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Induction of a VLA-2 (CD49b)-Expressing Effector T Cell Population by a Cell-Based Neuroblastoma Vaccine Expressing CD137L

Xiaocai Yan,*† Bryon D. Johnson,*† and Rimas J. Orentas2*†

In malignancies where no universally expressed dominant Ag exists, the use of tumor cell-based vaccines has been proposed. We have modified a mouse neuroblastoma cell line to express either CD80 (B7.1), CD137L (4-1BBL), or both receptors on the tumor cell surface. Vaccines expressing both induce a strong T cell response that is unique in that among responding CD8 T cells, a T effector memory cell (TEM) response arises in which a large number of the TEM express the α-chain of VLA-2, CD49b. We demonstrate using both in vitro and in vivo assays that the CD49b+ CD8 T cell population is a far more potent antitumor effector cell population than nonfractionated CD8 or CD49b+CD8 T cells and that CD49b on vaccine-induced CD8 T cells mediates invasion of a collagen matrix. In in vivo rechallenge studies, CD49b+ T cells no longer expanded, indicating that CD49b TEM expansion is restricted to the initial response to vaccine. To demonstrate a mechanistic link between the expression of costimulatory molecules on the vaccine and CD49b on responding T cells, we stimulated naive T cells in vitro with artificial APC expressing different combinations of anti-CD3, anti-CD28, and CD137L. Although some mRNA encoding CD49b was induced by combining anti-CD3 with anti-CD28 or CD137L, the highest level was induced when all three signals were present. This indicates that CD49b expression results from additive costimulation and that the level of CD49b message serves as an indicator of the effectiveness of T cell activation by a cell-based vaccine. The Journal of Immunology, 2008, 181: 4621–4631.

Neuroblastoma is the most common extracranial solid cancer in childhood and accounts for 15% of childhood cancer deaths (1). Survival for children with advanced disease is <40% despite aggressive therapy (2). Clinical research has provided clues that the immune system can be marshaled against this disease. For example, hematopoietic stem cell transplantation has been demonstrated to be superior to other treatment regimens that do not overtly manipulate the immune system and may induce a graft-versus-tumor effect (3, 4). Based on clinical approaches that manipulate the immune system, as well as experimental animal models, a number of immune-based strategies have been tested in clinical trials for neuroblastoma. These have included using allogeneic or autologous patient-derived cancer cells as a vaccine, or a modification of these cell-based vaccines by the introduction of IL-2 and/or lymphotactin gene expression vectors (5, 6). However, no clear clinical benefit was seen resulting in the need to reevaluate how best to modify these cells for an increased immunostimulatory effect (7). The evaluation of the effectiveness of these vaccines, as well as of other cancer vaccines, is complicated by the lack of well-defined readouts of efficacy, beyond that of relapse of disease.

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immunological ignorance (where CTL were not deleted or anergized, but simply not activated), allowing immunization with tumor-derived peptide to induce a protective CTL response (17).

VLA-2 (as detected by the DX5 Ab) was originally considered to be a NK cell marker. However, in addition to our studies, others have reported increased adhesion of CD137- and CD80-expressing cells to extracellular matrix components, such as collagen type I and IV and homotypic adhesion (24). In the current study, we link the reported increased adhesion of CD137-expressing cells to extracellular matrix components to the expression of VLA-2. We show that VLA-2 serves as a marker for activation by CD137L-expressing cell-based vaccines, and we demonstrate that the CD49b<sup>+</sup> population is far more active than CD49b<sup>-</sup> CD8<sup>T</sup> cells. We propose that the expression of CD49b (VLA-2) may serve as a surrogate marker for successful tumor vaccination.

**Materials and Methods**

**Animals, cell lines, Abs, and microbeads**

A/J mice were purchased from The Jackson Laboratory and used at 5–8 wk of age. All experiments were performed under approved protocols in a pathogen-free environment at the Medical College of Wisconsin, which contains an American Association for the Accreditation of Laboratory Animal Care-certified facility. Anti-CD8-conjugated immunomagnetic beads used for automated cell separation (AutoMACS) were purchased from Miltenyi Biotec. A Dynal mouse CD8-negative isolation kit was purchased from Invitrogen Dynal. Anti-CD8 (16-10A1), anti-CD4 (OX-35), anti-CD49b (HM2), and anti-CD82 (37.51) were purchased from BD Pharmingen. Anti-CD28 (37.51), anti-CD137L (TKS-1) and anti-CD32 (37.51) were purchased from BD Biosciences Pharmingen. Anti-CD8 (16-10A1) was purchased from Miltenyi Biotec. A Dynal mouse CD8-negative isolation kit was purchased from Invitrogen Dynal. Anti-CD8 (16-10A1), anti-CD4 (OX-35), anti-CD49b (HM2), and anti-CD82 (37.51) were purchased from BD Pharmingen. Anti-CD28 (37.51), anti-CD137L (TKS-1) and anti-CD32 (37.51) were purchased from BD Biosciences Pharmingen.

**Lymphocyte activation and analysis by quantitative real-time PCR**

CD8<sup>T</sup> T cells were purified from A/J splenocytes by immunomagnetic sorting using AutoMACS (purity > 95%). Production of the mouse CD32- and/or CD137L-expressing K562 artificial APC lines (aAPC) was described previously (11). CD3- and/or CD28 signals were provided by coating the CD32 and CD28 receptors with mAbs or anti-CD3 and anti-CD28 Abs. The expression of CD49b (VLA-2) may serve as a surrogate marker for successful tumor vaccination.

**Abbreviations used in this paper:** aAPC, artificial APC; HPRT, hypoxanthine phosphoribosyltransferase; BM, bone marrow; dLN, draining lymph node; DP, double positive; TEM, effector memory T cell; TCM, central memory T cell.

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**Figure 1.** Impact of vaccination on CD4 and CD8 cells. Mice were vaccinated s.c. with 2 × 10⁶ AGN2a/CD80, AGN2a/CD137L, or AGN2a/CD80/137L (AGN2a/DP) cells twice weekly and sacrificed 5 days after the second vaccination. A group of nonvaccinated (naive) controls were included in the experiments. Percentages of CD49b<sup>+</sup> cells in gated CD4 and CD8 expression of CD4/CD8 in naive control, AGN2a/CD80-, or AGN2a/CD137L-vaccinated groups; **p < 0.05 when compared with naive control, AGN2a/CD80, or AGN2a/CD137L vaccinated groups; *p < 0.05 when compared with naive controls; p values were determined by the Student t test). Data are the means ± SDs from one of three independent experiments (n = 3 mice/group).

**Figure 2.** CD49b expression on CD4 and CD8 cells after vaccination. Mice were vaccinated s.c. with 2 × 10⁶ AGN2a/CD80, AGN2a/CD137L, or AGN2a/CD80/137L (AGN2a/DP) cells twice weekly and sacrificed 5 days after the second vaccination. A group of nonvaccinated (naive) controls were included in the experiments. Percentages of CD49b<sup>+</sup> cells in gated CD4 and CD8 cell subsets from naive control, AGN2a/CD80-, AGN2a/CD137L-, or AGN2a/CD80/137L (DP)-immunized mice were determined by flow cytometry. Tissues analyzed were peripheral blood (PBL), spleen, BM, and vaccine dLN (*, p < 0.05 when compared with naive control, AGN2a/CD80-, or AGN2a/CD137L-vaccinated groups; **, p values were determined by the Student t test). Data are the means ± SDs from one of three independent experiments (n = 3 mice/group).
APC were irradiated with 4000 rad from a cesium source and cocultured with purified CD8 T cells at a ratio of 1:2 (2.5 × 10⁵ aAPC plus 5 × 10⁵ T cells in 24-well plates containing complete DMEM and 20 U/ml human rIL-2 (Proleukin; Chiron)). 5 × 10⁶ viable cells (as assessed by trypan blue exclusion) were harvested after 72 h of culture, total RNA was isolated with TRIzol (Invitrogen), and cDNA was generated using SSII reverse transcriptase and oligo(dT) (12–18) primer (Invitrogen). CD49b copy number was determined by real-time PCR in the presence of Brilliant SYBR Green (diluted 1/2000; Stratagene). Briefly, Taq polymerase, standard buffers (50 mM MgCl₂, 8% glycerol, 3% DMSO, and 25 mM dNTPs) and primers CD49b forward, 5'-CCGGGTGCTACAAGTCAT; reverse, 5'-GTCGGCCACATTGAAAAAGT and hypoxanthine phosphoribosyltransferase (HPRT) forward, 5'-TGAAGAGCTACTGTAATGATCAGTCAA; reverse, 5'-AGCAAGCTTGCAACCTTAACCA were mixed, and the cDNA was amplified at 50°C for 2 min, 95°C for 10 min, and then cycled 40 times between 95°C for 15 s and 60°C for 1 min using a DNA Engine Opticon2 (Bio-Rad). A single peak was seen on melting curves conducted at the end of each run to ensure single product amplification. The amount of CD49b increase was normalized to HPRT as an internal standard and calculated as fold increase seen over stimulation of T cells with anti-CD3.

**FIGURE 3.** Antitumor effector function of CD49b⁺ cells. Mice were vaccinated with AGN2a-CD80/137L as in Fig. 1, and CD49b⁺ and CD49b⁻ CD8 T cells were purified by flow cytometric sorting and used as effectors in chromium release assays (A), IFN-γ ELISPOT assays (B), and soluble cytokine production assays (C) with either unmodified wild-type AGN2a (AGN2a/WT), vaccine (AGN2a-CD80/137L or AGN2a/DP), or NK-sensitive YAC-1 tumor cells as the cellular targets. A, Percent lysis at the indicated E:T ratios is shown (the data are representative of three separate experiments, average percent lysis ± SD for a representative experiment is shown). B, The numbers of spots per well at the indicated T cell numbers added per well are shown (data representative of four separate experiments, average spot number ± SD from a representative experiment is shown). C, The release of IFN-γ and TNF-α after 72 h of coculture with tumor cell targets is shown, and the results are from one of two independent experiments, values calculated using provided BD Biosciences software for fluorescent microbead analysis.

<table>
<thead>
<tr>
<th>Treatment a</th>
<th>Absolute Numbers b (×10⁶ ± SD)</th>
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<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Naïve</td>
<td>40.0 ± 1.72</td>
</tr>
<tr>
<td>AGN2a/CD80</td>
<td>42.6 ± 2.55</td>
</tr>
<tr>
<td>AGN2a/CD137L</td>
<td>42.9 ± 3.44</td>
</tr>
<tr>
<td>AGN2a/DP</td>
<td>44.7 ± 1.97</td>
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a Groups of mice were vaccinated s.c. with the indicated tumor cells as indicated in Fig. 1 and spleens were harvested 5 days after the second vaccination. A group of nonvaccinated controls (naïve) was also included. Splenocytes were stained with the indicated markers and absolute numbers of different cell subsets were calculated.

b The data were determined from one of two replicate experiments (n = 3 mice/group).

*, p < 0.05 vs the other three groups.

**, p < 0.005 vs the other three groups.
alone using the comparative cycle threshold method (Applied Biosystems User Bulletin 2; Applied Biosystems).

Vaccination protocol
AGN2a cells expressing CD80 (AGN2a/CD80), CD137L (AGN2a/CD137L), or both (AGN2a/CD80/137L) were derived and established as permanently transfected cell lines, and expression of costimulatory molecules was verified before each experimental procedure. For vaccination, 2 × 10⁶ vaccine cells, either viable or irradiated with 4000 rad, were injected s.c. twice weekly (days 0 and 7). For recall response analysis, previously vaccinated mice were revaccinated at day 60. On days 12, 19, 26, 33, 60, 67, 74, and 81 after the first vaccination, peripheral blood, spleen, bone marrow, and draining lymph node (dLN) single-cell suspensions were purified by AutoMACS sorting and cryopreserved for ELISPOT analysis.

Adoptive immunotherapy with purified CD49b⁺ cells
A/J mice were vaccinated with irradiated AGN2a-CD80/137L cells twice weekly. Five days after the second vaccination, splenocytes were harvested and CD8⁺ T cells were purified by negative selection (i.e., untouched). Purified CD8⁺ cells were then fractionated by AutoMACS after incubating the cells with PE-conjugated anti-CD49b Ab and anti-PE-conjugated microbeads. In brief, 2.5 × 10⁶ CD49b⁺, CD49b⁻, or total CD8⁺ T cells were then infused by tail vein injection into tumor-bearing A/J mice (injected with 1 × 10⁶ wild-type parental tumor, AGN2a, 60 days later. Animals were sacrificed, according to institutional protocol, when tumors reached 250 mm².

Cytokine analysis
Splenocytes from AGN2a-CD80/137L-vaccinated mice were purified by AutoMACS after incubation with anti-CD8 microbeads. CD49b⁺ and CD49b⁻ populations were separated by flow cytometric sorting (FACS-Diva; BD Biosciences) and purified populations were incubated in triplicate wells with or without 1 × 10⁶ irradiated AGN2a (unmodified, wild-type tumor) in 96-well plates. After 3 days of incubation, 50 μL of supernatant was collected and cytokines were measured using the BD Biosciences Th1/Th2 cytokine bead array kit.

Chromium release assays
Purified lymphocyte subsets were tested for the ability to lyse tumor cells in standard chromium release assays as described previously (11). Briefly, tumor cell targets (AGN2a, AGN2a-CD80/137L, or YAC-1) were incubated in 100 μCi of ⁵¹Cr (as sodium chromate; Amersham Biosciences) per million cells for 90 min at 37°C, washed extensively, and then coincubated with effector cell populations at the indicated E:T ratios in a final volume of 200 μL. At the indicated time points, 50 μL of supernatant was collected from individual wells and added to Lumaplate-96 microplates (Packard Instrument). The plates were dried and radioactivity was measured on a Packard TopCount. Percent lysis is reported as the ratio of experimental: total release, following subtraction of spontaneous release from each.

ELISPOT analysis
Enumeration of IFN-γ producing cells was conducted as described previously (11). Briefly, capture anti-mouse IFN-γ mAb (BD Biosciences) was used to coat 96-well polyvinylidene difluoride membrane plates (Millipore) overnight. On the following day, CD8 splenocytes were added in incremental 2-fold dilutions, starting at 5 × 10⁴ or 1 × 10⁵ cells/well, and coincubated with 10⁴ tumor cell stimulators (AGN2a, AGN2a-CD80/137L, or YAC-1) for 30–35 h at 37°C. Plates were then washed, incubated with matched biotinylated detection Ab (BD Biosciences) for 2 h at room temperature, washed again, and then incubated with extravidin-alkaline phosphatase conjugate (Sigma-Aldrich). Spots were developed by adding 5-bromo-4-chloro-3-indolyl phosphate/NBT substrate (Sigma-Aldrich). The number of spots per well, corresponding to IFN-γ-secreting cells, was determined by automated counting (ImmunoSpot; CTL).
In vitro migration analysis

AJ/J mice were immunized with AGN2a-CD80/137L cells twice weekly. Five days after the second immunization, CD8+ splenocytes were purified by AutoMACS sorting, immunostained for CD49b, CD62L, and CD44, and then flow sorted into CD49b+CD62L+CD44+, CD49b-CD62L+CD44+, and CD49b-CD62L-CD44+ populations. Cells (1 × 10⁶) in 100 μl of culture medium supplemented with 250 U/ml human rIL-2 were then layered on top of 100 μl of solidified collagen matrix (Matrigel, growth-factor reduced; BD Biosciences) in flat-bottom 96-well plates. After 48 h of culture, medium was removed from the Matrigel surface, the surface was rinsed, and then 100 μl of collagenase (collagenase D, 1 mg/ml in pH 7.0 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂; Roche) was added to each well, and the plate was incubated at 37°C for 6–8 h. The number of viable cells (by trypan blue exclusion) migrating into the collagen matrix was then counted.

Results

Expansion and activation of CD49b+CD8+ but not CD49b+CD4+ T cells upon AGN2a-CD80/137L vaccination

To determine the mechanism by which CD137L-expressing cell-based vaccines activate cellular immunity, we explored changes in the composition of immune cell populations in lymphoid tissues. Mice were immunized twice weekly with AGN2a/CD80, AGN2a/CD137L, or AGN2a-CD80/137L, and 5 days after the second s.c. vaccination, lymphocytes from spleen, bone marrow (BM), peripheral blood (PBL), and vaccine dLN were analyzed. When the ratio of CD4:CD8 cells was analyzed, all tissues showed an increase in the number of CD8 cells, with the most dramatic changes associated with CD137L expression on the vaccine in lymphocytes collected from the peripheral blood and spleen (Fig. 1A). The level of CD8 expression, down-regulation being associated with increased cell activation, was decreased to the greatest extent in mice immunized with AGN2a-CD80/137L (double positive, DP) in peripheral blood (Fig. 1B). In previous work, we demonstrated that cell-based vaccines induce a CD49b-expressing CD8+ T cell population. In Fig. 2, percentages of CD8 cells expressing CD49b in PBL, spleen, BM, and dLN were shown. In all lymphoid compartments, but most notably in PBL and spleen, the highest percentages of CD49b+CD8+ cells were associated with the presence of both CD80 and CD137L on the vaccine. Mice vaccinated with CD80/137L-expressing tumor cells (AGN2a/DP) also contained significantly higher numbers of splenic CD49b+CD8+ and total CD8+ T cells than nonvaccinated naive mice or mice vaccinated with tumor cells expressing CD80 or CD137L alone (Table I). These results indicate that expression of both molecules is required to see CD49b induction and that these signaling pathways activate CD8 T cells in a synergistic manner. Increased expression of CD49b was not seen on CD4 cells (Fig. 2 and Table I).

In vitro antitumor activity

To determine whether there were differences in the in vitro antitumor activity of CD8+CD49b+ and CD8+CD49b- populations, AJ/J mice were immunized with AGN2a-CD80/137L as described above and CD49b+ and CD49b- CD8+ T cells were isolated by flow cytometric sorting of splenocytes harvested 5 days after the second weekly s.c. vaccination. Using either unmodified AGN2a, the vaccine line (AGN2a-CD80/137L), or the NK-sensitive YAC-1 cell line as targets in chromium release assays, CD49b+ cells consistently showed higher levels of lytic activity (Fig. 3A). In IFN-γ ELISPOT assays, the CD49b+ population again showed greater levels of antitumor reactivity as judged by the number of cells producing IFN-γ in response to coculture with tumor (Fig. 3B). The CD49b+ cells did not lyse YAC-1 target cells or secrete IFN-γ in response to YAC-1 stimulators (Fig. 3, A and B), indicating that the CD49b+CD8+ were tumor specific and did not exhibit NK cell activity. Cocultures of CD49b+ cells and tumor also showed higher levels of IFN-γ and TNF-α production (Fig. 3C). Notably, the CD49b+ T cell population showed spontaneous IFN-γ production when cultured without stimulation. This may indicate that these cells were already engaged in an active immune response upon isolation. No increase in IL-2, IL-5, or IL-10 production was seen in either population (data not shown).

In vivo antitumor activity

To determine whether CD49b+ cells showed greater antitumor activity in vivo, we established an in vivo assay system for the control of tumor progression. Mice bearing s.c. tumors for as little as 24 h are no longer protected by cell-based vaccines (B. Johnson, R. Orentas, unpublished data). If this is due to a failure to activate and expand T cells, adoptive immunotherapy may be an effective means of facilitating an antitumor response. To test this, mice inoculated with viable AGN2a cells were infused 24 h later with 2.5 × 10⁶ CD8+CD49b+, CD8+CD49b−, or unfractionated CD8+ cells isolated from AGN2a-CD80/137L-vaccinated mice or left untreated (Fig. 4). Five of 10 mice treated by adoptive immunotherapy with CD49b+ T cells survived while 0 of 10 untreated or...
Two of 10 mice treated with unfractionated CD8 cells also survived. Notably, the five tumors that occurred in CD49b\^/H11001 treated mice occurred much later than those in untreated or CD49\^/H11002 treated mice (Fig. 4A). When the five surviving mice were rechallenged with 1 \times 10^5 AGN2a, all survived beyond 60 days (data not shown).

**Correlation of CD49b expression with T effector-memory marker expression and lytic function**

The high lytic and cytokine-producing activity of our vaccine-induced CD49b\^/CD8\^population led us to test whether these cells bore cell surface markers associated with T effector memory (TEM) populations. TEM are Ag-experienced cells (hence they express memory markers) that appear to be actively engaged in immune responses. To test for the correlation between CD49b expression and TEM phenotype, mice were immunized with AGN2a/CD80, AGN2a/CD137L, or AGN2a-CD80/137L twice weekly and, on day 5 after the second vaccination, lymphocytes from spleen, peripheral blood, BM, and vaccine dLN were analyzed by flow cytometry. Representative results from spleen are shown in Fig. 5. To analyze which CD8 cells were primarily CD49b positive, cells from AGN2a-CD80/137L-vaccinated mice were analyzed first. Gated CD8\^cells were initially analyzed for CD62L and CD44 expression or CD49b expression (Fig. 5, left histograms). The CD44\^CD62L\^cells were then analyzed for CD49b expression, while the CD49b\^CD8\^ cells were analyzed for CD44 and CD62L expression (Fig. 5, right histograms). This analysis demonstrated that when cells were gated for T\_\text{EM} markers (CD44\^CD62L\^) the CD8 cells in this population were primarily CD49b\^ (70, 79, 67, and 56% in PBL, spleen, BM, and dLN, respectively). Also, the majority of gated CD49b\^ cells were of the T\_\text{EM} phenotype (87, 88, 87, and 59% in PBL, spleen, BM, and dLN, respectively). The largest population of CD8\^CD49b\^ treated mice survived (Fig. 4B). Two of 10 mice treated with unfractionated CD8 cells also survived. Notably, the five tumors that occurred in CD49b\^ treated mice occurred much later than those in untreated or CD49\^ treated mice (Fig. 4A).

**FIGURE 5.** Functional analysis of CD49b\^CD8\^ cells. The following CD8\^ cell subsets were purified from the spleens of AGN2a-CD80/137L-vaccinated mice by flow cytometric sorting: CD49b\^CD62L\^CD44\^ (●), CD49b\^CD62L\^CD44\^- (▲), CD62L\^CD44\^- (▼), and CD62L\^CD44\^- (●). IFN-\gamma ELISPOT assays were done in the presence of either wild-type (A, WT) or vaccine (B, AGN2a/DP) tumor cell stimulators over a range of T cell numbers per well. The data (mean ± SD of triplicate wells) are representative of two separate experiments. *p < 0.01 when compared with CD49b\^CD62L\^CD44\^- cells. Values of p were determined by two-way ANOVA analysis.
lymphocytes in the CD49b⁺CD8⁺ gate were CD44⁺ and CD62L⁻ (60, 47, 49, and 58%, in PBL, spleen, BM, and dLN, respectively). This is considered a naive T cell phenotype. Lymphocytes with a T central memory (T CM) phenotype (CD44⁺ CD62L⁻) accounted for between 12% in BM and 23% in the spleen when CD49b⁺ cells were analyzed. Interestingly, in the dLN, the T CM population was 33% of the gated CD49b⁺ cells. Although still not the majority of the CD49b⁺ cells, the dLN was the only tissue in which the majority of T CM were CD49b⁺. This may indicate that in the dLN these cells are being activated.

Based on the results in Fig. 5, we then analyzed the comparative ability of each of our cell-based vaccines to induce the CD49b⁺ CD62L⁻ population in the CD4 and CD8 lymphocyte subsets (Fig. 6A). This analysis clearly demonstrated that it was our dual-expressing vaccine, i.e., both CD80 and CD137L, which induced a CD49b⁺ CD62L⁻ phenotype. This was true for each of the lymphoid tissues tested. When the CD44⁺ CD62L⁻ T CM population was examined in CD4 and CD8 subsets, similar results were seen in all tissues tested except for BM (Fig. 6B). In the BM, CD4 cells had a uniform elevation in CD44, which held true in the unvaccinated (naive) mice as well. This analysis illustrates that CD4 T CM are elevated in BM and to some degree in the spleens of nonvaccinated mice. In PBL, the expression was low and remained low. Only in the dLN did vaccination appear to slightly expand the CD4 T CM population (Fig. 6B). Thus, alterations in CD4 T CM do not correlate to the vaccine-induced response, highlighting that early immune responses after treatment with a cell-based vaccine are better tracked by following changes in differentiated CD8 cells.

To determine whether there was a functional difference in cells expressing CD49b and T EM markers, IFN-γ-ELISPOT assays were conducted on sorted populations using wild-type AGN2a stimulator cells (Fig. 7A) or AGN2a-CD80/137L (AGN2a/DP) vaccine cells as the stimulator (Fig. 7B). With either target, the CD62L⁻ populations failed to produce IFN-γ in response to coculture with tumor. We included both wild-type and the vaccine line as stimulators in the assays since previous work in our laboratory had demonstrated that the presence of the immune costimulatory molecules on the surface of AGN2a makes it a far more avid target. This can readily be seen by the increase in spot number seen with AGN2a/CD80/137L stimulators (Fig. 7B). The larger difference seen when wild-type AGN2a cells were used as the target may indicate that CD49b provides a direct cell-cell adhesion function, that the CD49b⁺ population expresses other adhesion and activation markers, or that the threshold of activation is lower when the CD80 and CD13L costimulatory molecules are expressed on the tumor.

**Analyzing the persistence of CD49b on CD8 cells after vaccination**

Thus far our data indicated that CD49b is a key marker for the most highly activated T EM induced by AGN2a/CD80/137L 5 days after the second of two weekly vaccinations (i.e., at the peak of

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**Figure 8.** Expression of CD49b on CD8 cells after primary vaccination, but not on CD8 cells after a recall immune response. Mice were vaccinated with AGN2a-CD80/137L on days 0 and 7 and boosted with the same vaccine on day 60. At each indicated time point, lymphocytes in peripheral blood (PBL), spleen, BM, and vaccine dLN were analyzed for percentages of CD8 cells expressing CD49b (■) or expressing a CD62L⁻CD44⁺ phenotype (▲). These data are from one of two separate experiments.

**Figure 9.** Collagen matrix invasion by CD49b⁺ cells. A. Splenocytes from AGN2a-CD80/137L-vaccinated mice were harvested and the following CD8⁺ cell subsets were purified by flow cytometric sorting: CD49b⁺ CD62L⁻ CD44⁺, CD49b⁻ CD62L⁻ CD44⁺, CD49b⁺ CD62L⁺ CD44⁺, CD62L⁻ CD44⁺, and CD62L⁺ CD44⁻. Cells (10⁵) of each purified population were plated on solid Matrigel, and the numbers of cells invading the matrix were counted after 48 h. *p < 0.01 when compared with the other three cell populations. B. CD49b⁺ CD62L⁻ CD44⁺ sorted cells were cultured on Matrigel with no Ab (control), isotype control Ab, or 5 µg/ml anti-CD49b (VLA-2) Ab. Cell migration was then determined after 48 h. * and **p < 0.01 when compared with the control cells. The results are the averages and SDs of triplicate wells that were repeated three times. Values of p were determined by the Student t test.
response). To determine whether CD49b expression is still increased at later stages in the immune response to our cell-based vaccine, we monitored CD49b expression at various time points after the vaccine was administered in peripheral blood, spleen, BM, and vaccine dLN tissues (Fig. 8). Tissues were harvested at set time points and CD8 cells were analyzed for either the expression of CD49b or the CD44⁺CD62L⁻ TEM phenotype. Consistent with our previous results, percentages of CD8 cells expressing CD49b were increased on day 12 (5 days after the second of two weekly vaccinations). This population returned to the levels seen in naive mice by day 60. However, when mice were boosted with a s.c. vaccination of AGN2a-CD80/137L on day 60, a less robust increase in CD49b expression was seen in peripheral blood and spleen, and little or no increase was seen in CD8 cells from BM or vaccine dLN. This was in contrast to the changes in percentages of CD8 cells expressing the CD44⁺CD62L⁻ TEM phenotype. This phenotype was expressed to a far greater degree upon rechallenge and even exceeded the initial response to our cell-based vaccine. These results indicate that the real value in CD49b lay in its ability to identify the initial activation of antitumor effector cells as opposed to those involved in a recall response.

CD8 T cell migration mediated by CD49b in vitro

One function of integrins such as VLA-2 is to bind extracellular matrix components such as laminin and collagen. To determine whether tumor vaccine-reactive CD8 cells express CD49b as part of a functional collagen receptor, we used an in vitro collagen migration assay. A/J mice were vaccinated with 5 × 10⁶ irradiated AGN2a-CD80/137L cells as in previous experiments, and CD8⁺ splenocytes from immunized animals were purified into four cell populations by flow cytometry: CD44⁺CD62L⁺, CD44⁺CD62L⁻, CD44⁺CD62L⁻ CD49b⁺, and CD44⁺CD62L⁻ CD49b⁻. Cells (1 × 10⁴) from each subset were cultured on top of a solid collagen matrix (Matrigel) in the presence of IL-2 (required for CD8 T cell viability). After 48 h of culture, the number of cells that had infiltrated the collagen matrix was counted. Collagen invasion by the CD49b TEM population was the most robust (Fig. 9A). The percentage invasion rates for all cells added to the wells were 30, 8, 0.4, and 0% for CD44⁺CD62L⁺ CD49b⁺, CD44⁺CD62L⁻ CD49b⁺, CD44⁺CD62L⁻ CD49b⁻, and CD44⁺CD62L⁻ cells, respectively. The experiment was repeated using the CD44⁺CD62L⁺ CD49b⁻ population in the presence of anti-CD8-Ab to more strictly define the VLA-2 dependence of collagen invasion by vaccine-responding CD8 T cells. Blockade of VLA-2 with Ab resulted in a decrease of ~66% of the matrix invasion capability (Fig. 9B).

**Induction of CD49b mRNA by costimulatory molecule activation**

Based on our observation that AGN2a-CD80/137L vaccination induced a CD49b⁺ TEM population in vivo, we determined whether T cell activation with these ligands could directly induce CD49b expression in vitro. Splenic CD8⁺ T cells were isolated from naive mice and then cocultured with K562-based aAPC. The aAPC expressed either CD32 (Fc receptor) or CD32 and CD137L. Irradiated aAPC were loaded with anti-CD3 and/or anti-CD28 Ab and cocultured with CD8 T cells in the presence of IL-2. Viable cells were harvested and counted 72 h later and cDNA was generated for real-time quantitative PCR analysis of CD49b mRNA expression levels (see Materials and Methods). When the signal for CD49b was normalized to that induced by anti-CD3 alone, the presence of either anti-CD28 or CD137L increased the level of CD49b message induced by anti-CD3 ~5-fold (Fig. 10). When all three signals were included (anti-CD3, anti-CD28, and CD137L) CD49b expression increased 12-fold. This establishes a direct link between costimulation with CD137L and anti-CD28 and the expression of CD49b on CD8⁺ T cells. Our experimental results suggest that during the initiation of an immune response that CD49b functions as an adhesion molecule on the most activated TEM and may thereby increase the ability of TEM to enter tissues and mediate tumor destruction.

**Discussion**

In an effort to discover how our cell-based vaccine, featuring the expression of both CD80 and CD137L activates highly active antitumor immunity, we found that a new population of CD49b⁺ CD8 T cells arose. Moreover, CD49b was expressed on the most active antitumor effector cells, the CD49b⁺ effectors were tumor specific, and they did not exhibit NK activity. Key to CD49b expression was the presence of both CD137L and CD80 on the vaccine cell surface. The addition of CD86 to CD80 did not induce a similar highly active antitumor effector cell population (11). CD137L triggers an independent signaling pathway that cooperatively signals with CD28 in T cell activation (12). Studies have shown that CD137 signaling in T cells induces an independent TNF-receptor-associated factor 2 signaling pathway from CD28, enhances cell cycle progression, potentiates CD8 T cell survival, and activates components of the TCR signaling pathway (25–29). In sum, CD49b expression appears to be up-regulated upon activation due to strong secondary costimulatory signals. Given the high level of antitumor activity that CD49b⁺ cells displayed upon immediate harvest from vaccinated mice, we saw this as an opportunity to explore the differences in lymphocyte populations induced by a cancer vaccine that could be monitored in future clinical cancer studies. To date, surrogate markers that indicate vaccine sufficiency in tumor models have been lacking. In looking at more global effects of the vaccine response after AGN2a-CD80/137L vaccination, we observed both decreased CD4:CD8 ratios and lower cell surface expression of CD8 (Fig. 1). This indicates that broad phenotypic changes as well as specific functional markers of immune activation could be monitored in our vaccinated animals.

CD49b is the α-chain of integrin VLA-2 (23). In the AGN2a-CD80/137L-vaccinated mice, a large population of CD8⁺ CD49b⁺...
cells was seen in the spleen (39%), BM (25%), peripheral blood (41%), and vaccine dLN cells (26%), as detected by either the DX5 or HM2. Ab. In an infectious disease model, Kassiotis et al. (30) found CD49b to be expressed on a CD4 T memory subset that was associated with IFN-γ production. Although in agreement with the finding that CD49b expression was associated with Th1-like responses, we did not observe increased expression of CD49b on CD4 cells upon AGN2a-CD80/137L vaccination. Our changes in populations expressing CD49b were restricted to CD8 cells.

To demonstrate that the vaccine-induced expression of CD49b is associated with the increased antitumor effectiveness seen in vivo, we tested in vitro antitumor activity by ELISPOT, the production of soluble cytokines, and direct cellular cytotoxicity assays (Fig. 3). In each assay the most active antitumor effectors were those that expressed CD49b, and the tumor-specific CD49b+ cells did not exhibit NK activity. The spontaneous production of IFN-γ by CD49b cells isolated from vaccinated animals and placed in culture, although lower than when tumor cells were present as stimulators, indicates that this population has already been activated and is in the process of responding to vaccine in vivo (Fig. 3C). When tested in vivo for antitumor activity, CD49b+ cells were again shown to be superior in mediating tumor destruction. When tumor-bearing mice were treated by direct adoptive immunotherapy with CD8 T cell populations from AGN2a-CD80/137L-treated mice, no tumor protection was seen in the CD49b− populations, while 50% of the mice treated with CD49b+ cells survived (Fig. 4). Since the T cells were administered by tail vein injection and the tumor was introduced by s.c. vaccination, this indicates that the CD49b+ cells have the ability to circulate, extravasate, and mediate strong antitumor activity in peripheral tissues. An intermediate level of protection (20% survival) was seen when unfractionated CD8 cells were tested, indicating the importance of the number of CD49b+ cells infused. One of the first roles described for VLA-2 was the finding that VLA-2 was expressed on polymorphonuclear neutrophils that had extravasated, in contrast to polymorphonuclear neutrophils found in the blood (31–33). Abs against VLA-1 and VLA-2, the major collagen-binding integrins, significantly inhibited the effector phase of the inflammatory immune response in mouse model systems, implying a requirement for these receptors (33). Thus, it appears that leukocyte attachment and extravasation through vascular endothelium cannot be separated from the immune reactions mediated by these cells.

VLA-2 mediates binding to collagen and also to laminin when expressed on certain cells types, but not when expressed on platelets or fibroblasts (34–36). In mammals, there are 19 different collagen types. To summarize a large body of work, it appears that α₁β₁ integrin may favor binding to fibrillar collagen (like type I), while α₇β₇ (VLA-1) may favor binding to basement membrane types (like type IV) (37–39). VLA-1 and VLA-2 differ directly in that VLA-1 can bind transmembrane type XIII collagen but not type II, while VLA-2 has the opposite binding characteristics with regard to these two collagen types (40, 41). The complexity of the extracellular matrix necessitates a large family of receptors to be present in multicellular organisms to mediate movement throughout the tissues and undoubtedly VLA-2 plays a role in this process. Collagen is one of the most abundant molecules in the tissue spaces through which cells must migrate, and would clearly serve to signal T cells as to their relative anatomic location (33). The possibility that VLA-2 serves as a specific addressin-like function for surveillance of the s.c. space is a compelling hypothesis and would support our finding of higher antitumor activity displayed by these cells.

VLA-2 also appears to have additional functional importance for T cell biology. VLA-2 induction on T cells in inflammatory lesions is proposed to prevent Fas-mediated apoptosis by preventing Fas ligand expression and the sparing of T cells from activation-induced cell death by culture on type I collagen (42). Amplification of TCR-mediated signaling has also been reported when functional assays are conducted on collagen-coated vessels (43, 44).

As Abs to NK cell surface markers are studied in greater detail, it has become clear that the expression of these markers is far more complex than initially thought. Abs to NK1.1 bind to none, or both proteins expressed from the Klrhb/NK-R-P1B and Klrh1c/NKR-P1C genes, depending on the mouse strain studied (45, 46). These epitopes can also be expressed on NKt cells and some cultured monocytes. Ab 1+ mice do not express either epitope and thus we began studies of NK populations in vaccinated mice with the DX5 Ab. However, we were soon struck by the fact that what we considered to be standard CD3+CD8+ CTLs expressed high levels of CD49b as indicated by DX5 staining. The importance of these CD8 effector cells in the immune response to neuroblastoma is highlighted in this study by our ability to eradicate tumors in mice by the simple transfer of CD49b+ CD8 cells from vaccinated mice (Fig. 4) and Ab depletion studies in our earlier work (9, 47). The in vivo biology of CD137L-induced effectors is clearly more complex as recent studies of human cells by Zhang et al. (48) have demonstrated that NKG2D is up-regulated on CTL expanded by aAPC expressing anti-CD3/4/1-BBL (CD137L). This is significant because NKG2D does directly impart killing capacity. Supportive of our findings, Zhang et al. (48) demonstrate that their procedure expands memory cells that may potentially target viral or self-Ags.

In exploring the biology of the T cell response to our cell-based vaccine, we found that the CD8 cells expressing CD49b could be classified as TEM based on CD44 and CD62L expression (Fig. 6). ELISPOT data demonstrated that if CD8 TTEM were fractionated according to CD49b expression, the CD49b− had the strongest IFN-γ ELISPOT activity (Fig. 7). This also held true for cytolytic activity (Fig. 3A). Although expression of TTEM memory markers and expression of CD49b were not absolutely linked, there was a large degree of overlap (Fig. 5). Given the high degree of effector function present in TTEM and the ability of CD49b to mediate binding to extracellular matrix components (as demonstrated in Fig. 9), we hypothesize that CD49b+ TTEM are poised to enter tissue and mediate Ag-specific antitumor immune function. This point is emphasized further by the ability of CD49b cells injected i.v. to abrogate growth of tumor cells that had been injected s.c. 24 h earlier.

The induction of CD49b on CD8 TTEM upon initial vaccination with AGN2a-CD80/137L was clear and compelling (Fig. 8, day 12). Given the relationship between vaccine-induced VLA-2+ cells and TTEM, we sought to determine whether CD49b expression was also a component of the recall response to our cell-based neuroblastoma vaccine. To our surprise, in all tissues tested (peripheral blood, spleen, BM, and vaccine dLN), CD49b+ cells were now a minor component of the response (Fig. 8). As expected, the total TEM response (CD62L+CD44+ cells) appeared to be as strong or stronger than the primary TEM response (Fig. 8). Thus, DX5/CD49b-expressing CD8 T cells are a hallmark of a strong initial response but do not appear to serve as a component of long-term recall memory responses. One hypothesis is that once cells express CD49b, they are terminally differentiated TEM and will not survive once effector function has been conducted. Alternatively, CD49b may confer the ability to enter peripheral tissues, and when TEM express this marker they reside in nonlymphoid tissues long-term where they can be rapidly activated upon Ag encounter.

In our final experiment, we sought to establish a mechanism linking the combined costimulation of T cells via CD28 and CD137 (4−1BB) with the expression of CD49b. The transcriptional
control region of VLA-2 is composed of a core promoter, a silencer, and a tissue-specific enhancer (22). The promoter core-binding domain shows Sp1, AP1, AP2, Gata3, and Pu.1 binding sites (49). In megakaryocytes, epithelial cells, and fibroblasts expression of α2 is regulated at the translational level, making the presence of mRNA a good measure of promoter activity (50, 51). Given the inducible control of VLA-2 expression, we are confident that our results with CD8 T cells establish a direct link between combined T cell stimulation via CD28 and CD137 and the surface expression of CD49b (Fig. 10). In short-term activation assays using aAPC, the combined signaling of CD28 and CD137 induced CD49b mRNA to a far greater degree than either costimulatory molecule alone when combined with CD3 signaling. From this we can conclude that strong T cell activation generated by multiple costimulatory ligands induces CD49b mRNA, which correlates to differentiation into the most highly activated antitumor effector cells. In future studies, we will determine whether the induction of this highly active cell population can occur in tumor-bearing animals and, if so, under what conditions. And although we have established a link to CD49b expression and the high level of antitumor immunity mediated by these cells, it is likely that other activation or effector molecules up-regulated in concert with CD49b are directly responsible for the enhanced tumor killing seen in our assays. The expression of CD49b on T Effg in human cancer vaccine trials may serve as an effective surrogate for the successful initial induction of a Th1-like effector cell population that should have strong antitumor activity. However, the lack of CD49b on cells engaged in a recall response may imply that alternate receptors now determine the migratory pattern of activated CD8 cells throughout the organism.

Disclosures

The authors have no financial conflict of interest.

References


