Humoral immune response induced by an engineered cell-based neuroblastoma vaccine with or without CD25 blockade

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Neuroblastoma is the most common extracranial solid cancer in childhood and it can develop in the nerve tissue of the adrenal gland, neck, chest, or spinal cord. A number of tumor-associated antigens (TAAs), which can elicit humoral immunity, have been identified in cancer patients. To investigate the humoral immunity during neuroblastoma development, we treated A/J mice with an aggressive clone of neuroblastoma (AGN2a) cells, then vaccinated the mice with cells expressing AGN2a-CD80/CD137L under the conditions with or without regulatory T cell blockade. Strong humoral immunity was induced by AGN2a-CD80/CD137L immunization in the context of regulatory T cell blockade. Sera from treated mice were used to screen an AGN2a cDNA expression library for identifying TAAs by SEREX (serological analysis of recombinant cDNA expression libraries). Clones were identified by sequencing and comparative analysis of gene pools. Further investigation of these gene products revealed that most of them play a role in the neuronal differentiation, cell metabolism, and are highly expressed in other types of malignancy. Asz1 (ankyrin repeat, SAM, and basic leucine zipper domain-containing protein) was found in all tumor-bearing groups. These results implicated that these candidates identified from tumor-bearing mice may be neuroblastoma-associated antigens, which can be used as biomarkers in early diagnosis of neuroblastoma, whereas those identified from vaccinated mice may be the potential therapeutic targets.

Keywords neuroblastoma; humoral immunity; tumor bearing; costimulator; Treg blockade

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Introduction

Neuroblastoma is a disease in which malignant cells begin in nerve tissues of the adrenal gland, neck, chest, or spinal cord. It most often begins during early childhood, usually in children younger than 5 years [1,2]. There are different types of treatment for patients with neuroblastoma. The standard treatment includes surgery, radiation therapy, chemotherapy, and biologic therapy. Immune therapy is a treatment that uses the patient’s immune system to fight cancer. The substances prepared from either patient’s body or laboratory are used to boost or restore the body’s natural defenses against cancer. This type of cancer treatment is termed as immunotherapy [3].

The use of tumor cell-based vaccines is an attractive way for the generation of anti-neuroblastoma immunity, which does not increase the toxicity of concurrent radio- or chemotherapy [3,4]. Clinical evidence suggested that the generation of an immune response to neuroblastoma is possible [5]. Recently, we have generated a series of cell-based vaccines. It was demonstrated that the expression of CD80 and CD86 or CD80, CD86, CD54, and CD137L in AGN2a neuroblastoma cells is able to generate a potent protective effect in challenging studies [6,7].

Interestingly, our data showed that the vaccine generated from AGN2a-CD80/CD137L cells induced a better tumor-protective effect than that from either AGN2a-CD80 alone or the AGN2a-CD80/CD86 cells. The protective effect was especially evident when AGN2a-CD80/CD137L cells-immunized mice were challenged with higher doses of viable wild-type tumor [8].

Suppressor/regulatory T cells (Treg) were first shown to have an impact on cancer progression in experimental tumor models during the 1970s [9,10]. Treg is a unique subset of CD4+ T cells expressing CD25 molecule (IL-2Rα chain), which plays a key role in the maintenance of immune tolerance to both self- and harmless foreign antigens [11].

The humoral immune response (HIR) is the aspect of immunity that is mediated by secreted antibodies. In mammals, there are five types of antibodies: IgA, IgD, IgE, IgG, and IgM [12]. IgG antibodies are predominantly
involved in the secondary immune response. The presence of specific IgG generally corresponds to the maturation of the antibody response. There are four IgG subclasses (IgG1, 2, 3, and 4), IgG1 being the most abundant (66%), and then IgG2 (23%) [13]. In addition to their neutralizing properties, antibodies can mediate host effector functions and facilitate the removal of a pathogen from a host. Specifically, the Fc portion of immunoglobulin G2a (IgG2a) antibodies interacts with complement components and Fc receptors at a high affinity [14–16]. This interaction can efficiently activate Fc receptor-mediated effector functions, which include the stimulation of antibody-dependent cell-mediated cytotoxicity and opsonophagocytosis by macrophages [17,18], and the latter has been shown to contribute to the clearance of influenza virus from the infected hosts [19]. The Fc portion of IgG1 antibodies mediates a lower-affinity interaction with Fc receptors and does not stimulate Fc receptor-mediated immune responses effectively [20,21].

In this report, we tried to further explore the HIR and tumor-associated antigen (TAA) induced by an engineered cell-based neuroblastoma vaccine, which co-expressed CD80 and CD137L (AGN2a-CD80/137L) with or without CD25 blockade in neuroblastoma-bearing mouse. Our findings may provide new biomarkers for the early diagnosis of neuroblastoma and identify therapeutic targets for clinical application in neuroblastoma treatment.

Materials and Methods

Mice and tumor cell lines
A/J mice, 6–8 weeks old, were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Mice were housed in the Medical College of Wisconsin Biomedical Resource Center (AALAC accredited) and all protocols were approved by the MCW Institutional Animal Care and Use Committee. An invasive clone of Neuro2a (AGN2a) was derived from succession in vivo passage, and AGN2a transfectants that permanently expressed CD80 and CD137L (AGN2a-CD80/137L) had been previously described [7].

Group design and treatment
A/J mice were divided into eight groups. Each group has nine mice. Mice from tumor-bearing group (T), tumor-bearing plus vaccine group (T + V), tumor-bearing with Pc61-treating group (T + P) and tumor-bearing plus vaccine with Pc61-treating group (T + V + P) were inoculated with $1 \times 10^6$ viable AGN2a cells on Day 0. For blockade/depletion of Treg cells, mice from Pc61-treated group (P), Group T + P, vaccine with Pc61-treating group (V + P) and Group T + V + P received 250 μg of bioreactor-generated (Integra Biosciences, Chur, Switzerland) anti-CD25 monoclonal antibody (mAb), clone Pc61, by intraperitoneal injection 3 days prior to the first vaccination. On Days 7 and 14, mice from the vaccine group (V), Group T + V, Group V + P, and Group T + V + P were immunized with $2 \times 10^8$ irradiated AGN2a CD80/137L (5000 Rad). Blood was collected on Day 19, incubated at 37°C for 30 min, centrifuged at 800 g for 10 min, and then stored at $-80°C$.

Flow cytometry analyses
AGN2a cells were harvested and washed twice with PBS. Non-specific antibody binding was blocked by incubation with 10% heat-inactivated human serum in PBS and 100 μg/ml anti-FcγRIIIa/IIb mAb (Fc block, clone 2.4G2, BD Biosciences, San Diego, USA) prior to incubation with sera from each group at a 1:100 dilution. Then the cells from each group were divided into three samples, each sample has $1 \times 10^5$ cells. For detecting the IgG level, cells were incubated with biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, USA), then stained with SAv-PE (streptavidin–phycoerythrin, BD PharmingenTM, Franklin Lakes, USA). For detecting the IgG1 and IgG2a level, cells were incubated with R-PE (R-phycoerythrin)-conjugated rat anti-mouse IgG, Fcγ Subclass 2a-specific antibody (Jackson ImmunoResearch Laboratories, Inc.), respectively. Purified Mouse IgG1 k (BD PharmingenTM, clone MOPC-31C) and IgG2a k (BD PharmingenTM, clone MOPC-173) isotype control antibodies were used as control. The antibody-stained cells were analyzed using a Becton Dickinson FACScalibur flow cytometer (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Inc., San Carlos, USA).

Western blot analyses
AGN2a cells were washed twice with PBS, resuspended in PBS at $5 \times 10^6$/ml, and diluted in three volumes 4 × sample buffer (NuPAGE LDS Sample Buffer, Invitrogen, Carlsbad, USA). Boiled AGN2a cell lysates were loaded to each well ($1 \times 10^5$ cell/well). After resolution of proteins by SDS-PAGE (12%, NuPAGE gel system, Invitrogen), proteins were transferred to PVDF membrane (Invitrogen, 0.45 μm) using a Bis-Tris electrophoresis buffer system (Invitrogen). The membrane were blocked by incubation for 1 h at room temperature in blocking buffer (5% of non-fat dry milk and 3% of BSA in Tris-buffered saline, pH 7.5), rinsed and cut into strips, and then incubated in sera from each group. The sera were diluted 1:100 in antibody dilution buffer (1% BSA in Tris-buffered saline, pH 7.5). Bound antibodies were detected with biotin-conjugated goat anti-mouse IgG diluted 1: 2500 in antibody dilution buffer. The membrane were washed, then...
incubated with AP labeled-ExtraAvidin (extravidin-alkaline phosphatase, Sigma, St. Louis, USA) and detected by staining with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (NBT/BCIP, picoBLUE Immunoscreening Kit, Stratagene, La Jolla, USA).

**Immunoscreening of the AGN2a cDNA library**

Proteins encoded by the cDNA expression library established before [22] were probed with sera from each group (pooled from nine mice). Recombinant phages at a concentration of 50,000 pfu per plate (150 mm²) were amplified for 4 h at 42°C until plaques were visible and then transferred to nitrocellulose membranes (Millipore, Billerica, USA) pre-wetted with 10 mM IPTG (Invitrogen) for an additional 3.5 h at 37°C. Membranes were then washed three times with TBST (20 mM Tris–HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5), blocked with 1% bovine serum albumin (BSA, Sigma A3803) in TBS, and then incubated with a 1:100 dilution of immune serum, which had been pre-adsorbed with *Escherichia coli* phage lysate following the manufacturer's protocol (Stratagene). Bound antibody was detected with biotin-conjugated goat anti-mouse IgG (as above) diluted 1:2500 in antibody dilution buffer, then incubated with AP-labeled-ExtraAvidin and visualized by staining with NBT/BCIP. Positive clones were subcloned and re-screened as above.

**In vivo excision** was carried out with plaques that proved positive upon secondary screening in order to generate the pBK-CMV phagemid containing the cloned insert. Phagemid was isolated with QIAprep columns (Qiagen, Hilden, Germany) and the size of the cDNA insert was analyzed by XhoI/EcoRI restriction digest. Inserts were sequenced using T7 and T3 primers by automatic DNA sequencing.

**Re-screening the unique antigen by individual serum**

The titer of recombinant phage containing the identified antigens was examined and then mixed with control phage (no cDNA insert) at a ratio of 1:25, the mixture were amplified at a concentration of 20,000 pfu per plate (150 mm²) for 4 h at 42°C until plaques were visible and then transferred to nitrocellulose membranes (Millipore) pre-wetted with 10 mM IPTG (Invitrogen) for an additional 3.5 h at 37°C. Membranes were then washed three times with TBST, blocked with 1% BSA in TBS, cut into nine squares, and each square was incubated with a 1:100 dilution of individual immune serum from which the antigen was found. The serum had been pre-adsorbed with *E. coli* phage lysate following the manufacturer's protocol (Stratagene). The membranes containing the control phage proteins were used as control. The subsequent procedures were the same as western blotting.

**Results**

**CD25 blockade and cell-based vaccine could inhibit tumor growth**

A/J mice were divided into eight groups randomly and given different treatment as shown in Fig. 1(A). For all the tumor-bearing groups, the tumor size was measured every 3 days. In the beginning, tumors grew slowly and no difference was observed between each group. Tumors on the mice in tumor-bearing group (T) grew up faster than those in other groups after Day 9 and the tumor size reached to 200 mm² on Day 15. On Day 12, the size of tumors on mice treated with the vaccine (T + V), Pc61 (T + P), and the vaccine plus Pc61 (T + V + P), was significantly smaller than those in Group T. Although there was no significant difference in tumor size between Groups T + V, T + P, and T + V + P, the tumors on mice in Group T + V + P were relatively smaller [Fig. 1(B)].

**Humoral responses of immunized mice**

Flow cytometry was used to investigate the HIR in tumor-bearing mice given a tumor cell-based vaccine with or without CD25 blockade. As shown in Fig. 2(A), the IgG level of sera from both Group V + P and Group T + V + P was the highest compared with the other groups.

![Figure 1](https://academic.oup.com/abbs/article-abstract/43/2/124/994) Figure 1 Tumor growth was inhibited by CD25 blockade and cell-based vaccine (A) Group design and treatment. (B) Tumor growth was inhibited by CD25 blockade and cell-based vaccine. n = 9, *P < 0.05 vs. Group T + V, Group T + P, and Group T + V + P. Data are expressed as mean ± SEM.
However, the sera from Group V ÷P has the highest level of IgG2a. To explore the effect of each treatment on immune response, the levels of IgG, IgG1, and IgG2a in the sera of these groups were compared by different combination. As shown in Fig. 3(A1,B1,C1), CD25 blockade could increase the IgG level but could not increase IgG1 and IgG2a levels. The IgG level of Pc61 treatment group was almost equal to that in the vaccine group [Fig. 3(A2)]. All of the IgG, IgG1, and IgG2a levels were increased after Pc61 treatment followed by vaccination with engineered tumor cells [Fig. 3(A2,B2,C2)]. Furthermore, the IgG, IgG1, and IgG2a levels were also increased in tumor-bearing mouse with Pc61 treatment and vaccine (Group T ÷V ÷P) [Fig. 3(A3,B3,C3)]. These results indicated that CD25 blockade and cell-based vaccine have a synergistic effect on immune responses and can reverse the inhibiting effect on immune system, which is induced by tumor growth.

Neuroblastoma-associated antigens identified by immune serum and SEREX

Antibody reactive plaques identified by serum from individual groups were selected and used to re-infect E. coli, and screened once more serologically. Each phagemid insert DNA was sequenced, verified, and confirmed in online databases (NCBI Genbank). As listed in Table 1, the genes that were identified by immune serum-SEREX screening encoded different proteins. But As1 was found in all tumor-bearing groups. Primers were generated for each identified gene. The expression of gene was verified by PCR analysis with AGN2a-derived cDNA (data not shown). Further analysis demonstrated that most transcripts have roles in neuronal differentiation and cell cycle control, or act as transcripts that are over-expressed in other malignancies.

Positive frequency of neuroblastoma-associated antigens

To investigate the positive frequency of neuroblastoma-associated antigens we found, the recombinant phage containing the identified antigens were mixed with the control phage (no cDNA insert). Proteins of the bacteria, which were lysed by phage, were transferred to nitrocellulose membrane. Membranes that contained the found antigens were incubated with the serum of individual mouse that belonged to the group from which the antigen was found. Membranes that contained only the control phage proteins were used as control and incubated with mixed sera from the same group. Here we only show the control, one of the negative responses and one of the positive responses (Fig. 4). As shown in Table 2, the positive frequency of neuroblastoma-associated antigens is more than 68%. It reflected that the found antigens were widely over expressed in the AGN2a-immunized mouse.

Western blot analyses

Western blot results showed that the sera from the tumor-bearing group, vaccine group or vaccine with CD25 blockade group had a strong immune response against tumor cell lysates, except the Pc61-treated group. Each reaction was different. Some protein bands of individual group were in the same size, some were not [Fig. 5(A)]. We also analyzed the positive and negative sera of immunized mice with AGN2a cell lysates. As shown in Fig. 5(B), more positive bands appeared on the positive sera-incubated membrane.

Discussion

Aberrant levels of mutated or modified forms of proteins that are associated with malignant growth can express in tumors. Such proteins can be immunogenic and stimulate humoral and cellular immune responses [23]. A number of TAAs that elicit humoral immunity have been identified in cancer patients [24–26]. Serum antibodies are stable, and
can be readily detected with well-validated secondary antibodies [27].

Furthermore, B cells can produce specific antibodies in large amounts after stimulation by a small amount of tumor antigens [28]. As a result, TAA-specific serum antibodies can be detected at a high titer in patients with early stages of cancer [10].

CD80, also known as B7.1, is a molecule which is found in activated B cells and monocytes and provides a co-stimulatory signal necessary for T cell activation and survival [29]. The principal mode of CD80 action is its binding to CD28. Along with CD86, these molecules provide the necessary stimuli for prime T cells against antigens presented by antigen-presenting cells [30]. CD80 and CD86 also bind to CTLA-4, a cell surface molecule expressed on activated T cells. The interactions between CD80 or CD86 and CTLA-4 decrease the response of T cells [31]. CD137L (4-1BBL, TNFSF9), the receptor for CD137 (4-1BB), is a member of the TNF superfamily and also serves as a secondary signal to activated T cells. CD137L is expressed almost exclusively on T cells. T cell receptor will be stimulated after CD137L interacts with CD137, leading to T cell proliferation and cytokine production [32]. CD137 ligation also promotes the T cell survival and inhibits the apoptosis via the induction of the anti-apoptotic molecule Bcl-XL [33,34]. Although CD28/B7 interactions play a key role in the early phases of antigen recognition, 4-1BBL stimulation might be implicated in the later stage of the primary immune response and during antigen re-exposure [35]. Moreover, a report by Melero et al. [36] highlighted the importance of 4-1BB/4-1BBL interactions in anti-tumor immunity by showing that the administration of agonistic anti-4-1BB to the mouse resulted in dramatic tumor regressions, even in weakly immunogenic tumors.

Regulatory T cells are a specialized subpopulation of T cells that express CD4, CD25, and Foxp3, which can suppress the activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens [11]. The tolerance of host against self-antigens can be abolished by Treg depletion, which may be functionally analogous to the anti-tumor effect seen in allogeneic bone marrow transplantation, whose primary side-effect (graft vs. host disease) is evident so that tolerance to normal self-antigens has been modified [37]. In previous studies, we found that CD25+ regulatory T cell
inhibition could enhance the vaccine-induced immunity to neuroblastoma [38]. We also found that AGN2a-CD80/137L was an efficient vaccine which could induce the strong immune responses in live tumour vaccine challenge experiments, by which live tumor cell vaccines did not only fail to grow in mice, but also result in robust immunity [8]. In this study, in order to explore the HIR and TAA induced by AGN2a-CD80/137L with or without CD25 blockade in the context of tumor bearing, the animals were given $1 \times 10^6$ live tumor cells, followed by vaccination with AGN2a-CD80/137L with or without CD25 blockade. Our data showed that both AGN2a-CD80/137L vaccination and CD25 blockade could inhibit the tumor growth, and the best effect was found when binding these two ways together [Fig. 1(B)]. Because the inoculated tumor cell density is a bit high ($1 \times 10^6$/mouse), the significant differences were only found among tumor-bearing group (T) and other groups including tumor-bearing plus vaccine group (T+V), tumor-bearing with Pc61 treatment group (T+P) and tumor-bearing plus

Table 1 Neuroblastoma antigens identified by immune serum and SEREX

<table>
<thead>
<tr>
<th>Groups</th>
<th>Identity of cDNA SEREX hit/times</th>
<th>Accession number</th>
<th>Identity and description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor-bearing group</td>
<td>Asz1</td>
<td>NM_023729</td>
<td><em>Mus musculus</em> ankyrin repeat, SAM, and basic leucine zipper domain-containing 1 [41,42]</td>
</tr>
<tr>
<td></td>
<td>Ppp2ca</td>
<td>NM_019411</td>
<td><em>Mus musculus</em> protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform [43]</td>
</tr>
<tr>
<td>Vaccine group</td>
<td>Golga4</td>
<td>NM_018748</td>
<td><em>Mus musculus</em> golgi autoantigen, golgin subfamily a, 4, was found from Sjogren syndrome [44]</td>
</tr>
<tr>
<td></td>
<td>Ncl</td>
<td>NM_010880</td>
<td><em>Mus musculus</em> nucleolin, also known as C23, is an abundantly expressed acidic phosphoprotein of exponentially growing cells and is located mainly in dense fibrillar regions of the nucleolus. It is involved in the control of transcription of ribosomal RNA genes by RNA polymerase I, in ribosome maturation and assembly, and in nucleocytoplasmic transportation of ribosomal components [45]</td>
</tr>
<tr>
<td>Tumor-bearing plus vaccine group</td>
<td>Asz1</td>
<td>NM_023729</td>
<td><em>Mus musculus</em> ankyrin repeat, SAM and basic leucine zipper domain containing 1 [41,42]</td>
</tr>
<tr>
<td></td>
<td>Ftl1</td>
<td>NM_010240</td>
<td><em>Mus musculus</em> ferritin light chain 1 [46]</td>
</tr>
<tr>
<td></td>
<td>Cbr4</td>
<td>NM_145595</td>
<td><em>Mus musculus</em> carbonyl reductase 4. Carbonyl reductase is one of the several monomeric, NADPH-dependent oxidoreductases having wide specificity for carbonyl compounds that are generally referred to as aldo-keto reductases. Others include aldehyde reductase and aldose reductase [47]</td>
</tr>
<tr>
<td>Tumor bearing with Pc61-treated group</td>
<td>DEK</td>
<td>NM_025900.1</td>
<td><em>Mus musculus</em> DEK, first identified in a fusion with the CAN nucleoprotein in a specific subtype of acute myelogenous leukemia, DEK has also been shown to be an autoantigen in patients with pauciarticular onset juvenile rheumatoid arthritis [48]</td>
</tr>
<tr>
<td></td>
<td>Snx4</td>
<td>NM_080557</td>
<td><em>Mus musculus</em> sorting nexin 4 [49]</td>
</tr>
<tr>
<td></td>
<td>Asz1</td>
<td>NM_023729</td>
<td><em>Mus musculus</em> ankyrin repeat, SAM and basic leucine zipper domain-containing 1 [41,42]</td>
</tr>
<tr>
<td>Vaccine with Pc61 treated group</td>
<td>Mest</td>
<td>NM_008590.1</td>
<td><em>Mus musculus</em> mesoderm specific transcript mRNA [50]</td>
</tr>
<tr>
<td></td>
<td>Snx9</td>
<td>XM_984215</td>
<td><em>Mus musculus</em> sorting nexin 9 [51]</td>
</tr>
<tr>
<td></td>
<td>Prkar1a</td>
<td>NM_021880</td>
<td><em>Mus musculus</em> protein kinase, cAMP-dependent regulatory, Type I, alpha [52]</td>
</tr>
<tr>
<td>Tumor-bearing plus vaccine with Pc61 treated group</td>
<td>Asz1</td>
<td>NM_023729</td>
<td><em>Mus musculus</em> ankyrin repeat, SAM and basic leucine zipper domain-containing 1 [41,42]</td>
</tr>
<tr>
<td></td>
<td>Golph3l</td>
<td>NM_146133</td>
<td><em>Mus musculus</em> golgi phosphoprotein 3-like [53]</td>
</tr>
</tbody>
</table>

All information are derived from publicly available databases including Entrez Gene, National Center for Biotechnology Information, National Library of Medicine, and US National Institutes of Health.
It has been reported that rodents typically respond to vaccines and subunit vaccines with a Th2-type immune response that is associated with the stimulation of IgG1 antibodies, and with a Th1-type immune response that is associated with the stimulation of IgG2a antibodies [39]. Stimulation of IgG2a antibodies has been associated with increased efficacy of vaccination. Additionally, monoclonal antibodies of the IgG2a isotype are more efficient in clearing virus infections than the IgG1 isotype displaying similar antigenic specificities [40]. In this report, we found that vaccination with AGN2a-CD80/137L or Pc61 treatment alone could not efficiently increase IgG1 and IgG2a. However, a strong immune response was induced when these two ways were combined. This result was identical with the tumor growth data as shown in Fig. 1(B).

Using SEREX (the serological analysis of recombinant cDNA expression libraries) techniques, we also identified some neuroblastoma-associated antigens that play a role in the neuronal differentiation and cell cycle control and are overexpressed in other malignancies. Most importantly, Asz1 is found in all tumors-bearing mice. Asz1 is also named as C7orf7, CT1.19, GASZ (germ cell-specific ankyrin, SAM and basic leucine zipper domain-containing protein), MGC26634, and Orf3. GASZ is essential for male meiosis and suppression of retrotransposon expression in the male germline. It is composed of 475 amino acids and is a germ cell-specific protein with four Ankyrin repeats, a sterile α motif, and a basic leucine zipper domain that is conserved across vertebrate evolution in amphibians, fish, birds, and mammals [41,42]. In this report, Asz1 is found only in all tumor-bearing groups. We suppose that it may be a biomarker in early stages of neuroblastoma, for the antigens that are identified from the vaccine group may have potential applications in tumor treatment.

Western blot data showed that both the sera from the tumor-bearing group or vaccine group with/without Pc61 treatment have immune responses to tumor cells. More bands were found when animals were treated with the vaccine or Pc61. These results further confirmed the increased level of IgG1 and IgG2a in groups treated with vaccine and Pc61.

Not all the immunized sera contained the TAAs. As shown in Table 2, the positive frequencies of found antigens were over 68%, suggesting that the antigens found from different groups were wildly overexpressed in AGN2a-immunized mice. On the other hand, it suggested...
that the strong anti-tumor immune responses were not induced by vaccine and CD25 blockade in all animals. This phenomenon was also reflected in western blot. As shown in Fig. 5(B), more positive bands appeared on the membrane that was incubated with positive sera. This phenomenon exactly demonstrated the individual differences in the same species.

In conclusion, by the A/J mouse tumor-bearing model treated with cell-based AGN2a-CD80/CD137L and T regulatory cell blockade, a very strong humoral immunity was induced. Some neuroblastoma-associated antigens were exposed by SEREX screening. Asz1 that was detected in all tumor-bearing groups may be a biomarker in early stages of neuroblastoma. The antigen found in the vaccine group may have potential applications in tumor treatment.

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**References**

Immune response induced by a neuroblastoma vaccine


50 Kaneko-Ishino T, Kuroiwa Y, Miyoshi N, Kohda T, Suzuki R, Yokoyama J and Ito D, et al. Monoclonal antibodies against the 4–1BB T-cell acti-

