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T cells sensitized with breast tumor progenitor cell vaccine have therapeutic activity against spontaneous HER2/*neu* tumors

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Abstract

Cancer progenitor cells are critical for tumor initiation and recurrence so they are an important therapeutic target. We tested whether T cells could recognize tumor antigens expressed by breast cancer progenitor cells and acquire therapeutic activity against established metastases or delay onset of spontaneous tumors. Breast tumors were derived from HER2/*neu* transgenic mice and propagated *in vitro* under conditions that selected progenitor cells which were then used as an irradiated whole cell vaccine. A minor subset of recently-sensitized T cells was isolated from vaccine-draining lymph nodes then activated *in vitro* to achieve numerical expansion. We show that the tumor progenitor cell vaccines reversed tolerance to a known HER2/*neu* epitope, otherwise inhibited by Treg cells. Additional shared tumor antigens were recognized because a Neu^{neg} subclone also induced a Th1 type immune response against breast tumors. Adoptive transfer of *in vitro* activated lymph node T cells mediated regression of established metastases from multiple independently derived breast tumor lines. Moreover, adoptive transfer of effector T cells into Neu-tolerant mice, months before the onset of spontaneous tumors, significantly postponed tumor development. Interestingly, T-cell mediated lysis of metastases stimulated an IgG response to HER2/*neu* as well as other shared antigens. In summary, tumor progenitor cells contain shared antigens which can lead to a cross-protective T cell response. Moreover, antigens acquired during immune-mediated tumor destruction are presented in a manner conducive to reversal of tolerance and Ig class switching. These complementary effector mechanisms might augment therapy by eliminating refractory breast cancer stem cells.

Keywords

T cell; Immunotherapy; Her2/*neu*; breast cancer; animal tumor model

Introduction

There is substantial evidence that a subset of progenitor cells within tumors possesses features shared with normal stem cells such as self-renewal, intermittent cycling, and resistance to chemotherapy [1-3]. The cancer stem cell (CSC) concept was first established in acute myeloid leukemia where it was shown that a subset of transformed cells with stem cell-like properties were capable of regenerating tumor following serial transplantation into SCID mice [4, 5]. Likewise, this concept was validated in human breast cancer [6-8]. Conditions for *in vitro* culture of tumorspheres containing breast cancer stem cells have been

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based on studies identifying neural stem cells known as the neurosphere assay using serum-free medium containing EGF and FGF-2 [9-11]. This makes it possible to study the effectiveness of immune-mediated response to purified progenitor cells. There is evidence that cancer stem cells are susceptible to destruction by a specific immune response [12-14]. Thus, targeting tumor antigens that are expressed by cancer stem cells could overcome limitations of current therapy. Conversely, tumor vaccines focused on antigens found only in more differentiated tumor cells might result in a partial response without impacting cancer stem cell viability and eventual disease progression.

One promising cancer progenitor cell antigen is *HER2/neu*, a transmembrane receptor tyrosine kinase of the EGF receptor family, which is overexpressed in approximately 20% of human breast cancers and sporadically in other cancers [15-17]. Exogenous *HER2* expression in normal mammary epithelial cells or tumor lines induces features of stem cells while pathology of early neoplastic lesions link *HER2/neu* to carcinogenesis, tumor growth and invasion [18, 19]. *HER2* is an attractive target for active immunotherapy since antibody and T cell responses are naturally present in patients with *HER2* expressing breast cancer indicating its potential immunogenicity [20-22]. Early clinical studies with single HLA-A2 binding peptide demonstrated transient peptide-specific immune responses but no difference in long-term disease progression [23]. However, encouraging results have been obtained by use of multiple longer peptides that can be processed to yield MHC I and MHC II epitopes [24]. To examine T cell immunotherapy, we used FVB/*neuN* transgenic (*neu-N*) mice which express rat *neu* from an MMTV promoter and develop undifferentiated breast tumors at five months of age [25].

In previous studies with carcinogen-induced tumors we demonstrated that a minor subset of CD62L low T cells in vaccine-draining lymph nodes could be expanded to large numbers *in vitro* to mediate regression of established tumors [26-29]. Here, we adapted our approach of T cell adoptive transfer to explore whether spontaneously arising *HER2/neu* breast tumors could be targeted in the presence of host tolerance. We found that progenitor cell vaccines induced a superior sensitization of T cells reactive against *HER2/neu* and other tumor antigens. Interestingly, treated mice developed a humoral immune response raising the prospect that the transient efficacy of passive immunotherapy with trastuzumab could be augmented by induction of a sustained host T cell and Ig response.

Materials and methods

Mice and derivation of tumor lines

FVB-*neuN* transgenic (*neu-N*) mice were purchased (Jackson laboratory) and breeding colonies were established. Mice were housed in a specific pathogen-free environment according to National Institute of Health guidelines using an Institutional Animal Care and Use Committee approved protocol.

Five mouse mammary carcinoma (MMC) cell lines were derived from spontaneous tumors arising in *neu-N* mice harvested at 7 to 10 months of age. Cell lines were maintained *in vivo* by serial s.c. transplantation in *neu-N* transgenic mice and as *in vitro* cell lines. Tumor progenitor cells were selected by growing minced tumor as non-adherent tumorspheres in serum-free DMEM:F12 supplemented with EGF (20 ng/ml), FGF-2 (20 ng/ml) (PeproTech Inc), and 1:50 B27 (Invitrogen). Minced tumor was alternatively cultured in RPMI 1640 supplemented with 10% fetal bovine serum to generate matched adherent cell lines. A *HER2/neu* negative antigen-loss variant cell line (C2D1) was clonally selected *in vitro* for use in experiments testing the immunogenicity of antigens other than *HER2/neu*. In one experiment, we separated the MMC10 tumorsphere cell into CD44^{pos}CD24^{pos} and

CD44^{pos}CD24^{neg} subsets by MACS column (Miltenyi Biotec) to test the relative immunogenicity of these two populations.

FACS analysis and intracellular IFN- γ staining (ICS)

FITC, PE, or Cychrome-conjugated mAb against CD4, CD8, TCR- β , CD62L, CD24, CD44, Sca-1, H-2K^a, H-D^a/H-2L^a, I-A^a, IFN- γ , and isotype-matched Ig were purchased (BD Biosciences, Mountain View, CA). PE-conjugated mAb against CD29 and CD133 were purchased from (eBioscience). Neu protein expressed by Tumorspheres or MMC was measured with indirect fluorescence staining (Ab-4Ab, Calbiochem, San Diego, CA). Stained cells were analyzed using the CellQuest Software. H-2D^a tetramer was provided by (NIH Tetramer Core Facility, Atlanta, GA) loaded with the known rNeu peptide epitope 420-429. T cell subsets were assayed for production of intracellular IFN- γ by stimulating T cells with a single cell suspension of *in vivo* tumor digest at a 1:1 ratio, or anti-CD3 mAb, or no stimulation. Brefeldin A was added at 5 hours and cells were harvested overnight, and then stained and analyzed by FACS as previously described [30].

In vitro activation of vaccine-draining LN T cells

Neu-N transgenic mice were inoculated s.c. with 2×10^7 irradiated tumor cells in the lower flank region bilaterally. Nine days later, vaccine-draining lymph nodes were removed and a single-cell suspension was prepared. CD62L^{low} T cells were isolated by depletion of CD62L^{high} cells with MACS beads (Miltenyi Biotec). CD4⁺ or CD8⁺ T cell subsets were separated with MACS beads. T cells were suspended in CM at 2×10^6 /ml and activated with plate-bound anti-CD3 monoclonal antibody for 48 hours then were cultured in CM supplemented with IL-2 (4U/ml) and rmIL-7 (10ng/ml) in 24-well plates. On day 5, the cells were diluted to 2×10^5 /ml and incubated for additional 4 days, or extended to 23 days following re-stimulation according to our previous methods [29].

Adoptive immunotherapy and serum IgG immunoreactivity

Therapeutic efficacy of culture-activated T cell subsets was assessed in 8-week old neu-N transgenic mice. Pulmonary metastases were generated by i.v. injection of 8×10^5 MMC tumor digest cells. In some experiments mice received TBI (5 Gy) from a ¹³⁷Cs Irradiator prior to T cell therapy. On day 3 post inoculation groups of 3 to 4 mice each received CD4⁺, CD8⁺ T cells or combined subsets, or no cells. Mice were sacrificed on days 30 to 34 after inoculation of tumor, and the lungs were insufflated with India ink and the number of tumor nodules on the surface was enumerated. In some experiments, blood was obtained before lungs were harvested. Serum IgG reactive against neu^{pos} or neu^{neg} progenitor cells or MMC was determined by flow cytometry. Efficacy against development of primary tumors was tested by T cell adoptive transfer into 6 week old Neu-N mice.

Statistical analysis

The significance of differences between groups is analyzed by the Kaplan-Meier Log-Rank test, or by the Student's t test. A two-tailed p value of <0.05 is considered significant.

Results

Progenitor cells in primary breast tumors and tumorsphere cultures

Cell lines derived from single cell digest of mouse mammary carcinomas (MMC) formed a monolayer with tight junctions and epithelial morphology when cultured in serum-containing medium (**Figure 1A**). By contrast, the same tumor digest cultured in serum-free medium supplemented with EGF and FGF-2 developed tumorspheres (**Figure 1B**). The primary tumors as well as *in vivo* passaged tumors demonstrated high uniform expression of

neu, CD24, and CD29 but only a subset of tumor cells expressed other progenitor cell-associated markers CD44 and CD133 (**Figure 1C**). Besides the morphologic difference between adherent and tumorsphere cultures, CD44 was only expressed on tumorspheres and CD133 was upregulated (**Figure 1D**). Moreover, as few as 2×10^2 tumorsphere cells formed tumors in immunocompetent neu-N hosts (2/3 mice). The MMC tumors have been maintained *in vivo* by serial subcutaneous transplantation in syngeneic neu-N transgenic mice similar to our previous approach [30].

MMC tumors prime T cells against neu and other cross-reactive tumor antigens

Neu antigen and MHC I molecules are stably expressed at high levels following repeated passage *in vivo* in Neu-N mice indicating that there is no immune-selective pressure in tolerant mice. By contrast, neu-positive tumors are rejected in the non-transgenic parental FVB mice through the immunodominant H-2D^q restricted rat neu epitope 420-429 which differs from mouse neu at 3/10 amino acids [31, 32]. We inoculated neu-N mice s.c. with 2×10^7 irradiated MMC10-Adherent tumor cells and vaccine-draining lymph nodes were removed 9 days later from which CD62L^{low} or CD62L^{high} subsets were separated then activated with anti-CD3/IL-2/IL-7 for 9 days [30]. Activated cells consisted of nearly a 1:1 mixture of CD4⁺ and CD8⁺ T cells in each of the CD62L^{low} and CD62L^{high} cultures. As shown in **Figure 2A**, adoptive transfer of 5×10^7 vaccine-primed CD62L^{low} T cells, eradicated MMC10 lung metastases whereas 5×10^7 CD62L^{high} T cells had no therapeutic effect. There was also a dramatic, nearly 10-fold difference, in the percentage of CD62L^{low} T cells which produced IFN- γ when stimulated with MMC10 tumor digest compared with the CD62L^{high} T cell cultures (**Figure 2B**).

We next tested whether irradiated MMC10 tumorsphere cells could stimulate a response in vaccine-draining lymph nodes. In this experiment, CD8⁺ and CD4⁺ T cell subsets were separated prior to culture activation and then mixed prior to adoptive transfer into recipients bearing 3-day established lung metastases of MMC10 or MMC1. As demonstrated (**Figure 2C**) there was nearly complete eradication of lung tumors in treated mice. All of the MMC tumor lines express neu and the T cells showed significant cross-reactivity when stimulated with MMC4 tumor digest *in vitro* or *in vivo*. Additional experiments demonstrated cross-reactivity among MMC4, MMC10, and MMC15 tumors. Cross-reactivity is at least partially attributable to neu antigen because 2.6% of the CD8⁺ T cells bind H-2D^q tetramer loaded with the known peptide epitope 420-429 (**Figure 2D**) [33]. Thus, activated CD62L^{low} T cells derived from LNs draining neu^{pos} tumor vaccines have therapeutic effects and mediate the regression of established neu^{pos} pulmonary metastasis despite the presence of tolerance to neu during T cell sensitization in neu-N transgenic mice.

To determine whether neu was the exclusive cross-reactive antigen, we vaccinated mice with single cell tumor digest from the neu^{neg} antigen-loss variant line C2D1. Although mice were not primed against neu, they developed strong responses against other antigens on C2D1 tumor. T cells were cross-reactive with the MMC10 tumor when tested in the intracellular IFN- γ production assay. More importantly, a cross-protective response was observed against established MMC10 pulmonary metastases, or when T cells were co-inoculated with tumor subcutaneously (**Figure 3**). This indicates that there are other tumor rejection antigens besides neu which are expressed by multiple independently arising breast carcinomas in neu-N mice.

Enhanced potency of tumorsphere vaccines

Ideally, a tumor vaccine should stimulate a response that will eliminate progenitor cells. Because they are usually a minor population in tumor biopsies or conventional tumor cell lines it raises the question whether the most abundant antigens in bulk tumor preparations

sufficiently represent progenitor cells. We tested whether irradiated whole cell tumor vaccines prepared from tumorsphere cultures had equivalent immunogenicity compared with the same tumor cell line cultured as an adherent monolayer. We used irradiated tumor cells to avoid any changes in antigen expression that might occur during tumor cell proliferation *in vivo*. The yield of total cells per vaccine-draining LN was not substantially different (10.5×10^6 vs. 10.9×10^6 for MMC10-Tumorspheres or MMC10-Adherent respectively). Likewise, the yield of sensitized CD62L^{low} cells was similar (0.6×10^6 vs. 0.8×10^6 per LN). However, there was a significant augmentation of anti-tumor efficacy against lung metastases when tumorsphere vaccine was used to prime either CD4⁺ ($p < 0.05$) or CD8⁺ ($p < 0.01$) T cells vs. untreated control or adherent-vaccine cells (**Figure 4 A**). An important feature of this experiment is that the recipients did not receive lymphodepletion with TBI prior to T cell transfer so effector responses occurred in the presence of host regulatory cells. This experiment was repeated with similar findings. The anti-tumor efficacy of CD4⁺ T cells against the MHC II negative MMC tumors is similar to our findings with other tumor models and is mediated through cross-presentation of tumor antigens by APC residing within the tumors [34]. It is notable that the T cells derived from tumorsphere-primed lymph nodes contained a higher percentage of CD8⁺ and CD4⁺ T cells that produce IFN- γ than do the adherent cell vaccine derived T cells (**Figure 4B**). However, the percentage of T cells responding to the defined Neu epitope 420-429 was similar (**Figure 4C**) suggesting that a broader immunologic response was elicited by the tumorsphere cells.

Although CD44 and CD133 are commonly accepted markers for tumor stem cells, there may be other markers that define additional subsets. For example, in human breast cancers the CD44^{high}CD24^{low} phenotype has been associated with stem-like cells [6]. It is not known whether CD24^{low} precursor cells define progenitor cells in mouse breast cancers driven by HER2/*neu*. As depicted in **figure 5A**, a small population of CD44^{high}CD24^{low} cells (<1%) is present in tumorsphere cultures. After two cycles of enrichment through negative selection, tumor cells were effectively separated into stable CD44^{high}CD24^{low} and CD44^{high}CD24^{high} subsets (**Figure 5A**). To test their immunogenicity, we inoculated 2×10^7 irradiated CD44^{high}CD24^{low} or CD44^{high}CD24^{high} cells subcutaneously to sensitize draining lymph nodes in neu-N transgenic mice. T cell subsets were purified from vaccine-draining LNs then activated *in vitro* and 3×10^7 CD4⁺ or CD8⁺ T cells were adoptively transferred into non-lymphodepleted hosts bearing 3-day MMC10 pulmonary metastasis. In two independent experiments, T cells primed with either CD44^{high}CD24^{low} or CD44^{high}CD24^{high} vaccines had equivalent therapeutic activity against MMC10 pulmonary metastases (**Figure 5B**). In addition, each vaccine primed a similar frequency of CD8⁺ or CD4⁺ cells that could produce IFN- γ in response to neu^{pos} MMC10, MMC1 or MMC15 tumor digest (**Figure 5C**). Thus, we did not observe a substantial effect on the immunologic response induced by CD44^{high}CD24^{low} versus CD44^{high}CD24^{high} tumor cells. This finding is consistent with xenograft experiments by other investigators demonstrating that CD24^{pos} cells have tumor initiating properties of progenitor cells [35].

T-cell mediated tumor regression also induces a serologic response

Immunologically-mediated regression of tumor is anticipated to liberate tumor antigens under inflammatory conditions which might prime additional T cells or B cells. In our previous adoptive immunotherapy studies using carcinogen induced tumors with unique antigens, we observed that T cell-mediated tumor regression led to a secondary wave of host T cells that recognized cross-reactive tumor antigens [36]. We pooled serum from mice harboring lung metastases and treated with adoptive transfer of either CD4 or CD8 T cells. Tumor cells were blocked with anti-FcR antibody and incubated with serum then stained with anti-IgG and analyzed by FACS (**Figure 6A**). The mice treated with either CD4⁺ or CD8⁺ T cells subsequently developed IgG which bound to MMC10 targets derived from

adherent or tumorsphere cultures. Cell surface antigens other than neu were recognized because IgG from treated mice also bound the neu-negative C2D1 cells. There was no development of antibody in controls which did not receive T cells indicating that this response was associated with the process of tumor regression rather than exposure of hosts to tumor antigens during progressive growth of lung metastases. CD4 help for host B cells could have come directly from adoptively transferred CD4⁺ T cells but was mediated through a secondary wave of host CD4⁺ T cells in the recipients of transferred CD8⁺ cells.

Adoptive transfer of T cells postpones the development of spontaneous primary tumors

The ability to generate T cell reactivity against neu and other shared tumor antigens led us to test whether adoptive transfer of tumor-reactive T cells could delay the subsequent development of spontaneous breast tumors in neu-N mice. We activated MMC10-tumorsphere primed lymph node T cells *in vitro* as in previous experiments. We then transferred a combination of CD4⁺ and CD8⁺ T cells into neu-N mice at 6 weeks of age, well before overt tumor development and at a time when neu is not actively expressed. Whereas untreated control mice developed tumors at a median of 29 weeks, the treated mice remained tumor free for a median of 36 weeks and overall tumor onset was delayed ($p < 0.01$) (**Figure 6B**). Prevention of primary tumor growth in non-lymphodepleted hosts is a stringent experimental model because it requires the transferred T cells to establish memory and then respond during early tumor growth in peripheral tissue in the presence of host Treg cells.

Discussion

This study demonstrates that a therapeutic immune response can be generated against HER-2/*neu* and other shared antigens on breast cancer progenitor cells. An important aspect of the Neu-N tumor model is its relevance for strategies to overcome tolerance to self antigens expressed by tumors. We were able to isolate recently-sensitized CD62L^{low} T cells during the initial stages of vaccine priming that could subsequently be activated *in vitro* with anti-CD3 mAb and IL-2/IL-7 to acquire therapeutic efficacy. This indicates that separating T regulatory cells from antigen-primed cells prior to *ex vivo* activation allowed full acquisition of effector function. The H-2D^d tetramer allowed us to document this process for a known immunodominant T cell determinant but an analogous process likely occurred for other tumor antigens. Although it is possible that some of the other T cells were reactive to subdominant HER-2/*neu* epitopes that have been described [37-39] our data using the Neu^{neg} subclone C2D1 prove that other shared antigens contribute to the immune response.

The protective immune response elicited by HER2/*neu* and other shared tumor antigens has translational relevance. Sequencing and CGH studies in human breast cancer indicate that, although the majority of changes in each tumor are unique, there is a high frequency of recurrent mutations in known oncogenes [40, 41]. Thus cancer vaccines directed against shared tumor antigens such as *HER2/neu* that are functionally linked to malignancy-specific gene expression programs will be more broadly applicable [18]. Cancer-testes antigens are another example of shared tumor antigens expressed by cancer stem cells which have the desirable property of minimal expression in normal tissue stem cells [42]. We are currently trying to identify the other shared tumor antigens recognized by IgG or T cells in our experiments because they may have important functional roles in breast cancer development.

The delay in onset of primary tumors by adoptive transfer of effector T cells is a stringent test of the ability of T cells to establish memory and then respond to tumor during early stages of initiation. Moreover, the observation that mice treated with tumorsphere-primed CD4⁺ or CD8⁺ T cells developed a humoral response, whereas non-treated tumor-bearing

mice were non-reactive is noteworthy. The development of an IgG serologic response following effective T cell-mediated therapy indicates that CD4⁺ T cells provided cognate helper function and immunoglobulin class switch signals to B cells and reversed tolerance to neu as well as other shared tumor antigens. It has been reported that collaboration of cellular and humoral immune response can provide effective elimination of *HER2/neu* disseminated positive tumor cells in humans [43]. Experiments in BALB-neuT mice have also demonstrated that prime-boost vaccination strategies against *HER-2/neu* generate immunoprotection which is dependent on T cell production of IFN- γ and a humoral response [44]. CD8⁺ T cells would be expected to mediate direct killing of target cells but our previous studies indicate synergistic efficacy when anti-tumor CD4⁺ T cells are also provided. The CD4⁺ T cells also have effects on tumor-associated macrophages which can present antigens via MHC-II molecules. Local cytokine production by activated CD4⁺ T cells can also enhance phagocytic activity of macrophages and prime them for ADCC mediated through tumor-reactive IgG.

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Figure1. Phenotype of MMC primary tumors and cell lines

A. MMC10-Adherent cells cultured with serum grow as a polygonal monolayer with tight junctions (100X). **B.** MMC10-BCSC cultured in serum-free medium with EGF and FGF2 display tumorsphere morphology (100X). **C.** MMC15 primary tumor digest and *in vivo* passage 3 digest stained with the indicated antibodies and analyzed by FACS. The number represents the percentage of positive cells in the dot-plot quadrant. **D.** MMC10-Adherent or Tumorsphere cultures were stained for the indicated markers. The mean fluorescence intensity (MFI) is indicated for each histogram.

Figure 2. Immunologic reactivity of T cells primed with adherent or tumorsphere vaccines

A. Lymph node T cells primed with MMC10-Adherent cells were segregated into CD62L^{low} or CD62L^{high} subsets then activated *in vitro* for 9 days. T cells were transferred i.v. to recipients bearing 3-day lung metastases and conditioned with 5 Gy TBI (n=3 per group). Lung metastases were counted on day 34 with too numerous to count assigned a value of 250. **B.** Intracellular IFN- γ assay of T cells was performed at the time of adoptive transfer. T cells were incubated with single cell tumor digest of the indicated tumor or anti-CD3 mAb. Numbers indicate the percentage of IFN- γ positive cells (upper right quadrant) for CD8⁺ T cells (left two panels) or CD4⁺ T cells (right two panels). **C.** Lymph node T cells primed with MMC10-Tumorsphere cells were selected for CD62L^{low} then separated into CD4⁺ and CD8⁺ subsets and separately activated. T cells were transferred i.v. to recipients with 3-day lung metastases. **D.** Cultured LN T cells were incubated with H-2D^d tetramer loaded with rat HER2/*neu* 420-429 decamer peptide and analyzed by FACS.

Figure 3. Neu^{neg} subclone sensitizes T cells against other shared tumor antigens

A. C2D1 (neu^{neg}) or MMC10 (neu^{pos}) tumor digest was stained for HER2/*neu* expression.

B. Lymph node T cells sensitized with C2D1 (neu^{neg}) vaccine were separated into CD62L^{low} CD4⁺ or CD8⁺ subsets and activated *in vitro* then were incubated with the indicated tumor digest and stained for intracellular IFN-γ with percentage of responding cells indicated in the right upper quadrant. **C.** T cells were transferred i.v. to hosts bearing 3-day lung metastases or were **D.** co-inoculated with tumor cells subcutaneously and monitored for tumor size (n=3 per group).

Figure 4. Tumorsphere cells are more immunogenic than adherent cell vaccines

A. Vaccine-primed CD62L^{low} T cells were segregated into CD4⁺ or CD8⁺ subsets and activated *in vitro* for 2 cycles of stimulation then adoptively transferred to non-lymphodepleted mice bearing 3-day MMC10 lung metastases. On day 34 the number of lung metastases was counted, each dot represents one animal. Tumorsphere-primed CD4⁺ ($p < 0.05$) vs. Adherent-primed CD4⁺. Tumorsphere-primed CD8⁺ ($p < 0.01$) vs. Adherent-primed CD8⁺. **B.** CD62L^{low} CD4⁺ or CD8⁺ lymph node T cells primed by MMC10-Tumorspheres or MMC10-Adherent cells were incubated with a single cell tumor digest of MMC1, MMC10, or anti-CD3 mAb, overnight then stained for intracellular IFN- γ with percentage of responding cells indicated in the right upper quadrant. **C.** CD8⁺ T cell cultures were incubated with H-2D^d tetramer loaded with rat neu 420-429 decamer peptide and analyzed by FACS.

Figure 5. CD44^{pos}CD24^{pos} and CD44^{pos}CD24^{neg} Tumorsphere subsets are both immunogenic
A. MMC10-tumorsphere cells were separated into CD24^{low} or CD24^{high} subsets by MACS on day 1 and day 17 and maintenance of this phenotype *in vitro* was monitored. **B.** Mice were primed with CD44^{pos}CD24^{pos} or CD44^{pos}CD24^{neg} tumor cells and draining LN CD62L^{low}, CD8⁺, or CD4⁺ T cell subsets were activated *in vitro* for two cycles. CD4⁺ or CD8⁺ T cells (3×10^7) were adoptively transferred into non-irradiated neu-N mice with established 3-day MMC10 lung metastasis. **C.** Intracellular IFN- γ production by cultured CD8⁺ or CD4⁺ T cells from LNs primed with CD24^{high} tumorsphere vaccine (Black bar) or CD24^{low} tumorsphere vaccine (gray bar).

Figure 6. *In vivo* priming of an IgG response and memory cell prevention of primary tumor
A. Neu^{pos} MMC10-Adherent, MMC10-Tumorsphere, or neu^{neg} C2D1 cells were mixed with serum from tumor-bearing mice (1:4 dilution) (filled histogram) or 6 week old control Neu-N mice (gray line) then FITC-conjugated anti-mouse IgG. Serum was obtained from neu-N mice with established pulmonary tumors without treatment (upper row) or following CD8⁺ (middle row) or CD4⁺ (lower row) T cell therapy. Numbers indicate percentage of positive cells. **B.** Neu-N mice at 6 weeks of age received a combination of CD4⁺ and CD8⁺ T cells derived from MMC10 tumorsphere primed LNs that were activated *in vitro*. Control mice (N=7) or treated mice (N=6) were monitored for palpable mammary tumors.