T cells targeting a neuronal paraneoplastic antigen mediate tumor rejection and trigger CNS autoimmunity with humoral activation

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Abstract

Paraneoplastic neurologic diseases (PND) involving immune responses directed toward intracellular antigens are poorly understood. Here, we examine immunity to the PND antigen Nova2, which is expressed exclusively in central nervous system (CNS) neurons. In our model, CNS antigen expression limits antigen specific CD4+ and CD8+ T cell expansion. Chimera experiments demonstrate that this tolerance is mediated by antigen expression in non-hematopoietic cells. CNS antigen expression does not limit tumor rejection by adoptively transferred transgenic T cells but does limit the generation of a memory population that can be expanded upon secondary challenge in vivo. Despite mediating tumor rejection, adoptively transferred transgenic T cells do not lead to paraneoplastic neuronal targeting. Preliminary experiments suggest an additional requirement for humoral activation to induce CNS autoimmunity. These data provide rationale for B cell targeting therapy, such as rituximab, to treat PND.

Keywords

Paraneoplastic Neurologic Disease; autoimmunity; cellular immunity; humoral immunity; cancer

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Introduction

Paraneoplastic neurologic diseases (PND) are unusual but highly informative disorders for understanding both tumor immunity and autoimmune neurologic conditions. PND occur in cancer patients who develop immune responses to onconeural antigens expressed uniquely by tumor and neurons [1]. When the target antigen is intracellular, PND is characterized by severe and progressive neurologic degeneration. Though they are diagnosed by high titer autoantibodies, the pathogenesis of disease has been attributed to antigen specific CTL [2], as there is no known capacity for antibodies to access intracellular neuronal antigens. While the autoimmune brain disease is devastating, patients benefit from naturally occurring tumor immunity with improved responses to tumor treatment [3] or, in rare cases, complete eradication of tumor without tumor treatment [4, 5].

Since autoantibodies are the hallmark of and part of the diagnostic criteria for PND, initial attempts to generate animal models tested passive transfer of autoantibodies [6]. However, even direct injection of patient serum into mouse brain parenchyma does not cause neuronal death [6], nor does endogenous generation of anti-Purkinje cell antibodies [7]. We had instead hypothesized that antigen-specific killer T cells are primarily responsible for neuronal targeting [2, 8], and this was supported by demonstrating antigen-specific CTL in the blood and cerebrospinal fluid (CSF) [2, 8, 9] of patients with several different PND in which intracellular antigens were targeted. However others report that mice harboring functional cytotoxic Purkinje cell specific T cells [10] or with transferred Purkinje antigen activated monocytes [11] did not develop CNS disease. Consistent with these findings, increasing evidence suggests that the immune system has potent default mechanisms that confer tolerance to onconeural [12, 13] and other brain antigens [14].

To better understand how tolerance to neuronal antigen is broken in PND, we established a transgenic mouse, N2-LacZ, which expresses the intracellular model antigen beta-galactosidase (b-gal) under the control of the CNS neuron-restricted Nova2 promoter. Nova2 is an intracellular RNA-binding protein expressed throughout the CNS, including the forebrain, midbrain, hindbrain and ventral spinal cord, but not peripheral organs [15]. Patients with paraneoplastic opsoclonus myoclonus (POMA) harbor high titer antibodies (>1:1000) to Nova1 and/or Nova2 expressed in their neurons and tumors (breast, fallopian tube, bladder or small cell lung cancer) [16]. POMA demonstrates that tolerance can be broken to Nova2 in humans [15–17]. Using b-gal as a model neuronal antigen offered a multitude of reagents including well defined high and low avidity epitopes, transgenic CD4+ and CD8+ T cells, tetramers, monoclonal antibodies and a tumor cell line expressing the antigen. We hypothesized that activation of immune responses in the periphery could break CNS tolerance. We tested this hypothesis by stimulating b-gal specific humoral and cellular immunity in N2-LacZ and WT hosts and discovered a previously unknown synergy between these adaptive immune components in triggering neuronal autoimmunity.
Results

Limited clinical and immunologic responses to peripheral immunization against a model PND antigen

N2-LacZ mice, which selectively express b-gal in CNS neurons, were generated from crosses between Nova2-Cre[18] with chicken β-actin-LacZ mice[19] (Fig. 1A). F1 progeny, N2-LacZ, robustly express b-gal protein and mRNA in the brain (Fig. 1B and 1C). Despite low levels of mRNA detected in other cell types, there was no evidence of b-gal protein in any organ tested outside of the brain by immunohistochemistry or colorimetric assay (Fig. 1D and data not shown). Furthermore, the immunologic impact of any potential expression of b-gal by DCs, which had the largest amount of mRNA detected by qPCR after the brain, was ruled out in chimera experiments (Fig. 4D). To explore tolerance to b-gal in this model, we first immunized mice harboring LacZ expressing tumors with b-gal emulsified in Complete Freunds Adjuvant (CFA). 21 days later, an established time for generation of antibody responses, b-gal IgG could be detected in both N2-LacZ hosts and non-b-gal expressing littermates (Fig. 2A). Despite high titer autoantibodies, N2-LacZ mice exhibited no evidence of neurologic dysfunction (such as ataxia, hunched posturing or death for one year of follow up) or tumor rejection (n=5 mice per group in two experiments; data not shown). We conclude that high titer antibodies are not sufficient to generate autoimmune targeting of intracellular neuronal antigen or tumor rejection.

We next immunized mice with recombinant adenovirus expressing b-gal (AdV-b-gal), a well-established method for activating peak CD4+ T cell responses 13 days later, but not humoral immune responses. Neither host developed IgG antibodies to b-gal after this immunization (data not shown). To test CD4 T cell responses, we first confirmed that b-gal p726 peptide is the immunodominant epitope and is naturally processed and presented (Supporting information Fig. 1A and 1B) [20]. Immunization with AdV-b-gal resulted in significantly fewer IFNγ producing CD4+ T cell responses in N2-LacZ hosts compared to littermate controls (Figure 2B). Cytokine bead array of culture supernatants did not detect appreciable levels of IL-17, IL-4, IL-2, IL-10 (Supporting information Fig. 2) indicating no skewing to another T cell helper phenotype. Taken together, these data demonstrate that N2-LacZ mice CD4+ T cells are tolerized to the immunodominant b-gal epitope.

N2-LacZ and littermate control mice were immunized with AdV-b-gal. Fewer CD8+ T cells specific to MHCI immunodominant b-gal epitopes p96 [21] and p497 [22] were detected in N2-LacZ mice after immunization. The most pronounced reproducible difference between the genotypes was seen on day 22 (Fig. 2C and 2D). N2-LacZ CD8+ T cells produced IFNγ in response to b-gal endogenously processed and presented in E22 cells. Although they responded to b-gal p497 pulsed target cells, they did not secrete IFNγ in response to b-gal p96 pulsed target cells at any peptide concentration (Fig. 2E and data not shown). Avidity of b-gal p497-specific T cells from N2-LacZ hosts was identical to littermate controls (Fig. 2F). Among littermate controls, the avidity of b-gal p96 CD8+ T cells was 500-fold stronger than those specific to b-gal p497 (Fig. 2F), likely explaining the increased tolerance to b-gal p96, the higher avidity epitope [23, 24]. Thus, N2-LacZ mice display markedly fewer circulating b-gal specific CD8+ T cells after AdV-b-gal primary immune challenge that
secrete IFNγ ex vivo in response to the lower-avidity epitope but are tolerized to the high-avidity epitope.

**N2-LacZ mice produce partial responses to b-gal in vivo**

We next assessed the ability of b-gal stimulated T cells to lyse target cells in vivo. While AdV-b-gal immunized littermate controls lysed peptide pulsed CFSE-labeled target cells, N2-LacZ hosts had fewer lysed b-gal p497 pulsed targets and no lysis of b-gal p96 pulsed targets (Fig. 3A), consistent with the tolerance to b-gal p96 seen in the ex vivo assays (Fig. 2C–E). Taken together, these data demonstrate that despite partial (p497) or complete (p96) tolerance to b-gal (Fig. 2E), N2-LacZ mice can mount lytic T cell responses to a neuronal antigen.

Considering N2-LacZ host T cells could lyse b-gal p497 antigen-specific targets (Fig. 3A), we tested whether these mice mount significant responses to tumor expressing b-gal antigen (WP4 cells). Interestingly, despite evidence for tolerance to b-gal, partial tumor rejection was achieved in N2-LacZ mice immunized with AdV-b-gal, although less than in littermate controls (Fig. 3B). Combined with ex vivo data in Figures 2C–E, this in vivo result suggests that low avidity b-gal p497 CD8+ T cell responses contribute to productive tumor immunity. However, b-gal mediated tumor immunity did not lead to evidence of neurologic dysfunction despite b-gal expression in the CNS (mice were examined for ataxia, paralysis and seizure; n=8 mice per group, two independent experiments, data not shown).

**T cell tolerance to b-gal in N2-LacZ mice is due to b-gal expression in non-hematopoietic cells**

We inferred that the difference in tumor rejection between N2-LacZ mice and littermate controls was the presence of high avidity T cells in the latter. Given the limited activity of low avidity T cells (Fig. 2C–E), and the partial tumor rejection generated by AdV-b-gal immunization of the endogenous repertoire (Fig. 3B), we attempted to increase the sensitivity of our assay to evaluate tolerance and potentially induce autoimmunity by transferring high avidity transgenic T cells to N2-LacZ mice. BG1 and BG2 mice produce clonal T cells with transgenic T cell receptors specific to b-gal presented in the context of either MHCI (b-gal p96) or MHCII (b-gal p726), respectively. BG1 and BG2 T cells demonstrate the same avidity and lysis of target cells as T cells produced by AdV-b-gal immunization of WT mice (Supporting information Fig. 4A–C).

We used BG1 and BG2 T cells to address the possibility that low levels of b-gal mRNA, detected in Fig 1C, in medullary thymic epithelial cells (mTECs) of N2-LacZ mice could account for tolerance. We generated bone marrow chimeras using BG1 and BG2 bone marrow to reconstitute WT and N2-LacZ recipients. We tested for survival of BG1 and BG2 cells in the mature T cell repertoire by FACS analysis of Vβ7 (marker for BG1 T cells) and Vα11 (marker for BG2 T cells). This analysis indicated similar cell numbers in WT and N2-LacZ recipients (Fig. 4A and 4B). Without evidence of deletional tolerance in the N2-LacZ host, we also assessed whether there were increased circulating T regulatory cells. However, FACS analysis of Vα11+ or Vα11- CD25+/Foxp3+ T regulatory cells in N2-LacZ hosts indicated no such expansion (Fig. 4C). Taken together, these experiments indicate that the
resting peripheral T cell repertoire is ignorant of b-gal expressed in the CNS neurons in the N2-LacZ mouse in the steady state.

To test if a hematopoietic cell is responsible for the observed tolerance after active immunization, we repopulated WT or N2-LacZ hosts with either WT or N2-LacZ bone marrow mixed with BG1 bone marrow, and challenged mice with AdV-b-gal. N2-LacZ bone marrow did not limit expansion of BG1 cells in WT hosts (Fig. 4D), indicating that hematopoietic cells do not confer tolerance to b-gal. As a control, we found that N2-LacZ hosts repopulated with N2-LacZ and BG1 bone marrow were tolerant to the b-gal protein. N2-LacZ hosts repopulated with WT and BG1 bone marrow were also tolerant to antigen challenge, indicating that b-gal expression in a non-hematopoietic cell is sufficient to induce tolerance. In conclusion, in the steady state, prior to antigen challenge, T cells are ignorant to b-gal (Fig. 4A and B). The induction of tolerance is seen upon T cell activation due to the recognition of b-gal antigen expressed in a non-hematopoietic cell (Fig. 4D).

**BG1 and BG2 T cells reject tumor but do not cause autoimmune brain disease**

To test tumor rejection by normally deleted high avidity b-gal T cells in the N2-LacZ host, BG1 and BG2 T cells were transferred into either N2-LacZ or WT hosts and challenged with b-gal expressing WP4 cells (Fig. 5A). In contrast to partial tumor immunity seen in AdV-b-gal primed N2-LacZ hosts (Fig. 3B), transferred BG1 and BG2 cells completely rejected b-gal expressing tumor equally well in both N2-LacZ and WT hosts (Fig. 5B), indicating that despite tolerance during activation of endogenous b-gal T cells, transferred T cells reject tumor in the periphery. Consistent with a prior report [25], transfer of BG1 without BG2 T cells is insufficient for tumor rejection, indicating both CD4+ and CD8+ T cells are required (Fig. 5B). WP4 clearance is b-gal specific because transferred WT CD4+ and CD8+ T cells do not confer tumor rejection (Fig. 5B). WP4 cells lack MHCII (confirmed by FACS), indicating an MHCII expressing professional antigen presenting cell is critical in acquiring and presenting b-gal to CD4+ T cells.

After tumor rejection, similar amounts of tetramer positive b-gal p96 CD8+ T cells were detected in N2-LacZ and WT hosts (Fig. 5C). Although N2-LacZ hosts rejected tumors equivalently to WT hosts, they produced markedly less antibody (Fig. 5D). This result is surprising because there was little humoral tolerance in N2-LacZ mice challenged with CFA b-gal (Fig. 2A). One possible explanation for this discrepancy is that the amount of antigen expressed in a tumor is lower, and more transient (with tumor clearance), than the amount delivered to mice challenged with CFA b-gal. With less antigen, subtle differences between the genotypes might be more apparent. Nevertheless, this result indicates that rejection of b-gal expressing tumor does not require antibody.

While transferred T cells reject tumor in either host, secondary challenge with AdV-b-gal produced less b-gal p96 specific CD8+ T cell expansion in N2-LacZ hosts compared to littermates (Fig. 5E). Analysis of Vβ7+ (b-gal specific) CD8+ T cells demonstrated that memory and effector subsets were reduced by 67% and 75% respectively, while the naïve subset was only reduced by 37% in N2-LacZ hosts compared to WT (Fig. 5F). Despite clear indication of T cell activation as evidenced by tumor rejection, no mouse developed signs of neurologic dysfunction in over one year of follow up. To ensure there was no subclinical
neurologic disease, N2-LacZ and WT mice were submitted for blinded pathologic analysis of brain and spinal cords. No neuronal death was observed in any mouse (n=3 mice per group, Supporting information Fig. 5). Taken together, these data demonstrate that transferred high avidity neuron-specific T cells are sufficient to mediate functional tumor rejection but not autoimmune brain disease. Brain restricted antigen expression prevented the generation of CD8 T cell memory recall responses as well as antibody production.

**T cells may require B cell collaboration to generate neuronal targeting**

The absence of neuronal targeting with the effective rejection of a peripheral tumor by transferred T cells was surprising. Additionally, transfer of activated CD8+ T cells with adjuvants such as Pertussis toxin (PTx) and IL2 did not produce clinical evidence of neurologic dysfunction or detectable T cell infiltration or inflammation in N2-LacZ brains (Supporting information Fig. 6) despite robust tumor rejection (Fig. 5). We also tested whether transfer of T cells activated in a WT host would be more effective in mediating CNS disease. WT AdV-b-gal primed CD8+ T cells were transferred with Rag\(^{-/-}\) BG2 CD4+ T cells into N2-LacZ and littermate control mice. Within one week of transfer and tumor challenge, 1 of 4 N2-LacZ mice developed seizures and died, while no littermate controls died, nor did any mouse that was not given Rag\(^{-/-}\) BG2 CD4+ T cells (Table 1, Experiment A). When this experiment was replicated, 1 of 5 N2-LacZ mice transferred with AdV-b-gal CD8+ T cells died, again requiring co-transfer of Rag\(^{-/-}\) BG2 CD4+ T cells (Table 1, Experiment B). These results demonstrate that both CD4+ and CD8+ T cells were necessary to produce autoimmune disease.

A well-established helper function of CD4+ T cells is to enable B cell activation, and we hypothesized that this was their role in disease induction. We tested this by transferring BG1 and BG2 T cells and challenging mice with CFA-b-gal to induce a strong antibody response to b-gal; this led 1 of 4 N2-LacZ mice to die. No WT mice died after CFA-b-gal challenge nor did any N2-LacZ mouse that received BG1 and BG2 T cells but was not further challenged with CFA-b-gal (Table 1, Experiment C). We repeated Experiment C, which revealed that 1 of 3 BG1/BG2 N2-LacZ chimeras challenged with WP4 cells mounted a high titer (>1:1000) b-gal antibody response (Table 1, Experiment D), and this mouse, but not any other of 3 WT mice with high titer antibodies, became neurologically ill. The mouse was hunched and ataxic, and brain histopathology was characterized by a perivascular and intraparenchymal neutrophilic infiltrate in the middle and lateral thalamic nuclei as well as evidence of recent (TUNEL+) neuronal death hippocampal neurons (Fig. 6A–C). Complete necropsy of all other major organs revealed no significant pathology (data not shown). These pathologic findings indicate specific neuronal targeting. In summary, 4 of 16 N2-LacZ mice that received b-gal specific CD4+ and CD8+ T cells developed neurologic disease. In contrast, none of the 10 N2-LacZ mice that received CD8+ T cells alone developed disease. Together, these four experiments demonstrate that neither B cells, CD4+ nor CD8+ T cells alone are sufficient to produce autoimmune brain disease, however the combination tilts tumor immunity toward autoimmune neuronal targeting, resulting in severe neurologic dysfunction or death in 25% of mice.
Discussion

Priming a robust b-gal antibody response is not sufficient to cause disease in N2-LacZ mice (Fig. 2A), likely because antibodies cannot efficiently access intracellular antigen. Endogenous CD8+ T cell activation, capable of lysing target cells and providing some tumor protection, also fails to cause neurologic degeneration (Fig. 3A and B). Further, transfer of transgenic b-gal T cells mediates complete tumor immunity, but fails to induce CNS disease. Future efforts could take advantage of novel methods to track the fate of such adoptively transferred cells [26]. Therefore, CD8+ T cells targeting intracellular antigen, in conjunction with CD4+ T cell help, are sufficient for tumor immunity but not autoimmune neurologic disease.

Importantly, we provide unexpected evidence to support a more complex hypothesis, that CNS antigen-specific CD8+ T cells work in conjunction with CD4+ T cells and antibodies to induce autoimmune CNS disease. The pathologic findings at necropsy imply specific neuronal targeting and it is possible that a small primary attack by antigen-specific T cells could cause death of neurons followed by secondary recruitment of innate immune cells. It is also possible that neuronal injury could activate microglia to further propagate CD8+ T cell responses [27]. This model provides insight into the mechanisms by which tumor immunity and overt neurologic disease are associated in PND. PND is characterized by high titer autoantibodies. Comparably, CNS tolerance only breaks down in N2-LacZ mice that mount high titer antibody responses in conjunction with T cell responses, implying that it is the combination of cellular and humoral immunity that results in PND. One implication is that both B and T cells may be rational targets for treatment of PND. Recent work supports this as targeting T cells with tacrolimus [28], or B cells or rituximab [29], can attenuate neurologic decline in some PND patients. Future work will investigate whether antibodies directly contribute to pathogenesis or only mark B cell activation and antigen presentation.

Patients harboring intermediate titer antibodies (<1:1000) to an onconeural antigen have improved tumor responses without autoimmune complications [3, 30] suggesting that tumor immunity and autoimmunity can be uncoupled, and that these antigens may be good targets for immunotherapy [4]. Adoptive T cell transfer has been tested and produced very promising results for the treatment of leukemia [31] and melanoma [32]. Unfortunately, a recent clinical trial of adoptive transfer of anti-MAGE-A3 T cells in patients with melanoma led to acute mental status changes, coma and death that was accompanied by infiltrating CD8+ T cells in the brain in 2 of 9 patients. The receptor of this clone also recognizes MAGE-A12, which was subsequently detected in CNS neurons [33]. Our work suggests that prescreening for anti-MAGE autoantibodies prior to adoptive transfer of anti-MAGE-A3 T cells may help identify patients at risk for iatrogenic autoimmunity since humoral immunity may tip the balance from tumor immunity to autoimmunity.

Finally, our work reveals that CNS neuronal antigen expression exerts partial tolerance to low avidity T cells and striking deletional tolerance to high avidity T cells with immunologic challenge. While low avidity T cells survive, they are relatively inefficient in mounting tumor rejection. This presents a major obstacle to cancer vaccination strategies that aim to augment the endogenous T cell response. Transfer of T cells with high avidity
tumor antigen-specific receptors can overcome the problem of deletional tolerance. Additionally, because neuronal antigen expression prevents the generation of CD8+ T cell memory, strategies involving adoptive T cell transfer may require repeated administration.

**Materials and Methods**

**Mice**

N2-LacZ mice are offspring of crosses between Nova2-Cre with β-actin LacZ (The Jackson Laboratory, stock No. 002982), both crossed more than 10 generations onto C57BL/6. BG1 and BG2 mice were a gift from Nicolas Restifo. C57BL/6 mice (Jackson Laboratory) were housed in pathogen-free facilities at Rockefeller University. All experiments were performed in compliance with the Rockefeller University Institutional Animal Care and Use Committee regulations.

**Peptides, antibody, tumor challenge**

A/PR/8 NP p366, b-gal p96, b-gal p497, ova p257, b-gal p726, ova p323 (American Peptide, Inc). FACS antibodies (BD Pharmingen, Inc). ELISPOT antibodies (Mabtech). WP4.F2 (WP4) cell line, is a clone of the retrovirally transduced methylcholanthrene-induced MCA 205 fibrosarcoma cell with LSXN-based retrovirus encoding the *Escherichia coli* LacZ gene 98% of the cells stain positive for expression of b-gal by X-gal staining (Millipore). To challenge mice, 200,000 live cells were implanted intradermally on the right flank. Tumors were measured every 2–3 days.

**X-gal staining of brains**

Mice were perfused with 40 ml of heparinized PBS, brains were fixed and placed in X-gal staining solution (Millipore) for 6 hours at 37°C.

**B-gal immunohistochemistry**

Epitope retrieval in citrate buffer was performed on fixed, paraffin embedded 4 um sections. Slides were stained 1:4000 with anti-b-gal chicken polyclonal antibody (ab9361, Abcam) and then with avidin-biotin detection system, DAB chromogen, and hematoxylin counterstain.

**Quantitative-PCR of mouse organs**

Mice were perfused and organs snap frozen. Total RNA from tissues was extracted using TRIzol (invitrogen) and High Pure RNA Isolation Kit with DNase I digestion (Roche). Relative amounts of mRNA copies was determined by calculating the difference (Δ) in cycle time (Ct) between LacZ and β-actin (ΔCt). The difference between LacZ ΔCt values for N2-LacZ and WT mice was calculated (ΔΔCt) and 2^ΔΔCt represents the normalized LacZ fold change between N2-LacZ and WT mice. Primers: β-actin F, TCCACACCCGCCACCAG; β-actin R, CCATTCCCACCATCACACCCTG, LacZ F, GTGCGGATTGAAAATGGTCT, and LacZ R, GACCTGACCATGCAGAGGAT.
Generation of b-gal antibody in mice

10mg/ml b-galactosidase protein (Roche) or 3.3 mg/ml ovalbumin (Sigma-Aldrich) were emulsified 1:1 with CFA. Each mouse received 4×50 ul emulsate. Three weeks later, mice were bled and serum was analyzed by Western blot using a multiscreen apparatus (Biorad). Density was compared between bands from samples and a known concentration of monoclonal b-gal antibody (Clone DC1 4C7, Thermo Scientific) using ImageJ64 software.

Immunization of mice with AdV-b-gal

100 ul of serum free AdV-b-gal (Puresyn, Inc) [10^9 particles/ml] was injected intradermally. Pertussis toxin (PTx, Sigma-Aldrich) 400 ng was injected intraperitoneally on day 0 and day 2.

T cell isolation and ELISpot Assay

CD4+ and CD8+ T cells were positively selected using MACS (Miltenyi Biotech). ELISpot assays were performed as previously described [34]. Briefly, 200,000 isolated T cells were plated with 10,000 RMA-S, EL4, or E22 cells (EL4 cells transfected with the b-gal gene) in a well of a 96-well pre-anti-IFNγ-coated and blocked ELISpot plate for 40 hours. IFNγ production by individual cells was detected using Vectastain Elite Kit and substrate. Zellnet Consulting evaluated the ELISpot plates using an automated ELISpot reader (Zeiss) and KS ELISpot 4.8 software.

Tetramer staining

CD8+ T cells, or ACK (Gibco®) red blood cell lysed blood was FcR(CD16/CD32) blocked and stained using 1ul of H2-Kb b-gal p96, H2-Kb b-gal p497 or H-2Kb ova tetramer (Becton Coulter). Cells were evaluated by FACS using FACS Calibur instrument (BD) and analyzed using FlowJo software (Tree Star).

In vivo cytotoxicity assay

Mice were immunized with 100 ul purified Adv-b-gal adenovirus and PTx (400 ng / mouse) intraperitoneally (days 0 and 2). Thirteen days later mice were infused with 3×10^7 CFSE labeled syngeneic targets pulsed with 1uM Flu NP p366 (CFSE hi), b-gal p96 (CFSE intermediate) and b-gal p497 (CFSE low) intravenously. Sixteen hours later splenocytes were harvested and analyzed by FACS.

Bone marrow chimeras

Host mice were lethally irradiated with 900 cGy (2×450 cGy). Four hours later, host mice were reconstituted with a total of 10×10^6 bone marrow (Thy1.2+ cell depleted) cells or 5×10^6 of each donor bone marrow cells for mixed chimera experiments. 70–95 days post reconstitution, mice were injected with 100 ul purified AdV-b-gal intradermally and PTx toxin (400 ng / mouse) intraperitoneally on days 0 and 2. 13 days later splenic T cells were enumerated by FACS.
Transfer of transgenic T cells for the generation of tumor immunity

5×10^6 CD8+ and CD4+ cells from BG1 and BG2 transgenic mice were positively selected using MACS (Miltenyi Biotec) and transferred into non-irradiated C57BL/6 hosts or N2-LacZ hosts. Mice were simultaneously tumor challenged. 30 days later, mice were treated with AdV-b-gal and PTx. On day 38, the number of tetramer positive cells in blood was enumerated. On day 40 naïve (CD62L hi, CD44 lo), effector memory (CD62L lo, CD44 hi) and central memory T cells (CD62L hi, CD44 hi) were determined.

Generation of mouse models of PND

Experiment A: Mice were sub-lethally irradiated (500cGy) and 24 hours later intravenously given cells positively selected by MACS (Miltenyi Biotec). Mice were given 5×10^6 CD8+ T cells from AdV-b-gal sex-matched C57BL/6 immunized mice in combination with, where indicated, 10^6 CD4+ T cells from BG2 RAG−/− mice. Mice were concurrently challenged with 200,000 live WP4 cells. Nine days post challenge, one N2-LacZ mouse died.

Experiment B: Five N2-LacZ and five littermate control mice were sub-lethally irradiated (500 cGy) and 24 hours later were intravenously given 5–10^6 CD8+ T cells MACS isolated from sex-matched C57BL/6 AdV-b-gal immunized mice and concurrently challenged with 200,000 live WP4 cells. At the same time, five N2-LacZ and five littermate control mice were sub-lethally irradiated (500 cGy) and 24 hours later mice were intravenously given 5×10^6 CD8+ T cells MACS isolated from sex-matched C57BL/6 AdV-b-gal immunized mice, 10^6 CD4+ T cells MACS isolated from BG2 RAG−/− mice, PTx (400 ng/mouse on day 0 and day2), and Hamster IgG from BioXcell (500 ug day 0 and 250 ug days 2, 4, and 6). On the day of T cell transfer, mice also received 200,000 apoptotic (10,000 cGy irradiated) WP4 cells on the left flank and 200,000 live WP4 on the right flank. One mouse in the N2-LacZ group died twelve days post tumor challenge. Experiment C: Non-irradiated N2-LacZ or WT hosts intravenously received a mix of 5×10^6 BG1 mouse CD8+ and 5×10^6 BG2 mouse CD4+ T cells. On the day of T cell transfer, mice were challenged with 200,000 live WP4 cells. By day 28, all mice rejected tumors and were given AdV-b-gal and PTx (400 ng/mouse day 28 and day 30). On day 50 (22 days later), mice were challenged with CFA+b-gal to mount antibody responses, measured on day 71. Experiment D: Bone marrow chimeras were established by transferring 5×10^6 BG1 and 5×10^6 BG2 T cell depleted bone marrow cells into lethally irradiated hosts (2×500 cGy). 51 days post transfer, mice were challenged with 200,000 live WP4 cells on the right flank and 200,000 apoptotic WP4 cells on the left flank. On day 106, one N2-LacZ mouse was found sick and was bled prior to being perfused with 4% paraformaldehyde (PFA). On day 106, all remaining mice were given AdV-b-gal, PTx (400 ng/mouse, day 106 and day 108). Mice were bled and then given CFA-b-gal on day 120. Mice were bled again on day 122 and then perfused with 4% PFA.

Pathology

Mice were perfused with PFA and brain sections were stained with hematoxylin and eosin and TUNEL [35] and assessed by a board-certified veterinary pathologist as previously described.
Statistics

Repeated measure two-way ANOVA analysis was performed by Prism 5.0a (GraphPad Software). Data are presented as mean +/- SD; p-values were determined by two-tailed Student’s t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

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<tr>
<td>AdV</td>
<td>Adenovirus</td>
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<tr>
<td>CSF</td>
<td>cerebral spinal fluid</td>
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<td>PND</td>
<td>paraneoplastic neurologic diseases</td>
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<td>PTx</td>
<td>Pertussis toxin</td>
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References


Figure 1. Selective Expression of β-galactosidase in N2-LacZ mice

(A) Schematic diagram of the breeding strategy for N2-LacZ mice. Nova2-Cre-β-actin-LacZ (N2-LacZ) mice are double transgenic F1 offspring of crossing Nova2-Cre transgenic mice with chicken β-actin-LacZ transgenic mice. Upon induction of Cre activity in β-actin-LacZ X Nova2-Cre mice, the loxP-flanked STOP sequence is removed and LacZ is expressed in neurons expressing Nova2. (B) X-gal staining of WT, N2-Cre and N2-LacZ mouse brains. (C) qPCR analysis of LacZ mRNA in WT and N2-LacZ mouse organs normalized to the housekeeping gene, β-actin. Presented are the fold changes of LacZ expression in N2-LacZ organs.
mouse organs relative to the same tissue in littermate control mice. Data shown is mean+/−SD and is representative of three experiments. (D) β-gal staining by immunohistochemistry of organs of N2-LacZ and wild type mice. Arrows indicate β-gal expression (brown) in neurons. Magnification 600×: Bar indicates 20 μm. Hemotoxylin was used as a counterstain.
Figure 2. Testing of Humoral and Cellular tolerance to β-galactosidase in N2-LacZ mice
(A) Western blots of serum from N2-LacZ □ or Littermate control □ mice immunized with β-gal/CFA and PTx. Relative density was calculated by normalizing to a known commercial β-gal monoclonal antibody. (B–F) N2-LacZ or Littermate control mice were immunized with AdV-β-gal and PTx. (B) IFNγ ELISPOT responses of splenic CD4+ T cells cultured with irradiated and peptide pulsed splenocytes 13 days after immunization. (C) Representative FACS plots of CD8+ and tetramer positive cells 22 days after immunization. Gating strategy presented in Supporting information Fig. 3A. (D) Summary of (C), 8 mice...
per group. (E) IFN\(\gamma\) ELISPOT responses of splenic CD8+ T cells cultured with EL4 cells pulsed with 1 \(\mu\)M ova (irrelevant), b-gal p96, b-gal p497, or E22 cells, 13 days after immunization. (F) IFN\(\gamma\) ELISPOT responses of splenic CD8+ T cells to EL4 cells pulsed with decreasing amounts of b-gal p96 or p497 peptides 13 days post immunization. No IFN\(\gamma\) spots were detected from N2-LacZ mice at any p96 peptide concentration indicated by \(\circ\). Data are presented as mean+/−SD of three mice per group unless otherwise indicated. All data shown is representative of at least 3 experiments. NS indicates difference is not statistically significant by 2 tailed t-test. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. Mice exhibited no signs of ataxia, hunched posturing or death.
Figure 3. N2-lacZ mice respond to in vivo β-gal challenge in the periphery
(A) Percent cytotoxicity of peptide pulsed CFSE labeled splenocytes infused 13 days after AdV-β-gal and PTx immunization. Percent cytotoxicity was calculated by comparing survival of CFSE labeled cells in immunized mice with non-immunized controls. Data presented is from one of five representative mice. Gating strategy is presented in Supporting information Fig. 3C.
(B) Tumor diameter of N2-LacZ (blue) or littermate (red) mice (5 per group) immunized with AdV-β-gal and PTx and challenged with β-gal expressing WP4 cells 10 days later. Data presented are mean±SD. *** indicates p<0.001 by repeated measure two-way ANOVA. All data is representative of at least 3 experiments.
Figure 4. T cell tolerance to b-gal in N2-LacZ mice is not due to b-gal expression in peripheral radio-resistant cells or in hematopoietic cells

Lethally irradiated WT or N2-LacZ host mice were reconstituted with BG1 and BG2 bone marrow. Reconstitution was assessed by measuring (A) %Vβ7+ CD8+ T cells, (B) %Vα11+ CD4+ T cells and (C) %CD25+/Foxp3+ of Vα11+ and Vα11- fractions. Gating strategy presented in Supporting information Fig. 3B. (D) %p96 tetramer+ CD8+ T cells in lethally irradiated WT or N2-LacZ host mice reconstituted with a mix of BG1 and WT or BG1 and N2-LacZ bone marrow and challenged with AdV-b-gal and PTx and 13 days later (n=6 mice per group, mean+SD). All data shown is representative of at least 3 experiments. NS
indicates difference is not statistically significant by 2-tailed t-test. * indicates p<0.05, ** indicates p<0.01, *** indicates p<0.001. Mice exhibited no signs of ataxia, hunched posturing or death.
Figure 5. BG1 and BG2 T cells reject tumor but do not cause autoimmune brain disease

(A) Experimental design: 5 million CD8+ +/− 5 million CD4+ cells from BG1, BG2 or WT mice were transferred into WT or N2-LacZ hosts and challenged with b-gal expressing WP4 cells. 30 days later, mice were secondarily challenged with AdV-b-gal and PTx. (B) Tumor growth in N2-LacZ or WT hosts that received T cell transfer. (C) Circulating b-gal p96 tetramer+ cells and (D) Western blot of serum b-gal antibody 28 days post tumor challenge (primary challenge). (E) % b-gal p96 or ova tetramer+ CD8+ T cells 8 days after AdV-b-gal and PTx (secondary challenge). (F) b-gal p96 specific CD8+ Vβ7+ CD3 cells were assessed...
for markers of effector (CD62L$^{lo}$, CD44$^{hi}$), central memory (CD62L$^{hi}$, CD4+4$^{hi}$) and naïve T cells (CD62L$^{+}$, CD44$^{lo}$ and CD44$^{int}$) 10 days after secondary challenge (n=8 mice per group, mean+SD). Gating strategy for C–F is presented in Supporting information Fig. 3A and B. *** indicates p<0.001 by 2-tailed t-test. Data is representative of two experiments.
Figure 6. **T cells and B cells collaborate to generate neuronal targeting**

Perfused brain sections were stained with hematoxylin and eosin and TUNEL. (A) Cerebral cortex. (B) Hippocampus. In A and B, arrows indicate dying neurons in the dentate gyrus. Arrowheads indicate normal reference neurons. Magnification 600×: Bar indicates 20 μm. (C) Intraparenchymal blood vessel. Arrows indicate neutrophilic infiltrate.
### Table 1

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<th>Experiment</th>
<th>Transferred CD8+</th>
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^a In all experiments mice were challenged with 200,000 live b-gal expressing WP4 tumor cells

^a mouse that died mounted high titer antibody after tumor challenge

N/A: Not Applicable

BM: Bone Marrow Chimeras