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An autoimmune-mediated strategy for prophylactic breast cancer vaccination

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Although vaccination is most effective when used to prevent disease, cancer vaccine development has focused predominantly on providing therapy against established growing tumors[1]. The difficulty in developing prophylactic cancer vaccines is primarily due to the fact that tumor antigens are variations of self proteins and would probably mediate profound autoimmune complications if used in a preventive vaccine setting[2]. Here we use several mouse breast cancer models to define a prototypic strategy for prophylactic cancer vaccination. We selected α-lactalbumin as our target vaccine autoantigen because it is a breast-specific differentiation protein expressed in high amounts in the majority of human breast carcinomas[3–5] and in mammary epithelial cells only during lactation[6–9]. We found that immunoreactivity against α-lactalbumin provides substantial protection and therapy against growth of autochthonous tumors in transgenic mouse models of breast cancer and against 4T1 transplantable breast tumors in BALB/c mice. Because α-lactalbumin is conditionally expressed only during lactation, vaccination-induced prophylaxis occurs without any detectable inflammation in normal nonlactating breast tissue. Thus, α-lactalbumin vaccination may provide safe and effective protection against the development of breast cancer for women in their post–child-bearing, premenopausal years, when lactation is readily avoidable and risk for developing breast cancer is high[10].

Our overall hypothesis is that a full-strength autoimmune attack sufficient to induce organ-specific failure may provide protection and therapy against tumors derived from the targeted organ. Thus, our experimental plan involved generating a recombinant mouse mammary-specific differentiation protein antigen, determining the presence of breast autoimmunity following immunization with the generated protein and evaluating the efficacy of immunization with the breast-specific protein as both a prophylactic and therapeutic breast cancer vaccine. We generated the breast-specific α-lactalbumin cDNA from lactating mouse mammary tissue, expressed as a 6×-His–tagged fusion protein and purified it by nickel-nitrilotriacetic acid affinity chromatography followed by reverse-phase HPLC to produce an endotoxin-free protein[11] (**Fig. 1a**). Ten days after immunization of female SWXJ mice with recombinant mouse α-lactalbumin in complete Freund's adjuvant (CFA), lymph node cells (LNCs) showed dose-dependent proliferation in recall responses to α-lactalbumin and were unresponsive to recombinant human cochlin generated in a virtually identical manner[12] (**Fig. 1b**). Responsiveness to α-lactalbumin involved both $CD4^+$ and $CD8^+$ T cells (**Fig. 1c**) and showed a proinflammatory phenotype involving high production of interferon-γ (IFN-γ) and interleukin-2 (IL-2) and low production of IL-4, IL-5 and IL-10 (**Fig. 1d**).

Breast tissue from nonlactating mice immunized with α-lactalbumin never showed any inflammatory infiltration and instead consistently showed isolated individual $CD3^+$ T cells migrating through breast parenchyma in a classic surveillance pattern (**Fig. 1e**). In sharp contrast, extensive T cell infiltrates consistently occurred throughout mammary tissue of lactating mice immunized with α-lactalbumin (**Fig. 1f**). Breast tissue from lactating control mice immunized with CFA alone never showed any inflammatory T cell infiltration (**Fig. 1g**). Analysis of breast infiltrating T cells by flow cytometry showed high frequencies of $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells highly expressing the activation marker CD44 (**Fig. 1h**). Analysis by quantitative real-time RT-PCR showed that breast tissue from lactating mice immunized with α-lactalbumin had significantly elevated expression levels of IFN-γ ($P = 0.001$) but not IL-10 ($P > 0.10$) when compared to levels expressed in breast tissue from untreated normal nonlactating or lactating mice or lactating mice immunized with CFA alone (**Fig. 1i**). We subsequently found that the inflammation observed in breasts of lactating mice immunized with α-lactalbumin was T cell mediated and resulted in breast failure, which is characterized by pups showing diminished weight gain and failure to thrive, often with kwashiorkor-like signs of nutritional deficiency and runting (data not shown). This breast failure phenotype mimics that observed in α-lactalbumin–deficient mice[13].

Our focus quickly turned to the potential use of α-lactalbumin as a breast cancer vaccine. We examined whether early immunization with α-lactalbumin could prophylactically inhibit growth of autochthonous breast tumors. MMTV-neu mice express the unactivated *neu* protooncogene under the regulation of the long terminal repeat of mouse mammary tumor virus (MMTV) and show a 50% incidence of spontaneous mammary tumors by 205 d of age[14]. We immunized

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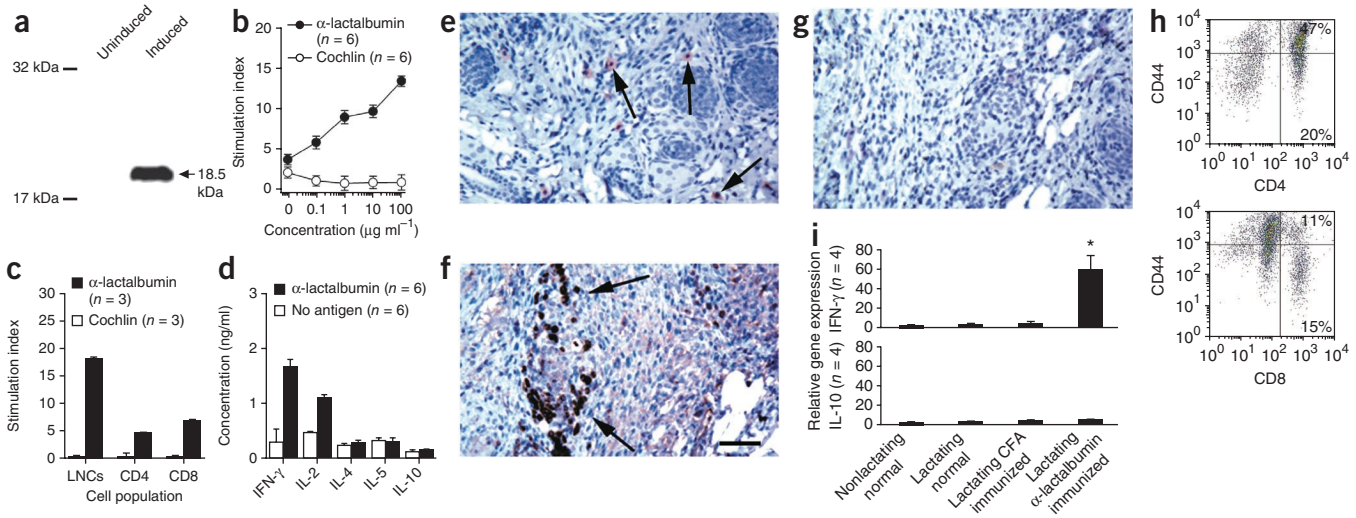

Figure 1 Immunogenicity of recombinant mouse a-lactalbumin. (**a**) Western blot showing purified recombinant mouse α-lactalbumin detected with His tag–specific antibody. (**b**) Recall responses to recombinant proteins elicited by whole LNCs taken 10 d after immunization of SWXJ female mice with α-lactalbumin. (**c**) Recall responses to recombinant proteins elicited by $CD4^+$ and $CD8^+$ T cells purified from LNCs taken 10 d after immunization of SWXJ female mice with α-lactalbumin. (**d**) Cytokine production in response to recombinant α-lactalbumin by LNCs taken 10 d after immunization of SWXJ mice with α-lactalbumin. (**e**–**g**) Immunocytochemical staining for CD8. In **e**, arrows show $CD3^+$ surveillance T cells in mammary parenchyma of nonlactating SWXJ female mice 6 weeks after immunization with α-lactalbumin. In **f**, arrows show extensive inflammatory infiltrates of $CD3^+$ T cells in mammary parenchyma of lactating SWXJ female mice 6 weeks after immunization with α-lactalbumin. In **g**, $CD3^+$ infiltrates were never observed in mammary tissue from lactating control mice immunized with CFA alone. Scale bar for **e**–**g**, 50 μm. (**h**) Flow cytometry analysis of breast infiltrates showing high frequencies of $CD3^+CD4^+$ (left) and $CD3^+CD8^+$ T cells (right) expressing the $CD44^{high}$ activated phenotype. (**i**) Real-time RT-PCR analysis of lactating mammary tissue showing significantly elevated expression of IFN-γ (*$P = 0.001$) but not IL-10 ($P > 0.10$). All error bars show means ± s.e.m.

8-week-old MMTV-neu mice with either α-lactalbumin in CFA or with CFA alone and killed all mice when the first tumor in any mouse reached 17 mm in diameter. Whereas all CFA-immunized control mice developed breast tumors when the experiment was terminated at 10 months of age, none of the mice immunized with α-lactalbumin had any detectable mammary tumors ($P = 0.0004$; **Fig. 2a**). Prophylactic vaccination with α-lactalbumin was also effective against transplantable 4T1 tumors. Highly significant ($P = 0.0006$) growth inhibition occurred in BALB/c mice immunized with α-lactalbumin 13 d before inoculation with 4T1 tumor cells (**Fig. 2b**).

We next determined whether α-lactalbumin immunization could therapeutically inhibit growth of established tumors. Although palpable tumors appear by 2–3 weeks after subcutaneous (s.c.) inoculation of BALB/c mice with 2×10^4 4T1 tumor cells, tumors are well established within 5 d after inoculation (**Fig. 2c**). We observed significant inhibition of tumor growth ($P < 0.01$ in each case) when α-lactalbumin vaccination occurred at 5 d (**Fig. 2d**) and 13 d (**Fig. 2e**) but not at 21 d (**Fig. 2f**) after inoculation with 4T1 tumor cells. However, the lack of tumor growth inhibition in mice immunized 21 d after inoculation may be due to the shortened 11-d observation period between the time of immunization and the time when tumors reached the maximum size mandating killing.

We also determined the effectiveness of α-lactalbumin vaccination on the growth of established autochthonous tumors. MMTV-PyVT transgenic mice constitutively express activated *neu* in breast tissues and develop very rapidly growing mammary tumors palpable by 5 weeks of age[15]. We observed significant inhibition ($P < 0.0006$) in the growth of very aggressive established autochthonous tumors in MMTV-PyVT transgenic mice vaccinated at 6 weeks of age with α-lactalbumin (**Fig. 2g**). Our results indicate that α-lactalbumin vaccination provides effective protection and therapy against breast tumor growth and is particularly effective when immunization occurs before the appearance of palpable tumors.

Tumors taken 32 d after inoculation with 4T1 tumor cells and immunization with α-lactalbumin showed extensive infiltration of $CD3^+$ T cells (**Fig. 3a**). Such inflammatory infiltrates did not occur in tumors from CFA-immunized control mice (**Fig. 3b**). Flow cytometry analysis of tumor infiltrating lymphocytes (TILs) showed a predominance of $CD4^+$ (64.3%) over $CD8^+$ (14.4%) T cells (**Fig. 3c**) and a type 1 proinflammatory phenotype involving high production of IFN-γ rather than IL-5 or IL-10, measured by ELISA, in response to α-lactalbumin (**Fig. 3d**). Enzyme-linked immunospot (ELISPOT) analysis of TILs showed that $CD4^+$ rather than $CD8^+$ T cells produced the IFN-γ, as its secretion by cultured T cells was inhibited by treatment with major histocompatibility complex (MHC) class II– but not MHC class I–specific antibodies (**Fig. 3e**). However, $CD8^+$ T cells mediated 4T1-specific cytotoxicity, as death of cultured 4T1 tumor cells was inhibited by treatment of cultured α-lactalbumin–primed LNCs with antibodies specific for mouse CD8 but not CD4 (**Fig. 3f**).

Transfer of α-lactalbumin–primed T cells but not primed B cells or sera induced breast failure in naive recipients (data not shown). Similarly, transfer of α-lactalbumin–primed LNCs into naive recipient BALB/c mice on the same day as inoculation with 4T1 tumors resulted in highly significant ($P < 0.0001$) inhibition of tumor growth (**Fig. 4a**), significantly lowered ($P < 0.03$) the number of tumor-bearing mice (**Fig. 4b**) and significantly decreased ($P < 0.0008$) final tumor weight (**Fig. 4c**). We also found that significant tumor growth inhibition occurred in naive mice receiving either $CD4^+$ (**Fig. 4d**; $P = 0.002$) or $CD8^+$ (**Fig. 4d**; $P = 0.003$) T cells enriched by magnetic bead separation from α-lactalbumin–primed LNCs. Overall, our data indicate that activated $CD4^+$ and $CD8^+$ TILs mediate the protective and therapeutic effects of α-lactalbumin vaccination on breast tumor growth.

Our study indicates that induction of autoimmune-mediated breast failure after a single immunization with α-lactalbumin provides effective breast cancer vaccination. However, the most noteworthy implication

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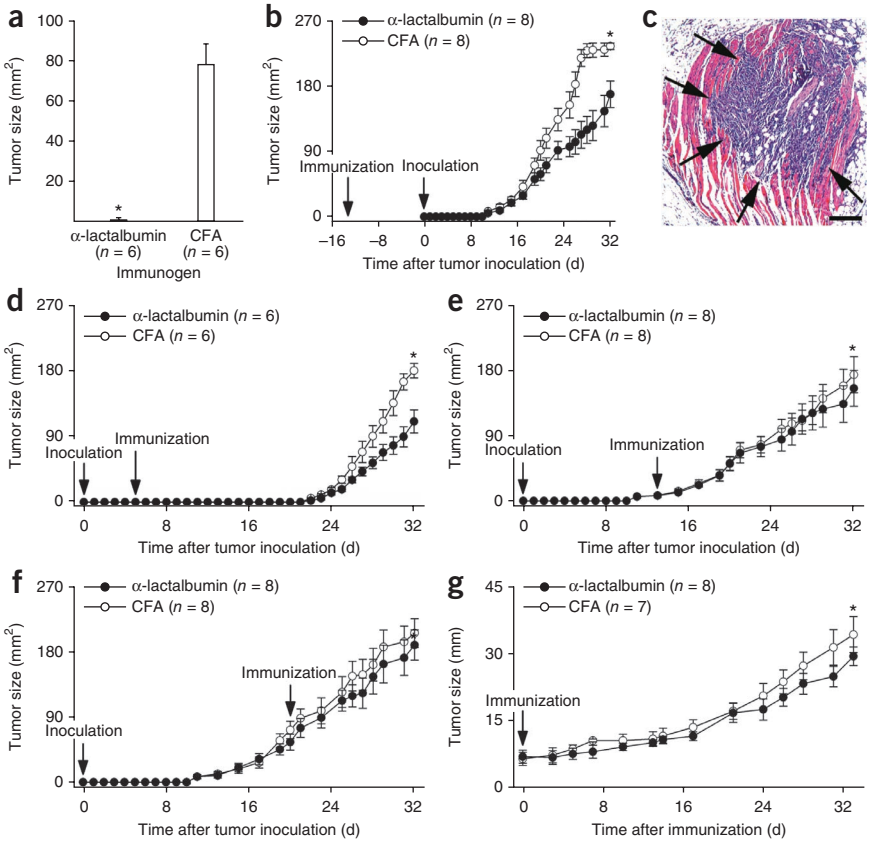

Figure 2 α-lactalbumin vaccination delays and treats breast tumor growth. (**a**) Growth of autochthonous breast tumors in 10-month-old MMTV-neu mice immunized with α-lactalbumin in CFA or CFA alone at 8 weeks of age. (**b**) Growth of transplanted 4T1 tumors after prophylactic immunization with α-lactalbumin in CFA or CFA alone 13 d before tumor inoculation. (**c**) H&E staining of tissue extracted from the flank of a representative mouse 5 d after s.c. inoculation of 2×10^4 4T1 tumor cells. Arrows outline a 4T1 tumor. Scale bar, 100 μm. (**d**–**f**) 4T1 tumor size after α-lactalbumin immunization 5 d after tumor inoculation ($P < 0.01$) (**d**), 13 d after tumor inoculation ($P < 0.01$) (**e**) and 21 d after tumor inoculation ($P > 0.10$) (**f**). (**g**) Tumor size of very aggressive autochthonous tumors after α-lactalbumin immunization of MMTV-PyVT transgenic mice at 6 weeks of age ($P < 0.0006$). Tumors in MMTV-PyVT mice were amenable to measurement in only one direction. All error bars show ± s.e.m. Each * indicates significance.

of our study is that α-lactalbumin vaccination provides prophylaxis against breast cancer in the absence of any detectable autoimmune-induced breast inflammation. Thus, our results may serve as a rational basis for the development of a safe and effective prophylactic vaccine against human breast cancer. In a broader sense, our study defines the criteria for antigen selection in the development of any targeted prophylactic cancer vaccine: the antigen must be constitutively overexpressed in the majority of targeted tumors, expression of the target antigen in normal tissue must be conditional and the condition determining expression of the target antigen in normal tissue must be readily avoidable. The lactation-dependent milk protein, α-lactalbumin, meets these criteria for prophylactic breast cancer vaccination.

Relative to most organs, the breast is metabolically dormant until lactation occurs, at which time the milk proteins, including α-lactalbumin, are strongly induced. Thus, α-lactalbumin availability in normal nonlactating breast tissue is insufficient to target any autoimmune inflammation. This unique conditional expression of a differentiation protein provides an opportunity for prophylactic breast cancer vaccination of normal healthy women who are either

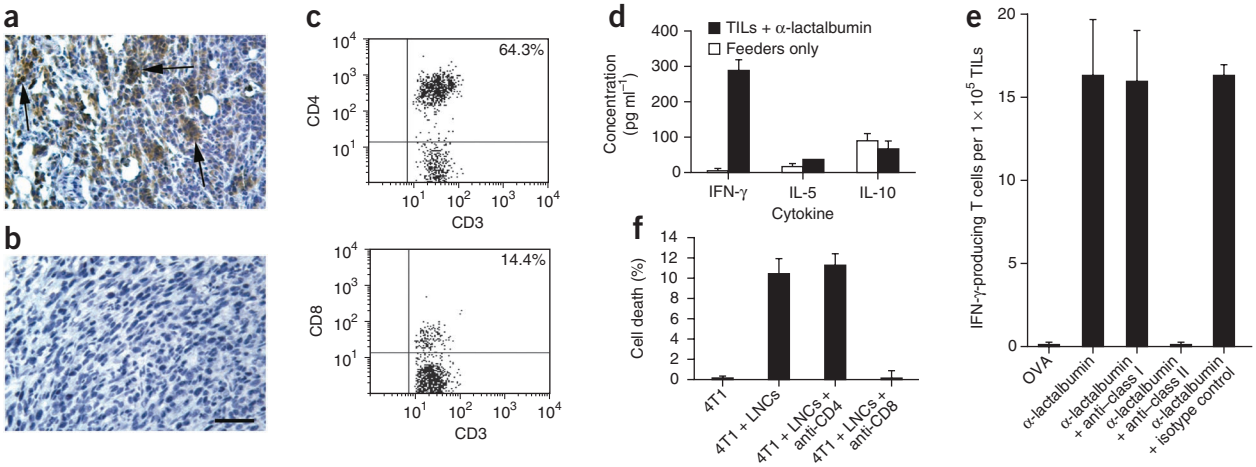

Figure 3 α-lactalbumin–specific T cells induce tumor inflammation and cytotoxicity. (**a**,**b**) Immunocytochemical staining for CD3. In **a**, arrows show extensive infiltration of tumors with $CD3^+$ T cells in BALB/c mice 32 d after vaccination with α-lactalbumin and inoculation with 4T1 cells. In **b**, T cell infiltrates were never observed in tumor-inoculated mice immunized with CFA alone. Scale bar, 50 μm. (**c**) Flow cytometry analysis of TILs showing a predominance of $CD4^+$ (64.3%) over $CD8^+$ (14.4%) T cells. (**d**) Cytokine production in response to 50 μg ml^{-1} α-lactalbumin by TILs taken from 4T1 tumors. (**e**) ELISPOT analysis of TILs showing production of IFN-γ in response to 50 μg ml^{-1} α-lactalbumin in the presence of MHC class I– and class II–specific antibodies (anti–class I and anti–class II). (**f**) Cytotoxicity of 4T1 tumor cells induced by LNCs taken from BALB/c female 10 d after immunization with α-lactalbumin. All data shown are representative of three experiments providing similar results. All error bars show means ± s.e.m.

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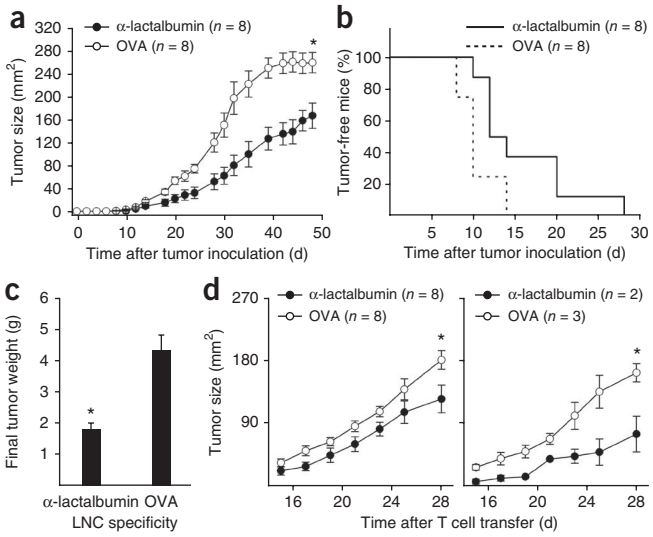

Figure 4 Inhibition of tumor growth by α-lactalbumin vaccination is mediated by T cells. (**a**–**c**) Growth of 4T1 tumors after transfer of α-lactalbumin–primed or OVA-primed LNCs into naive recipient BALB/c mice on the same day as inoculation with 4T1 tumor cells (**a**), numbers of tumor-bearing mice after transfer of α-lactalbumin–primed or OVA primed LNCs into naive recipient BALB/c mice on the same day as inoculation with 4T1 tumor cells (**b**) and final tumor weights after transfer of α-lactalbumin–primed or OVA primed LNCs into naive recipient BALB/c mice on the same day as inoculation with 4T1 tumor cells (**c**). (**d**) Growth of 4T1 tumors after transfer of α-lactalbumin–primed or OVA primed $CD4^+$ T cells (left) and $CD8^+$ T cells (right) into naive recipient BALB/c mice on the same day as inoculation with 4T1 tumor cells. All error bars show means ± s.e.m. Each * indicates significance.

willing to avoid lactation or are past their child-bearing years and at an increased risk for breast cancer. Thus, in a broader perspective, it is not difficult to envision an adult vaccination plan patterned on the childhood vaccine strategy that would provide prophylaxis against adult onset diseases including, but not limited to, breast cancer.

For successful cancer vaccination, it is essential that the selected immunogen is overexpressed in the targeted tumor. Although one early study was unable to show α-lactalbumin mRNA in human breast tumors[16], and other studies have shown that expression of α-lactalbumin protein by breast tumors may not render any substantial diagnostic or prognostic usefulness[3–5], several reports have shown that the α-lactalbumin protein is produced in the majority of human breast malignancies at levels sufficient for detection by immunocytochemical analysis[3–5]. Moreover, recent studies involving adenoviral-mediated gene delivery have shown that the promoter of the gene encoding α-lactalbumin can be used for specifically targeting human breast tumors for expression of several toxic factors, including the tumoricidal E1A transcriptional regulator[17] and the suicide genes thymidine kinase[18] and cytosine deaminase[19]. Although these studies indicate that α-lactalbumin is expressed in appreciable amounts in most human breast tumors, the ability of α-lactalbumin to be an effective target for breast cancer vaccination also depends on whether it is sufficiently immunogenic in humans. Our preliminary *in vitro* priming data clearly indicate that women have a substantial proinflammatory T cell repertoire available for responding to recombinant human α-lactalbumin (data not shown).

Although $CD4^+$ T cells often may be more efficient at tumor rejection than $CD8^+$ T cells[20], optimal tumoricidal activity typically occurs when tumor responses involve both $CD4^+$ and $CD8^+$ T cells[21]. Thus, it is noteworthy that the response to α-lactalbumin involves both $CD4^+$ and $CD8^+$ T cells. Nevertheless, it is not likely that our current data reflect an optimized cancer vaccination strategy. Incorporation of a dendritic cell vaccine approach[22] offers the potential for *in vitro* shaping of the autoimmune antitumor T cell repertoire, perhaps by enhancement of the autoaggressive T helper type 17 lineage[23] or by selective co-stimulatory and/or co-inhibitory manipulation[24]. A complementary and perhaps synergistic strategy may also involve partial inhibition or ablation of $FoxP3^+$ regulatory T cells that inhibit autoimmunity[25]. In any event, our data provide experimental support for developing safe and effective protection against breast tumors, and potentially tumors derived from other organs, by targeted vaccination against conditionally expressed, tissue-specific differentiation proteins.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

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AUTHOR CONTRIBUTIONS

R.J. performed the adoptive tumor immunotherapy studies and the TIL analysis and evaluated the effects of α-lactalbumin vaccination in MMTV-PyVT mice. P.K. evaluated the phenotype of autoimmune-mediated breast failure and performed the initial tumor immunotherapy studies. J.M.J. participated in the cloning and generation of α-lactalbumin. C.Z.A. and D.J.-w. provided technical assistance with molecular and immunocytochemical assays, and V.K.T. designed the experiments, supervised and obtained funding for the project, analyzed the data and wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Recombinant mouse α-lactalbumin. α-lactalbumin cDNA generated from lactating mouse mammary tissue was inserted into the pQE-82L expression vector (Qiagen) for producing a 6×-His–tagged fusion protein in SG13009 *Escherichia coli* (Stratagene). His-tagged α-lactalbumin was purified by nickel-nitrilotriacetic acid affinity chromatography followed by reverse-phase HPLC to yield endotoxin-free protein[11].

Mice and immunization. Female SWXJ (H-2^{q,s}), MMTV-neu, MMTV-PyVT and BALB/cJ mice were purchased from a commercial source (Jackson Laboratories) and immunized by s.c. injection in the abdominal flank with 100 μg α-lactalbumin in 200 μl of an emulsion of equal volumes of water and CFA (Difco). All protocols were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Proliferation assays. We cultured 3×10^5 primed LNCs in triplicate in 200 μl of DMEM (Mediatech Cellgro) supplemented as described previously[26] in 96-well flat-bottomed microtiter Falcon plates (BD Labware) in the presence of serial tenfold dilutions of immunogen. Wells were pulsed at 72 h with [methyl-^{3}H] thymidine (1 μCi per well, specific activity 6.7 Ci mmol^{-1}; New England Nuclear) and collected 16 h later for scintillation spectrometry. The stimulation index was determined as mean scintillation counts per minute from cultures with antigen divided by those from cultures without antigen. CD4$^+$ and CD8$^+$ T cells were positively enriched (>90%) from primed LNCs by magnetic bead separation with a MidiMACS cell separator (Miltenyi). We cultured 3×10^5 purified CD4$^+$ and CD8$^+$ T cells per well as described above in the presence of 2.5×10^5 γ-irradiated (2,000 rads) naive syngeneic splenocytes serving as antigen-presenting cells.

Adoptive transfer. Ten-day–primed LNCs or CD4$^+$ and CD8$^+$ T cells enriched (>90%) by magnetic bead separation (Miltenyi) from primed LNCs were cultured at 4–5 × 10^6 cells per well in supplemented DMEM at 2 ml per well in 24-well plates (BD Labware) containing immunogen at 25 μg ml^{-1}. Wells containing purified CD4$^+$ or CD8$^+$ T cells were supplemented with 4×10^6 γ-irradiated (2,000 rads) naive syngeneic splenocytes as antigen-presenting cells. At 72 h, 2–3 × 10^7 washed cells were injected intraperitoneally into naive recipients in 200 μl PBS.

Tumor inoculation. 4T1 mouse mammary carcinoma cells (American Type Culture Collection CRL-2539) were cultured in 75-cm^2 tissue culture flasks (BD Labware) in RPMI 1640 (Mediatech CellGro) supplemented and incubated as described above. At 70–75% confluence, cells were collected by treatment with 0.25% trypsin and 0.02% EDTA (Sigma Aldrich), and 2×10^4 washed cells were inoculated s.c. in the abdominal flank of 7- to 10-week-old BALB/c females. Mice were weighed and tumors were measured by Vernier caliper daily. Tumor area was calculated as length × breadth. Mice were killed 32 d after 4T1 inoculation. In tumor prophylaxis experiments involving MMTV-neu mice, all mice were killed on the day when the first tumor on any mouse reached a length of 17 mm. Due to massive multifocal tumor growth, tumors in MMTV-PyVT mice were amenable to measurement in only one direction. The length on all ten MMTV-PyVT tumors were added to calculate total tumor load in mm on each day.

Isolation of breast and tumor infiltrating lymphocytes. Lymphocytes were isolated from lactating breasts or 4T1 tumors by digestion of minced tissue for 30 min at 37 °C in HBSS containing 50 KU of DNase I (Sigma Aldrich) and 0.2 mg ml^{-1} collagenase II (Life Technologies). Cells were collected by discontinuous gradient centrifugation and further enriched for T cells by positive selection with Thy1.2-specific antibody–coated magnetic beads and a MidiMACS cell separator (Miltenyi). These enriched cells were used for flow cytometry analysis and in ELISA and ELISPOT assays.

Flow cytometry analysis. Enriched T cells obtained from digestion of lactating breast tissue were triple-stained with CD3-specific antibody conjugated to FITC and CD44-specific antibody conjugated to Cy5 as well as either CD4-specific antibody conjugated to phycoerythrin (PE) or CD8-specific antibody conjugated to PE (BD Biosciences). Enriched TILs were double stained with CD3-specific antibody conjugated to FITC and either CD4-specific antibody conjugated to PE or CD8-specific antibody conjugated to PE. Data collected on 30,000 total events were analyzed with FlowJo software (BD Biosciences) after gating on the CD3$^+$ population.

Enzyme linked immunosorbent assay and enzyme-linked immunosorbent spot analysis. As described previously[26], ELISAs were used to measure cytokine concentrations on 48-h supernatants of 10-d–primed LNCs cultured in supplemented DMEM at 4–5 × 10^6 cells per well in 24-well flat-bottom Falcon plates (BD Labware) in the presence of 25 μg ml^{-1} α-lactalbumin in a final volume of 2 ml per well. Purified capture-detection antibody pairs and recombinant cytokine standards were obtained from commercial sources (BD Biosciences). ELISAs were also used to measure cytokine concentrations in microtiter well cultures containing 1.5×10^5 Thy1.2-enriched TILs, 2×10^4 γ-irradiated (2,000 rads) syngeneic splenocyte feeder cells and 50 μg ml^{-1} α-lactalbumin in a total volume of 200 μl per well supplemented DMEM (Mediatech Cellgro). Identical culture conditions using ELISPOT plates (Millipore) and capture-detection mouse IFN-γ antibody pairs were used to determine frequencies of IFN-γ–producing TILs in response to 50 μg ml^{-1} α-lactalbumin or grade VII ovalbumin (Sigma Aldrich) as previously described[12]. Some ELISPOT cultures were treated at initiation with 20 μg ml^{-1} MHC class I–specific (H-2K^d and H-2D^d) or MHC class II–specific (IA^d and IE^d) blocking antibodies or their IgG isotype controls (eBiosciences).

T cell cytotoxicity assay. We cultured 2×10^4 LNCs from BALB/c mice immunized 10 d previously with α-lactalbumin in triplicate microtiter wells containing 200 μl per well supplemented DMEM in the presence of 1×10^4 4T1 tumor cells pretreated with 50 μg ml^{-1} colchicine (Sigma Aldrich) to arrest cell division. Cytotoxicity was determined at 96 h as percentage survival relative to maximum survival determined from wells containing 1×10^4 4T1 cells alone by the CellTiter 96 aqueous one solution cell proliferation assay (Promega). Some cultures were treated at initiation with 20 μg ml^{-1} of blocking antibodies specific for mouse CD4 or CD8 (eBiosciences).

Real time reverse transcription PCR. Breast tissue RNA was converted to cDNA and analyzed for gene expression by real-time RT-PCR with the following primer pairs: IFN-γ forward 5′-TCAAGTGGCATAGATGTGGAAGAA-3′, reverse 5′-TGGCTCTGCAGGATTTTCATG-3′; IL-10 forward 5′-GGTTG CCAAGCCTTATCGGA-3′, reverse 5′-ACCTGCTCCACTGCCTTGCT-3′; and GAPDH forward 5′-TTCACCACCATGGAGAAGGGC-3′, reverse 5′-GGCATCGACTGTCATGA-3′. Relative gene expression was determined as the ratio of test gene expression to GAPDH gene expression for each tissue using the comparative threshold cycle method.

Immunocytochemistry. Unmasked and blocked formalin-fixed, paraffin-embedded tissues at 6 μm were treated with a 1 in 50 dilution of rat antibody to mouse CD3 (Novacastra) followed by a 1 in 100 dilution of mouse-adsorbed biotinylated goat antibody to rat IgG (BD Biosciences). Slides were developed conventionally with streptavidin–horseradish peroxidase complex (Vector).

Statistical analyses. Differences between mRNA expression levels, mean tumor weights and mean tumor areas were compared with the Student's *t* test. Differences between tumor growth curves were compared by unweighted one-way analysis of variance for correlated samples. Differences between Kaplan-Meier curves were compared with the log-rank test.

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