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MHC class II-mediated spontaneous rejection of breast carcinomas expressing model neoantigens

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ABSTRACT

Background Cancers persist despite expression of immunogenic neoantigens and ongoing antitumor immune responses. While some occult tumors likely are cleared by effective antitumor immune responses, the targeted antigens are not easily identifiable as those tumors spontaneously disappear.

Methods We used mouse models with a defined antigenic protein mimicking tumor-specific neoantigens to address the nature of these spontaneous anti-tumor immune responses.

Results BALB/c (*H-2*^{d}) mice challenged with BALB/c breast tumors expressing the rat-erbB2 oncoprotein succumb to their tumors despite ongoing immune responses targeting tumor-specific model antigens. Meanwhile, congenic BALB.B (*H-2*^{b}) and *H-2*^{d}/*H-2*^{b} F1 hybrid mice spontaneously eliminate genetically matched tumors in a major histocompatibility complex (MHC)-II dependent manner. Adoptive transfer and immune cell depletion strategies revealed CD4+ T cells and CD20+ B cells are crucial mediators of the protective response in *H-2*^{b} mice. Furthermore, passive transfer of immune serum from mice rejecting their tumors confers resistance in tumor antigen-tolerant animals with an inversely proportional relationship between tumor outgrowth and the amount of rat-erbB2 specific antibody present in tumor-bearing mice. Introduction of the rat-erb2 ectodomain into other *H-2*^{b} tumor models also promotes their spontaneous tumor rejection. Notably, the tumor microenvironments differ in rat-erbB2+ tumor-bearing BALB.B and BALB/c mice at the time of fate decision in the models reflecting the differences between effective and ineffective tumor immune responses.

Conclusions We find that the effective antitumor immunity targeting neoantigens in these breast cancer models is determined by MHC-II-restricted presentation of optimal cancer-associated antigens. These responses are dependent on CD4+ T cells, B cells, and antigen-specific antibodies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Tumors develop and persist despite expressing numerous tumor-specific neoantigens that promote active immune responses targeting the tumor cells. Rarely, tumor neoantigens are generated that spontaneously invoke, without intervention, an effective clearance of the mutated tumor cells. The nature of such "optimal" antigens capable of inducing a protective response and how they work is not understood.

WHAT THIS STUDY ADDS

⇒ The present study develops and uses new mouse models expressing a model neoantigen to investigate the nature and mechanisms mediating effective spontaneous antitumor immunity. The study demonstrates the importance of major histocompatibility complex class II genotype in determining the outcome of the antitumor immune response and accompanying changes in the tumor microenvironment, defining protective CD4+ T cells, B cells, and resulting tumor antigen-specific antibodies as the basis of effective spontaneous immunity to these tumors. The introduction of this same antigen into other tumors that normally persist despite ongoing host immune responses also promotes spontaneous protective immunity demonstrating the generalizable relevance of the emerging findings.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ While much ongoing research seeks to boost the immune system to target cancer neoantigens, this study delineates a mechanism by which the immune system is naturally effective. The new mechanistic insights emerging from this study can be used to design new therapies mimicking naturally effective immune responses to overcome tumor defenses and promote tumor eradication.

BACKGROUND

While the immune system can target cancer, the range of mechanisms employed is not fully understood. Despite numerous efforts to use immunotherapy to treat cancer,[1–3] over 600,000 individuals lost their lives to cancer in 2024 (NCI Cancer Stat Facts). This indicates a need for a more complete understanding of how the immune system can be harnessed

for prevention and treatment. Shankaran *et al* demonstrated that that cancers developing in immunodeficient mice are more immunogenic than tumors developing in immunocompetent hosts, documenting the importance of the immune system in preventing or shaping developing tumors.[4] These seminal findings have been extended by delineating the different immune cells and molecules important for underlying anticancer protection.[5] The observation of spontaneous tumor regression or loss of tumor-associated antigens in human patients with multiple independent primary cancerous lesions in immunocompetent patients supports this perspective that spontaneous tumor immunity shapes cancer outcomes.[6–9]

While it is known that antigenic tumors are often present in immunocompetent individuals, the mechanisms determining why one immunocompetent individual succumbs to a tumor while a second individual does not are not understood.[10] The ability to determine the differences between individual immune responses against very similar tumors in a controlled setting would benefit our understanding of these mechanisms. Resulting new insights might also help decipher how immunity can be boosted using cancer vaccines to improve survival outcomes.[3 11]

Here we employ variants of the BALB/c-neuT mouse mammary tumor model which expresses an oncogenic form of the rat-erbB2/neu protein to address differences between effective and ineffective immunity in the spontaneous immune response to cancer antigens.[12 13] The rat-erbB2 protein differs by 66 amino acids from its mouse homologue,[14] providing a defined set of pseudo-neoantigens in this model. When a cell line derived from BALB/c-neuT breast tumors is transplanted into BALB/c wild type (WT) mice, carcinomas grow out despite the immunogenicity of the rat-neuT+ tumor cells and the ensuing antitumor immune response.[12] Thus, the scenario in which BALB/c mice are challenged with immunogenic BALB/c-neuT tumor cells models the usual failure of an active immune response to target a tumor antigen effectively and protect against developing cancer.

A new variant of this model exemplifies the successful mobilization of an effective and protective spontaneous response against breast ductal carcinomas expressing the same rat-*neuT* oncogene. The major histocompatibility complex (MHC) congenic variant BALB.B expresses the MHC *H-2*^{b} genes on the BALB/c background.[15] The *neuT* transgene was introduced onto the BALB.B *H-2*^{b} congenic line to generate mice which spontaneously develop tumors for transplantation into BALB.B parental mice to evaluate the contribution of MHC genes on the unfolding spontaneous antitumor immune response. We found that BALB.B-neuT tumors were spontaneously rejected by BALB.B-WT mice in an MHC-II dependent manner. This rejection was mediated by CD4+ T cells and B cells and protection was transferred passively with antibodies to permissive animals. We used the principles learned from our BALB-neuT models to modify other established tumor models to promote spontaneous tumor resistance. Comparison of the models provides a roadmap for understanding how spontaneous antitumor immune responses can result in either protective or non-protective immunity and could lead to generalizable insights into how an otherwise inadequate immune response can be manipulated to become protective.

METHODS

Antibodies and Reagents

Flow cytometry, CyTOF, depletion/purification antibodies and reagents, western blot, and ELISA reagents are annotated in online supplemental tables 1-5.

Mouse models

Mice on the BALB/c background transgenic for a rat-erbB2 oncogene (BALB/c-neuT) were bred at Mayo Clinic with permission from Guido Forni (University of Turin, Italy). BALB.B-neuT mice were derived from BALB/c-neuT mice by intercrossing with BALB.B mice, which along with BALB/cJ and CB6F1/J were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). BALB/c-rat-erbB2+ mice containing the WT rat-erbB2 coding sequence encompassing exons 2–27 stop with the SV40 late polyadenylation signal sequence inserted into the mouse *ERBB2* locus were generated in the Mayo Clinic Transgenic and Knockout Core. Rat-erbB2 tolerant mice, BALB.B-rat-erbB2, were derived subsequently by intercross. BALB.B-neuT, BALB/c-neuT, and BALB.B-rat-erbB2+ mice were maintained by breeding a heterozygous neuT+ or rat-erbB2+ male to a WT female. Genotypes were determined by PCR using oligonucleotide primers for neuT (GTAACACAGGCAGATG TAGGA and ATCGGTGATGTCGGCGATAT) or *ERBB2* (ATCCTCAGGACAATGTCGCCG and GCGCAGGCT-GCACACTGAT). MHC genotypes were determined by analyzing PBMCs for K^b or K^d by flow cytometry. Female mice (8–12 weeks) were used in all experiments. Males responded in a similar manner (data not shown). Animals identified by ear punch were dispersed in different treatment groups among litters.

Generation and transplantation of tumor cell lines

BALB.B-neuT and BALB/c-neuT female mice generate spontaneous tumors by 15–20 weeks of age. Tumors from female mice were dissociated, cultured in Roswell Park Memorial Institute 10% fetal bovine serum, and frozen. Thawed tumor cells were cultured until near confluence, harvested, washed and resuspended in phosphate-buffered saline (PBS). 4T1-rat-erbB2+/GFP+ cells were generated by transduction with a lentivirus and 4T1-rat-erbB2+/GFP−, mouse/rat chimeric erbB2 cell lines, and B16F10-rat-erbB2+ were generated by transduction with retroviruses. 10^6 cells (unless otherwise indicated) were injected subcutaneously in the second right mammary fat pad. Tumor diameters were estimated using a caliper as the square root of the width/length product.

Survival analysis

Mice were monitored for tumor growth weekly, until they reached a tumor diameter of 13 mm and then euthanized as specified by the Mayo Clinic Institutional Animal Care and Use Committee.

Flow cytometry

Rat-erbB2 expression was measured on tumor cell lines by staining cells with a viability dye followed by mouse monoclonal anti-rat-erbB2 antibody clone 7.16.4 or immune sera followed by secondary antibodies. Rat-erbB2 expression by the cell lines was checked prior to implantation of the tumor cell lines into recipient mice.

Serum was harvested from clotted blood, pooled, transferred to a new tube, and frozen once. Prior to the assay, serum was thawed and diluted in Fluoresence-Activated Cell Sorting buffer. 30 uL diluted serum was added to 10^5 4T1 cells expressing or not expressing rat-erbB2. Cells were washed 3× prior to secondary antibody staining. Median fluorescence intensity was read for both stained 4T1 and 4T1-rat-erbB2+ cells to verify the presence of rat-erbB2-specific antibodies.

For high parameter flow cytometry analysis of tumors, mice were euthanized and perfused with PBS to deplete circulating lymphocytes from recovered tumors, prior to their dissection and enzymatic dissociation. Tumor cells were stained with viability dye in PBS and antibodies in FACS buffer. Data were analyzed in FlowJo V.10 (BD Life Sciences) by gating first on CD45+ cells and then performing Uniform Manifold Approximation and Projection (UMAP) and Phenograph dimensionality reduction.

The frequencies of CD4+, CD8+, and B220+ cells per µL were assessed in whole mouse blood collected in heparinized test tubes. 20 µL whole blood was added to a master mix containing staining antibodies in PBS. Erythrocytes were lysed using Ammonium-chloride-potassium (ACK) lysis buffer for 10 min, and then an equal volume of PBS was added. Counting beads were added before recording samples.

CyTOF analysis of tumor microenvironments

Tumor cells were isolated from mice at 2.5 weeks post inoculation. Tumors were dissociated, and cells were resuspended in 1 mL of cell staining buffer (CSB). Each sample was incubated with 0.5 um Cisplatin solution in PBS. Samples were then washed twice with CSB. Samples were incubated with extracellular markers in CSB for 45 min. After washing twice with CSB, cells were permeabilized, washed, and resuspended in permeabilization buffer before adding intracellular markers and incubating at room temperature for 45 min. Cells were washed, fixed with 2% paraformaldehyde for 30 min, resuspended in intercalation solution, and incubated overnight at 4°C. Cells were washed with PBS and resuspended with calibration beads and cell acquisition solution.

After acquisition, intrafile signal drift was normalized to the calibration bead signal. File cleanup was performed using FlowJo. Fcs files were analyzed using Cytofkit V.3.8 in R.[16] Clustering and dimensionality reduction were performed using R Phenograph.[17 18] Clusters were visualized on a t-distributed stochastic neighbor embedded (tSNE) plot.[17 18] For CD45+ reanalysis of this data, CD45+ cells were identified by gating on the cleaned fcs files and analyzed performing UMAP and Phenograph analyses in FlowJo V.10.

Adoptive transfer of splenocytes

BALB.B mice challenged with 10^6 tumor cells rejected their tumors in ~8 weeks and were rechallenged with tumor. After another 1–2 weeks, mice were euthanized, splenocytes harvested, depleted of red blood cells by ACK lysis for 1–3 min, and resuspended in PBS. Some splenocytes were depleted of CD4+, CD8+, or B cells according to manufacturer's protocols, while others were used directly for intravenous adoptive transfer. Cell depletions and isolations were performed using negative selection with kits listed in online supplemental table 4. Cell depletions and isolations were verified to be greater than 90% by flow cytometry. Cells were harvested 4 weeks after primary tumor challenge from BALB/c and BALB.B and used directly in adoptive transfer experiments to assess differences between permissive and resistant antitumor responses.

Sera adsorption and passive transfer

Serum was collected from tumor-challenged BALB.B-WT mice and stored until ready for use. For adsorption, sera were incubated with 10^7 4T1 cells or 4T1-rat-erbB2+ cells per mL for 1 hour at room temperature. Cells were spun out of serum, and the incubation was repeated 6×. Adsorbed sera were assayed to verify antigen-specific antibody depletion (approximately 66.7%). Sterile filtered sera (600 uL) were intraperitoneally transferred into mice 5, 9, and 13 days after tumor inoculation.

Antibody depletion of CD4+, CD8+, and CD20+ cells

CD4 and CD8 depleting antibody (100 µg) and CD20 depleting antibody (150 µg) were injected intraperitoneally into mice in PBS. Mice were injected 3 times at 3-day intervals before tumor inoculation and weekly after tumor inoculation.

Figures

Figures were created using GraphPad Prism V.10 and BioRender (biorender.com).

Statistical analysis

Statistical analyses were performed in GraphPad Prism V.10. Data were analyzed as indicated (α=0.05).

RESULTS

The BALB.B-neuT model

The BALB/c-neuT and BALB.B-neuT breast cancer models are both driven by the same rat-erbB2 oncogene (neuT) expressed under regulation of the MMTV

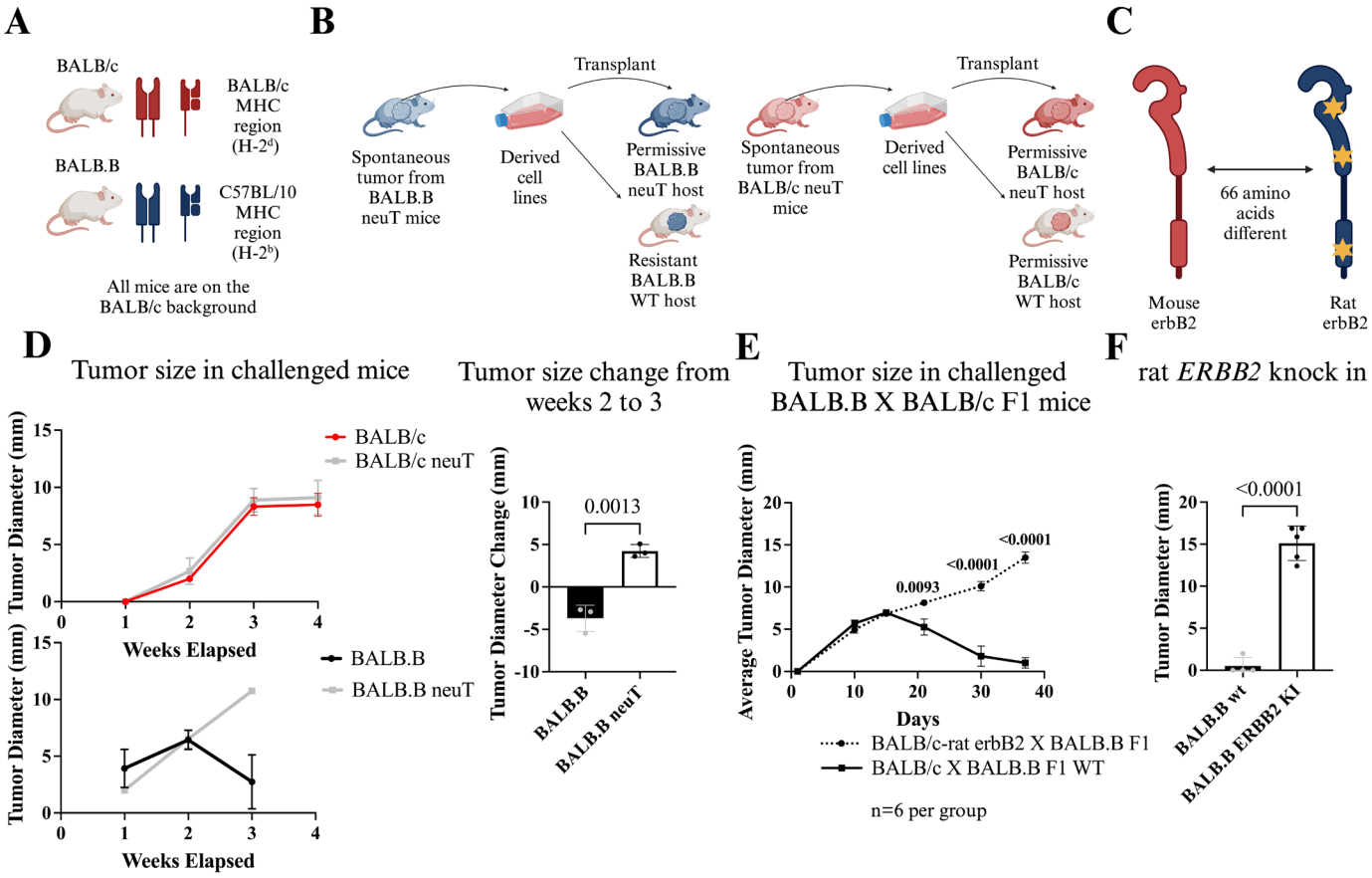

Figure 1 BALB.B-WT mice reject BALB.B-neuT tumors by targeting the rat neu antigen. (A) Schematic of BALB/c and BALB.B mice, with genetic differences in MHC-I and MHC-II genes. (B) Schematic of rat-neuT+ tumor challenge into genetically matched BALB/c and BALB.B hosts. (C) Schematic depicting 66 dispersed amino acid differences between the mouse and rat-erbB2 proteins. (D) Tumor growth of BALB/c-neuT (left top, n=4) or BALB.B-neuT (left bottom, n=3) tumors transplanted into the recipient mice as indicated. Tumor diameter changes from weeks 2 to 3 were measured and compared for BALB.B mice (right). Data were analyzed using the Student's unpaired t-test. (E) Tumor diameter of BALB.B X BALB/c-neuT tumors harvested in the same manner as depicted in B and transplanted into the indicated recipients. Data were analyzed by multiple t-tests accounting for multiple comparisons. (F) Tumor diameter of BALB.B-neuT tumors measured 40 days after transplantation into BALB.B-WT mice or BALB.B-rat-erbB2 knock-in mice were analyzed as in D. MHC, major histocompatibility complex; WT, wild-type.

promoter.[19] The two models differ with respect to alleles linked to the MHC gene cluster on chromosome 17 (figure 1A).[15] Multiple independent neuT tumors were generated from each mouse line (figure 1B) expressing oncogenic rat-erbB2 (figure 1C) and transplanted into recipient MHC matched WT and 8-week-old tumor-free neuT+ mice (figure 1D–E). The neuT+ recipients are fully syngeneic for the tumor and presumably tolerant to rat-erbB2, while the WT recipients lacking the rat-erbB2 neu oncogene should respond to the tumor-specific antigen.[12 19] Unlike the BALB/c-WT recipients, whose transplanted tumors grew at approximately the same rate despite the presence of the rat-erbB2 antigen as reported previously,[12] BALB.B-WT recipients began rejecting their tumors after approximately 2 weeks (figure 1D). Both BALB.B-neuT and BALB/c-neuT syngeneic recipients grew out the transplanted neuT+ tumors and were euthanized appropriately (figure 1D).

To verify that BALB.B hosts were targeting the neuT oncoprotein in the rejection response, the rat-erbB2 coding sequence was knocked into the mouse *ERBB2* locus of BALB/c mice. Transgenic second-generation knock-in mice were crossed to BALB.B mice and screened for tolerance to a BALB.B X BALB/c F1 neuT+ tumor graft. While BALB.B X BALB/c-neuT+ mice grew out their tumors, the neuT- mice were protected, demonstrating that MHC H-2^b mediated tumor rejection is a dominant trait (figure 1E). Tolerance of BALB.B-neuT mice to neu+ tumor challenge was recapitulated in the knock-in mice expressing non-oncogenic rat-erbB2 as self. On backcross to BALB.B mice, the mouse line BALB.B–rat-erbB2 was established. These neuT+ BALB.B tumor permissive mice demonstrate that neither the expression of the oncogenic transmembrane mutation nor the presence of endogenous neuT+ tumors are essential for tolerance (figure 1F).

BALB.B and BALB/c-WT mice challenged with their respective MHC-matched tumors both generated spontaneous immune responses targeting rat-erbB2. Specifically, both strains produced isotype class switched 4T1 rat-erbB2-specific antibodies after tumor challenge (figure 2A–C), a clear indication that both the congenic

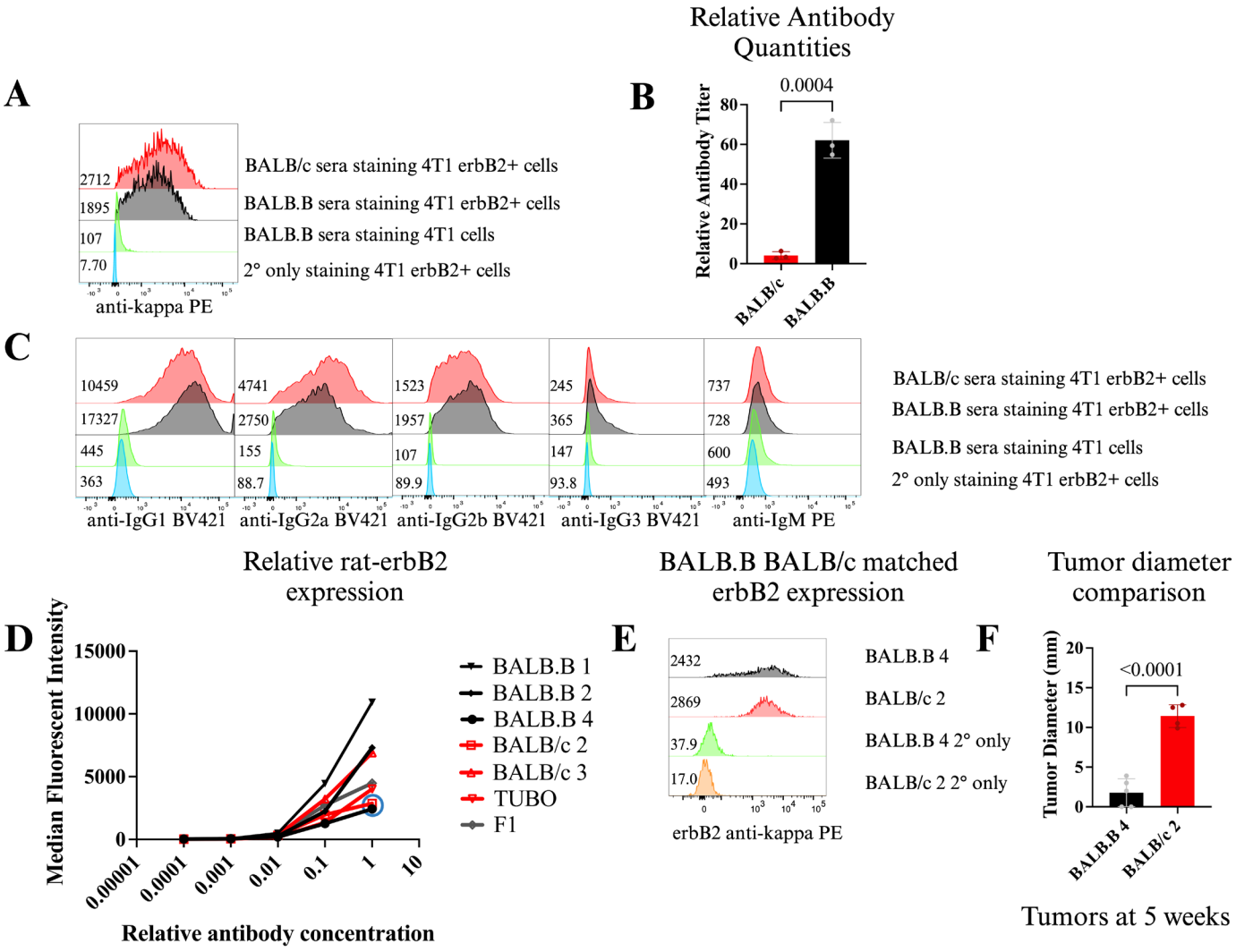

Figure 2 BALB.B and BALB/c neuT+ tumors are immunogenic. Sera from BALB.B and BALB/c mice (4 weeks post-tumor inoculation) were incubated with 4T1 or 4T1-rat-erbB2+ cells to assess antigen-specific antibody binding using fluorescently tagged secondary kappa antibodies (A–B) or antibodies of the indicated isotype by flow cytometry (C). (B) Relative titers of BALB/c and BALB.B antibody levels, calculated by the dilution required to achieve the same median fluorescent intensity. Data were analyzed using the Student's unpaired t-test. (D) Rat-erbB2 expression by independent BALB.B, BALB/c, and BALB.B X BALB/c F1 tumors were stained with the monoclonal rat-erbB2 antibody clone 7.16.4. (E) Matched erbB2 expression on the selected tumors designated by the circle in D. (F) Direct growth comparison of tumors from E following transplantation into BALB.B and BALB/c hosts. Data were analyzed using the Student's unpaired t-test.

lines developed CD4+ T cell and B cell responses to the neuT+ tumors. Western blot, ELISA, and competition assays validate the specificity of these antibodies in the immune sera (online supplemental figure 1A–C). Analysis using 4T1 cells transduced with chimeric mouse/rat erbB2 proteins revealed that rat domain 3 is bound by antibodies generated in BALB.B mice (online supplemental figure 1D).

The antigen-specific antibody titer in tumor-challenged BALB.B mice was approximately 15-fold greater than in BALB/c responding mice (figure 2B). Together, the BALB.B and BALB/c models comprise scenarios where the anticancer immunity generated against a tumor-specific antigen is sufficient to clear one tumor (BALB.B) but not another (BALB/c). To understand the difference, we examined the antitumor immune responses in both models. To test whether the level of erbB2 protein expression was a distinguishing factor, erbB2 expression levels on BALB.B and BALB/c tumor cell lines were analyzed. Overlapping ranges of rat-erbB2 expression were found among the BALB.B and BALB/c tumors (figure 2D). A direct comparison of two closely matched low neuT-expressing tumors, BALB.B-4 and BALB/c-2 confirmed that MHC genotype but not rat-erbB2 expression levels was a defining determinant of rejection (figure 2E–F).

Tumor microenvironments differ between BALB.B and BALB/c tumor-bearing mice

BALB.B and BALB/c-WT mice were challenged with MHC-matched tumors and analyzed for immune and tumor cell markers by CyTOF 17 days postchallenge, at the point where BALB.B tumors begin to reject while

BALB/c tumors continue growing (figure 1D). Phenograph analysis revealed multiple clusters, including immune and tumor cells (online supplemental figures 2–3A). CD8+ T cells were largely absent from the tumor microenvironment of BALB/c mice and present in small numbers in tumors from BALB.B mice (online supplemental figure 3B). Early reports accentuate the importance of the CD8+ T cell response in immunologic resistance to breast carcinomas.[20 21] To evaluate the potential role of CD8+ T cells in the spontaneous immune attack against the neuT+ breast carcinoma cells, BALB/c mice were challenged with tumor cells derived from a MHC-I mismatched BALB.B X BALB/c-neuT host, where the graft would be expected to recognize the MHC-I major antigens H2-K^b and H2-D^b as foreign. As expected, tumor-infiltrating CD8+ T cells were prominent in the recovered tumor microenvironment, paired with a decrease in neuT+ tumor cells. This demonstrates CD8+ T cell tumor killing capacity in the model should they be activated and tumor infiltrating (online supplemental figure 3B).

To assess whether the cellular composition of the immune cells within the tumor microenvironment reflects the tumor rejection phenotype of the mice, we conducted an analysis of CD45+ tumor-infiltrating cells. An analysis of independent tumors implanted into BALB.B, BALB.B rat-erbB2+, and BALB/c mice was conducted using a Cytek Aurora platform (figure 3). These results showed a significant increase in the major populations of CD8+ cells, CD4+ cells, CD335+ cells, and significant decreases in CD11b+F4/80+ and Ly-6G+ cells compared in BALB.B compared with BALB.B rat-erbB2+ and BALB/c tumors. A reanalysis of the CD45+ cells from the CyTOF analysis in (online supplemental figures 2–3) confirmed the initial observation made in the Cytek analysis (online supplemental figure 4).

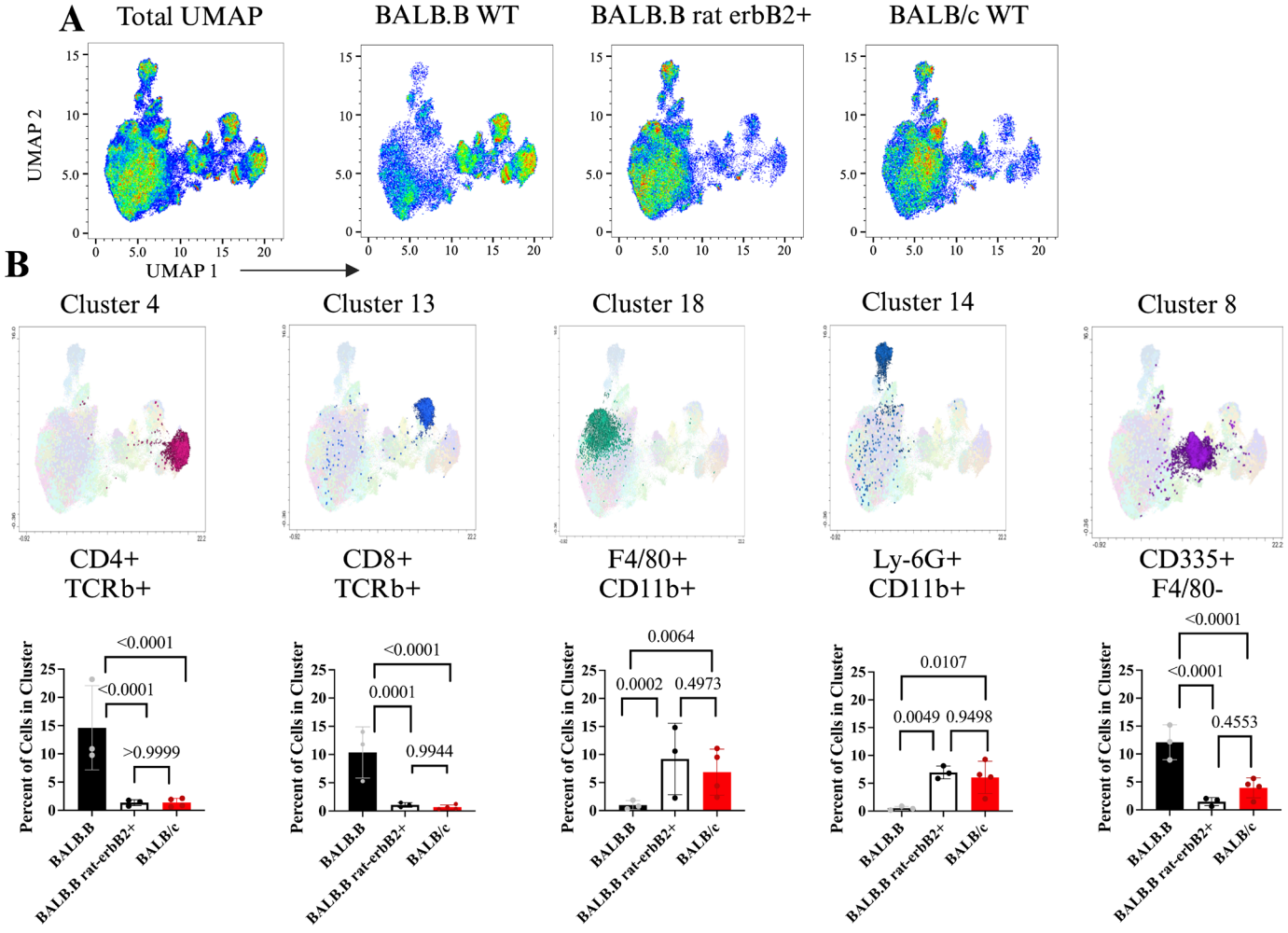

Figure 3 Analysis of CD45+ cells present in breast carcinomas shows immune compartment changes among BALB.B, BALB.B rat-erbB2, and BALB/c mice using the Cytek platform. BALB.B, BALB.B rat erbB2+, and BALB/c mice were challenged with MHC matched tumors. (A) Summary UMAP of immune cells of all mice (left), pooled BALB.B mice (center left), BALB.B-rat-erbB2+ mice (center right), or BALB/c mice (right) present in the tumor. (B) Location of the indicated cell clusters on the UMAP (top), and the frequency of cluster presence of total immune cells in each tumor by group (bottom). Data were analyzed by a two-way ANOVA focusing on the cell clusters for CD4+, CD8+, F4/80+, Ly-6G+, and CD335+ cells ($p<0.0001$), followed by Šidák's multiple comparisons test. ANOVA, analysis of variance; MHC, major histocompatibility complex; UMAP, Uniform Manifold Approximation and Projection plot; WT, wild-type.

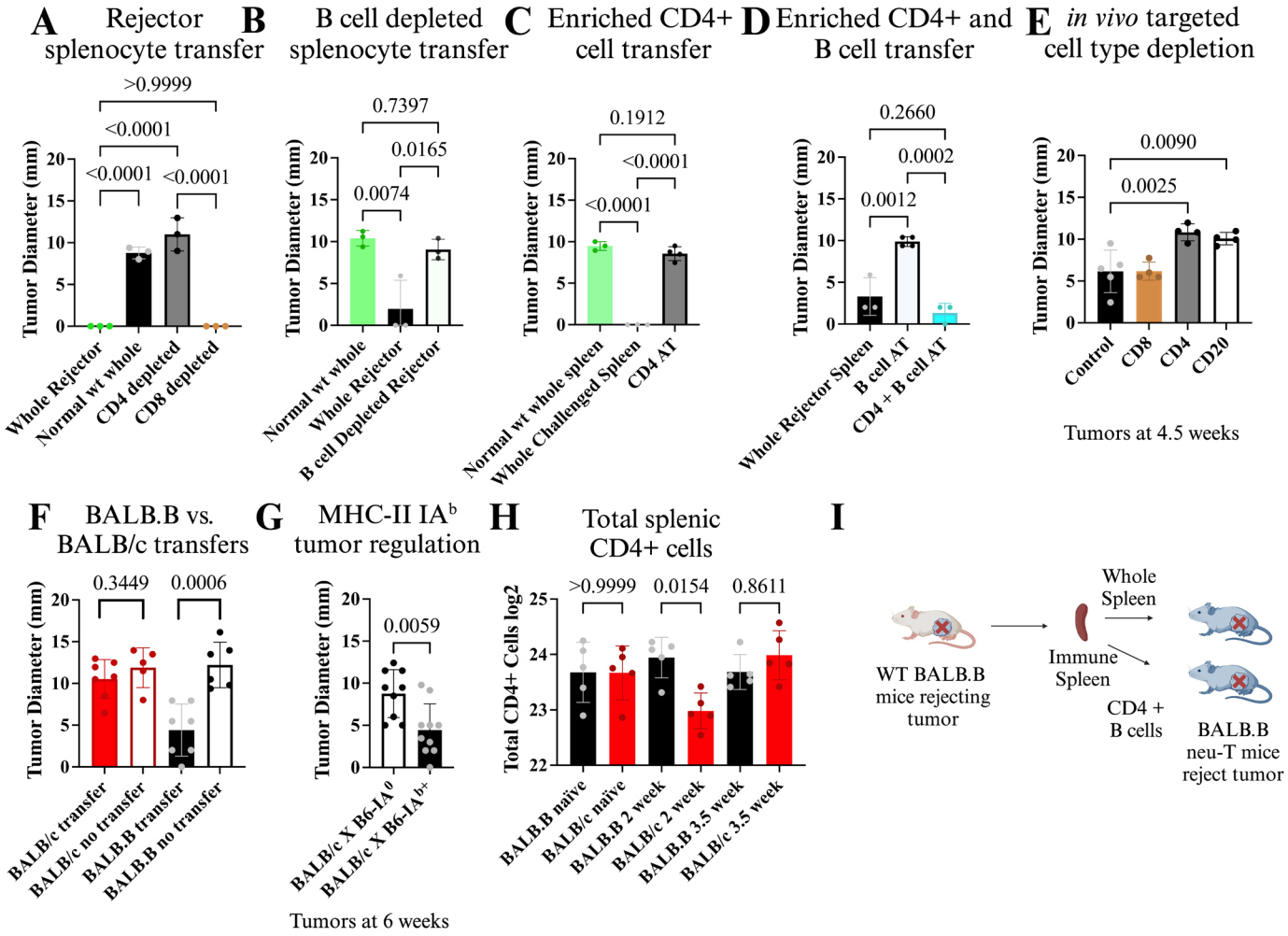

Figure 4 BALB.B immune cells convey tumor protection to permissive hosts in an MHC-II dependent manner. (A–D) Splenocytes were harvested from BALB.B-WT mice that were either unchallenged or had already rejected BALB.B-neuT tumors and adoptively transferred into tumor permissive recipient BALB.B-neuT mice along with a BALB.B-neuT tumor inoculation. Tumor diameters were measured 4 weeks later unless otherwise specified. (A) CD4 or CD8 cells were depleted from splenocytes prior to adoptive transfer. (A–D) Data were analyzed from a one-way ANOVA using a two-tailed Dunning test for multiple comparisons. (B) B-cells were depleted from splenocytes prior to adoptive transfer. (C, D) CD4 cells and B cells were purified from rejector splenocytes and adoptively transferred, alone or together. (E) BALB.B-WT mice were depleted of CD8, CD4, or B-cells and inoculated with BALB.B-neuT tumor cells. Data were analyzed as in A–D. (F) Immune splenocytes harvested from tumor-bearing BALB.B and BALB/c hosts at 4 weeks postchallenge were adoptively transferred into permissive BALB.B-neuT or BALB/c-neuT hosts prior to challenge with MHC-matched tumors and compared to tumor-challenged naïve animals. T-tests were conducted to compare groups of mice within each genotype. (G) IA^b-positive CB6F1 mice or IAb-negative BALB/c X B6-IA^0 F1 mice were challenged with BALB/c-neuT tumors. The Student's unpaired t-test was used to compare groups. (H) Total cell counts of splenic CD4+ T cells at 0, 2, and 4 weeks post-tumor inoculation. One-way ANOVA was performed to compare groups, with Tukey's multiple comparison test to compare all groups to each other. (I) Schematic of the conclusion, CD4 + B cells from the spleen of a BALB.B-WT mouse that has rejected tumor confers protection in a recipient BALB.B-neuT mouse. ANOVA, analysis of variance; MHC, major histocompatibility complex; WT, wild-type.

Immune mechanisms mediating rejection of breast ductal carcinoma in BALB.B-WT mice

To determine how BALB.B-WT mice reject BALB.B–neuT breast ductal carcinoma cells, splenocytes from BALB.B-WT mice that have rejected their tumors were intravenously transferred into naïve tumor permissive BALB.B-neuT or BALB.B-rat-erbB2 recipients. Rejector splenocyte transfer into recipient BALB.B-neuT mice conferred protection to the recipient mice, while transfer of naïve BALB.B splenocytes did not (figure 4A). Depletion of CD8+, CD4+, and B220+ cells from the protective immune spleen population used in the adoptive transfer showed that CD8+ T cells were dispensable for transferred protection but CD4+ T cells and B220+ B cells were necessary (figure 4A–B). While neither splenic CD4+ T cell nor B-cell adoptive transfer alone was sufficient, adoptive transfer of both cell types together was sufficient (figure 4C–D).

To determine whether the mechanism mediating spontaneous immune rejection reflects the requirement for CD4+ T cells and B cells highlighted in the adoptive transfer experiments, BALB.B WT mice were depleted

systemically of CD4+, CD8+, or CD20+ cells with depleting antibodies prior to tumor challenge (figure 4E). Depletion of CD4+ or CD20+ but not CD8+ cells resulted in an increased tumor burden relative to untreated mice (figure 4E), mirroring the adoptive transfer studies. The efficiency of the cell depletions was ascertained in the blood 0, 2, and 5 weeks post-tumor inoculation (online supplemental figure 5A). CD4+, CD8+, and CD20+ cells were stably depleted throughout the course of the experiment (online supplemental figure 5B–D).

To evaluate whether immune spleens from permissive BALB/c-WT mice could similarly confer tumor protection, splenocytes from BALB/c and BALB.B were compared for their ability to protect MHC-matched rat-erbB2 tolerant mice following adoptive transfer. While the BALB.B splenocytes conferred protection, the BALB/c splenocytes did not (figure 4F). Thus, BALB/c immune splenocytes responding to tumor antigens were insufficient to mobilize a protective response against the neuT+ tumors even after priming and generating rat-erbB2+ antibodies (figure 2). The critical contributions of CD4+ T cells and B cells in mediating tumor rejection imply a central role of MHC-II antigen presentation by the H-2 IA^b allele in determining the nature of the antitumor response. To test this possibility, we evaluated the ability of CB6F1 mice either containing or lacking the H-2^b IA^b MHC-II allele for their ability to mount a protective antitumor response. C57BL/6 IA^0 mice were used in the cross to generate mice expressing only the MHC-II H-2 IA^d and IE^d alleles derived from the BALB/c parent, but not the H-2 IA^b allele from the C57BL/6 parent. The IA^b+F1 mice rejected their transplanted tumor while the IA^b-negative F1 mice did not, demonstrating that the tumor rejection is indeed MHC-II IA^b dependent (figure 4G). We have also observed this same outcome comparing IA^b and IA^b-negative BALB.B X BALB/c F1 mice challenged with IA^b-negative BALB/c-neuT tumor (p=0.0457, n=5, not pictured). This indicates that MHC-II IA^b is critical for rejection independently of any expression of IA^b on the tumor cells. The critical role of CD4+ T cells in mediating the rejection phenotype is reflected by transiently decreased numbers of splenic CD4+ T cells in BALB/c mice compared with BALB.B mice at the tumor growth inflection point 2 weeks post-tumor inoculation (figure 4H). B cell numbers were equivalent across strains, with an increase in TCRβ−/B220− cell populations consistent with known tumor-associated increases in splenic myeloid-derived suppressor cells over time in breast carcinomas[22] (online supplemental figure 6).

Finally, to evaluate the relevance of the described mediators of immune rejection in the setting of spontaneously developing breast carcinomas, immune splenocytes from rejector BALB.B splenocytes were adoptively transferred into 8-week-old BALB.B-neuT mice, which display frank signs of ductal dysplasia throughout each of their mammary glands but no palpable tumors.[12] The transferred immune splenocytes significantly delayed tumor appearance, demonstrating the capacity of active immunity to control aggressive tumor development (online supplemental figure 7). We deduce from these experiments that immune CD4+ T cells and B cells are essential to confer resistance against the tumor challenge and once activated can be protective against spontaneous tumor development (figure 4I).

Role of antibodies in BALB.B rejection of tumors

We noted erbB2-specific antibodies decreased in BALB.B-WT mice following CD4+ or CD20+ but not CD8+ cell depletion, suggesting that antibodies may play a role in tumor clearance (figure 5A). Furthermore, tumor size negatively correlated with the amount of erbB2-specific antibody detected in the blood of tumor-bearing mice reinforcing the hypothesis that antibodies may mediate tumor protection (figure 5B). To test this hypothesis, immune serum was passively transferred from BALB.B mice into tumor-bearing permissive BALB.B-rat-erbB2 hosts. The transferred sera conferred protection (figure 5C). Furthermore, adsorbing erbB2 specific antibodies out of the immune sera with 4T1 cells expressing rat-erbB2 significantly diminished the protective activity (figure 5D). Together these data demonstrate the protective role of CD4+ T cells and B cells together and the resulting tumor-specific antibodies are determinants of immunity in this model of spontaneous tumor rejection using BALB.B-neuT tumors in BALB.B-WT mice (figure 5E).

Validation of antigenicity of rat-erbB2 model in B16F10 and 4T1 tumors

We hypothesized that optimal immune recognition of critical rat-neuT epitopes by CD4+ T cells evokes spontaneous antitumor immunity, and the introduction of these rat-erbB2 antigens as passenger proteins into IA^b-positive tumors would lead to spontaneous resistance as well. Accordingly, we generated virulent B16F10[23] and 4T1[24] tumor cell lines expressing the non-oncogenic rat-erbB2 ectodomain as the model neoantigen. Both IA^b+B6 and CB6F1 mice were more resistant to challenge with rat-erbB2+ tumors relative to their respective controls (online supplemental figure 8A,B). Anti-rat-erbB2-specific antibodies were evident in these tumor-resisting animals (online supplemental figure 8C,D). Analysis of rat domains 1 and 3 supported our hypothesis that an optimal IA^b-restricted tumor-associated peptide antigen(s) promotes a protective response (online supplemental figure 8D).

DISCUSSION/CONCLUSION

Tumors can develop mutations that promote a spontaneous protective antitumor immune response.[25] In methylcholanthrene (MCA)-induced tumors an average of 2,000 non-synonymous mutations were detected, whereas only approximately 40% of these tumors contain optimally immunogenic neoantigens.[4] While neoantigenic epitopes generated in MCA tumors can be targeted by

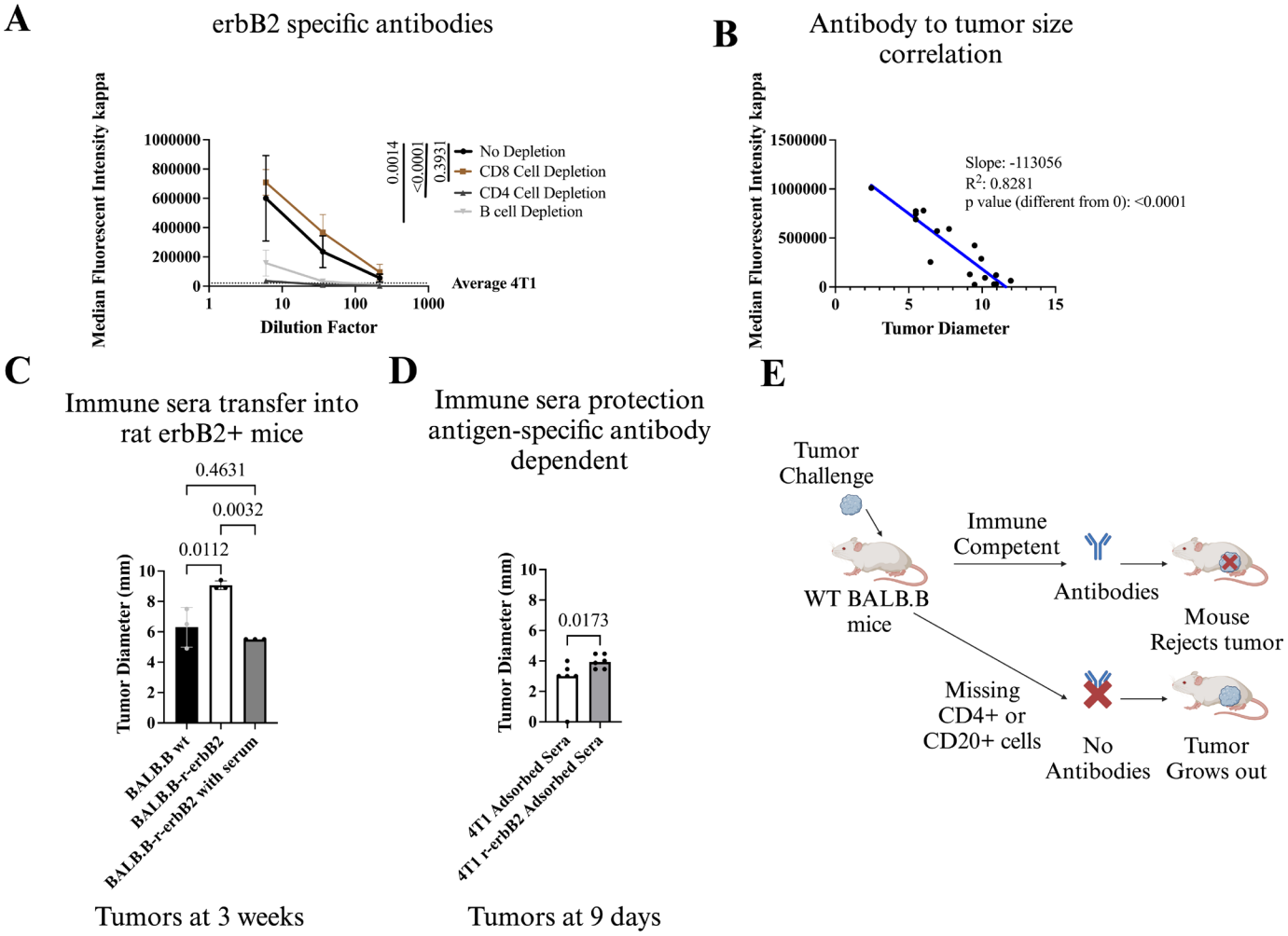

Figure 5 Rat-neu specific BALB.B antibodies confer tumor protection. (A) Rat-erbB2+ specific antibodies were measured from mice in figure 3E (n=5). Dotted line represents 4T1 background staining. Group compared using a two-way repeated measures ANOVA with a Tukey's test for multiple comparisons. (B) Correlation of specific antibody titer from A with tumor size for mice from figure 3E using a simple linear regression to test the null hypothesis that the slope was zero. (C) BALB.B-WT mice and BALB.B rat-erbB2+ mice were inoculated with BALB.B-neuT tumors. Serum from BALB.B rejector mice was passively transferred into one group of BALB.B-rat-erbB2+ mice. Data were analyzed using a one-way ANOVA using a two-tailed Dunning test for multiple comparisons. (D) Antigen-specific antibodies were adsorbed with 4T1-rat-erbB2+ or 4T1 cells and injected into recipient BALB.B-rat-erbB2 mice on day 5 post-tumor inoculation. Data were analyzed using a Mann-Whitney test. (E) Schematic of the conclusion of figure 4. BALB.B-WT mice that contain functional CD4+ and CD20+ cells secrete antibodies and reject their tumors, while mice lacking CD4+ or CD20+ cells do not secrete antibodies and succumb to their tumors. ANOVA, analysis of variance; WT, wild-type.

prior immunization,[10 12 21] antigens inducing spontaneous antitumor immunity appear to be culled by immunoselection. The present work establishes a clear tumor model for delineating the determinants governing spontaneous tumor rejection and permissive tumor growth in immunocompetent individuals. Whereas immunization was required to mobilize a protective response in the BALB/c mice,[12 20 26] tumors were rejected spontaneously in BALB.B mice (figure 1D).

The model features the defined neuT-specific tumor antigen, a protein that differs from its mouse homologue by multiple amino acids, containing epitopes that are representative of cancer mutations giving rise to neoantigens present in animal and human cancers.[27] A major unresolved question is why cancer neoantigens persist in immune-competent individuals. Our model allows this question to be addressed under genetically controlled conditions. In BALB.B mice, the mechanism mediating tumor rejection is due to CD4+ T cells and B cells and resulting antibodies (figures 3–4). While tumor-challenged BALB/c-WT mice also produce antitumor antibodies, their response is insufficient to protect against tumor challenge (figures 1–2). This likely reflects differences in antibody quantity or quality, representing cases where patients develop immune responses against their tumors but fail to reject.[10] In this model, notable differences in antibody titer generated in response to tumor challenge were observed (figure 2B). Possible qualitative differences distinguishing tumor-specific antibodies generated by the two strains will require further investigation.

Both MHC-II and the nature of neoantigens vary among individuals in natural settings.[19 28] Tumor antigens presented in the context of different MHC molecules likely result in variations of CD4 T-cell activation.[29 30] We will use this model to study differences between peptides that activate CD4 T cells to mobilize a protective response and ones that do not. Understanding what is necessary for an optimally activated CD4 T cell is the first step towards augmenting existing ineffective CD4 T cell responses to achieve productive antitumor immunity.

Given that the principles of MHC-T cell interaction are generally conserved between mice and humans, this work has potential to be generalizable to human cancer patients across different cancers.[31–35] The potential for this principle to be generalized is highlighted by the rejection of the B16F10 and 4T1 tumor lines expressing the rat-erbB2 non-oncogenic ectodomain, demonstrating that the targeted neoantigen need not be a driver mutation (online supplemental figure 8). In many human breast cancers, the erbB2 oncoprotein is antigenic and results in a suboptimal spontaneous immune response in patients like what we have observed in BALB/c mice[36] (figure 2A–C). Vaccination of these patients sometimes leads to a productive immune response similar to what has been observed in vaccinated BALB/c mice.[21]

The lack of literature describing a spontaneous productive immune response in human cancer patients as represented by BALB.B may be partially due to individuals who spontaneously rejected their cancer rarely being evaluated clinically. Immune-compromised individuals develop tumors at a much higher rate than the general population, suggesting that the human immune system does clear many tumors.[5] This view is supported by evidence that immunosuppressed individuals can clear Kaposi's sarcomas following reconstitution of CD4+ cells.[37–39] Furthermore, the correlation of improved outcomes with increased numbers of CD4+ T follicular helper cells and B cells in patients with breast and lung cancer together with improved outcomes in mice following the introduction of strong T follicular helper and B cell antigens is consistent with our thesis.[40–44] Our work has significant relevance to understanding how a human cancer could be productively recognized by the human immune system and that understanding such immunity can help us recapitulate it with future immunotherapies.

In conclusion, we present a new model which highlights the importance of MHC-II in the development of spontaneous antitumor immunity (online supplemental file 2). The model identifies a CD4+ T cell, B cell, and antibody requirement for successful defense against breast carcinoma challenge. The known role of MHC-II in mediating the interactions between T helper cells and B cells in the generation of T-dependent antibody responses underscores the dependence of developing immunity on class II genotype. Our model also provides a venue for identifying why some prototypical antigens have the capacity to mobilize spontaneously a protective immune response. Our ongoing studies are addressing how these emerging insights apply to human cancer and can be translated into new cancer therapies.

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Contributors JWJ and AP contributed equally to this work. JWJ, AP, VPVK, and LRP were responsible for overall conception and design of the study. JWJ performed original and validation experiments. MJH, SAC, VPVK, STF, PER-C, JY, SMO, and KDP performed experiments and data analysis. JWJ or LRP independently verified all the data analysis. SJF generated and characterized knock-in mice and viral constructs. AJ and LRP managed and provided mentoring for the project. JWJ and LRP wrote the manuscript. All authors reviewed, edited, and approved the manuscript. LRP is the guarantor.

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