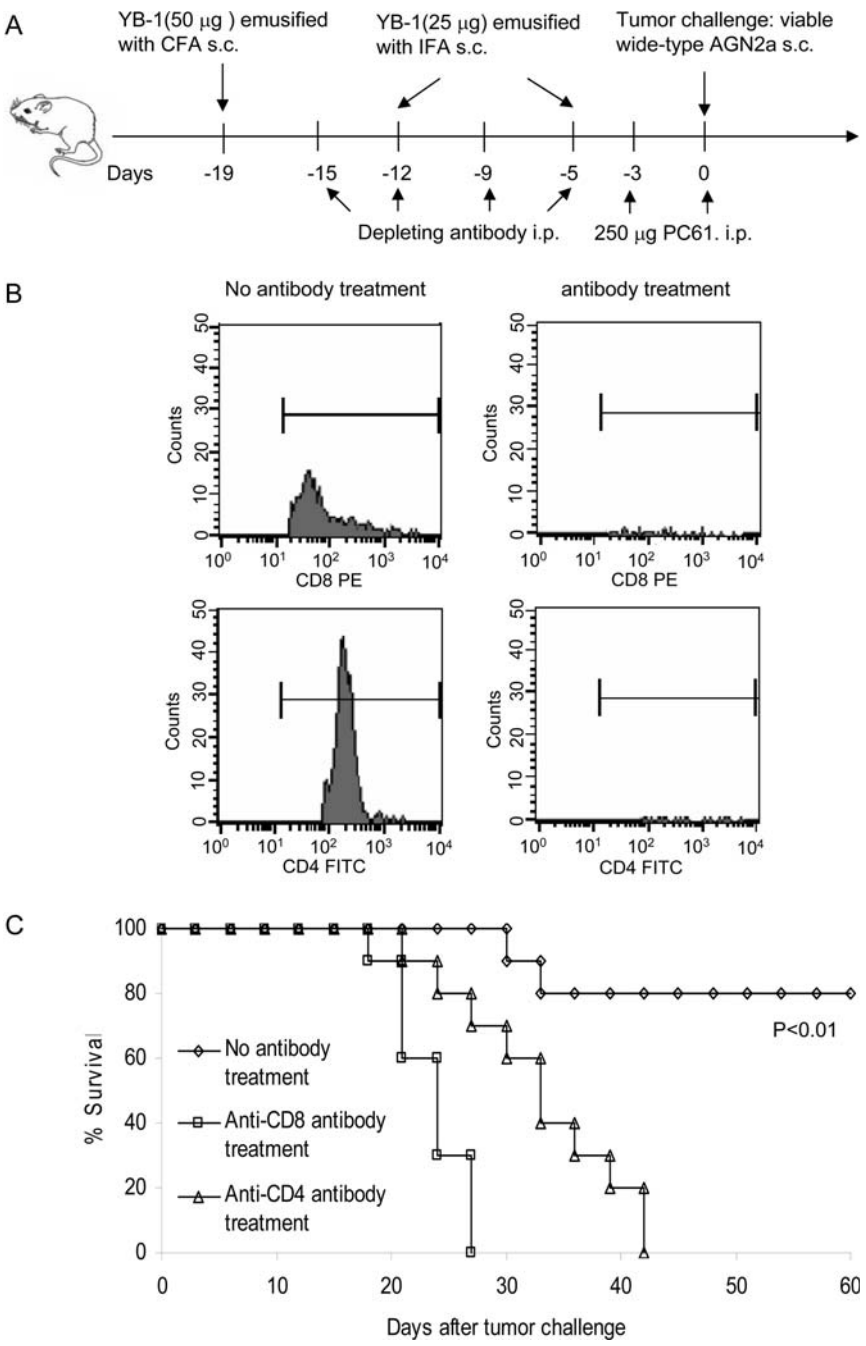

Figure 5 Both CD4$^+$ and CD8$^+$ T cells were involved in the anti-neuroblastoma responses induced by YB-1 immunization combined with anti-CD25 antibody treatment (A) Experiment design. (B) Flow cytometric analysis of CD8$^+$ and CD4$^+$ T cells in peripheral blood. More than 96% of CD8$^+$ and 95% of CD4$^+$ T cells were depleted by the mAb treatment. (C) Anti-CD4 or anti-CD8 mAb treatment significantly reduced the survival of vaccinated mice, $P < 0.01$ for both anti-CD4 and anti-CD8-treated mice compared with the no-antibody treatment group.

In summary, we firstly identified that anti-YB-1 CD8$^+$ T-cell responses could be induced by AGN2a immunization, and then the strongest CD8$^+$ T-cell responses against AGN2a were induced by YB-1-immunized mice in the context of Treg depletion. Furthermore, it was also shown that YB-1 immunization in the context of Treg depletion could prevent the growth of neuroblastoma, and adoptive transfer of T cells for the vaccination could also be effective to inhibit the tumor growth in the early stage. Both CD4$^+$ and CD8$^+$ T cells were involved in the anti-neuroblastoma responses induced by YB-1 immunization combined with anti-CD25 antibody treatment, indicating that both cellular and humoral immune response might play an important role in the suppression of tumor growth. Taken together, these results indicated that neuroblastoma-associated antigens immunization combined with Treg depletion could be a potential strategy for the prevention and treatment of neuroblastoma in the early stage.

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