Spontaneous Mammary Tumors Differ Widely in Their Inherent Sensitivity to Adoptively Transferred T Cells

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Abstract

Immunotherapy of cancer can lead to the selection of antigen loss variants, which provides strong rationale to target oncogenes that are essential for tumor growth or viability. To investigate this concept, we tagged the HER2/neu oncogene with epitopes from ovalbumin to confer recognition by T-cell receptor transgenic CD8+ (OT-I) and CD4+ (OT-II) T cells. Transgenic mice expressing neuOT-I/OT-II developed mammary adenocarcinomas at 6 to 10 months of age. Adoptively transferred naive OT-I cells (with or without OT-II cells) proliferated vigorously on encountering neuOT-I/OT-II-expressing tumors. This was followed by the complete regression of 37% of tumors, whereas others showed partial/stable responses (40%) or progressive disease (23%). Those tumors undergoing complete regression never recurred. In mice with multiple primary tumors, simultaneous regressions and non-regressions were often seen, indicating that immune evasion occurred at a local rather than systemic level. The majority of nonregressing tumors expressed NeuOT-I/OT-II and MHC class I, and many avoided rejection through a profound block to T-cell infiltration. Thus, T cells directed against an essential oncogene can permanently eradicate a subset of spontaneous, established mammary tumors. However, in other tumors, local barriers severely limit the therapeutic response. To maximize the efficacy of immunotherapy against spontaneous cancers, predictive strategies that take into account the heterogeneity of the tumor microenvironment will be required. [Cancer Res 2007;67(13):6442–50]

Introduction

Adoptive immunotherapy represents a promising strategy to induce T-cell responses against human cancer. In the past two decades, methods have been developed to identify, clone, and expand antigen-specific human T cells in vitro (1). On reinfusion into autologous hosts, such T cells can mount protective responses to EBV and cytomegalovirus in immunocompromised individuals (2). In the allogeneic transplantation setting, donor T cells can mount curative responses against host leukemic cells (graft-versus-leukemia effect; ref. 3). Similarly, adoptively transferred T cells can induce objective tumor responses in advanced melanoma patients (4). Despite many anecdotal successes, however, most immunotherapy trials continue to yield primarily negative results, likely owing to our incomplete understanding of the heterogeneity of human cancer at the immunologic level.

It can be argued that the availability of tumor-associated antigens and cognate T cells is no longer a major limitation for cancer immunotherapy. In human breast cancer, for example, several attractive target antigens for T cells are available, including HER2/neu, MUC-1, NY-BR-1, and MAGE family members (5, 6). Likewise, T-cell responses to such antigens can be successfully enhanced through vaccination or adoptive transfer (1, 7). If target antigens and cognate T cells are available, what then limits the therapeutic response? Human cancers show considerable variability with respect to immunologic factors, such as (a) cytokine profiles; (b) defects in antigen processing or presentation; (c) the presence of immunosuppressive myeloid cells or regulatory T cells; or (d) permissiveness to lymphocyte infiltration (8). Although tumors can easily be typed for expression of target antigens and MHC molecules, these other immunologic factors are not understood in a systematic way that allows one to predict whether a given tumor is likely to be sensitive or resistant to T-cell therapy. This could explain the unpredictable responses seen in human clinical immunotherapy trials to date.

Our understanding of breast cancer biology and treatment has been greatly accelerated by transgenic mouse models. For example, transgenic mice that express HER2/neu under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer (9) have been used extensively to study the molecular genetics of mammary tumorigenesis (10). They have also been subjected to various immune-based therapies, including neu-specific monoclonal antibodies (mAb; 11, 12), neu-specific vaccines (13–15), systemic interleukin (IL)-12 administration (16), and combined chemotherapy and vaccination (17). Many of these regimens show efficacy in delaying or reducing the incidence of tumor formation, suggesting that these tumors are somewhat susceptible to immune intervention, at least at early stages. However, at late stages, there are few examples of immunotherapeutic approaches eliminating MMTV/neu tumors, or any other experimental cancers. From that perspective, mouse models continue to provide similar therapeutic challenges as faced with human cancer.

We describe here a modified version of the MMTV/neu transgenic mouse model that allows for the first time precise monitoring of the responses of adoptively transferred CD8+ and CD4+ T cells to spontaneous mammary tumors. Our results show that the molecular and cellular heterogeneity of cancer is reflected also at the immunologic level. After adoptive transfer of tumorspecific CD4+ and CD8+ T cells, spontaneous tumors showed a continuum of responses ranging from complete regression to progressive disease. Responses were largely dictated by inherent factors in the local tumor environment rather than systemic properties. Thus, we provide a new model of breast cancer that will...
facilitate the development of predictive, personalized immunotherapeutic strategies based on the inherent properties of the tumor environment.

Materials and Methods

Mice. This study followed Canadian Council for Animal Care guidelines and was approved by the University of Victoria Animal Care Advisory Committee. All mice were C57BL/6 (H-2b). The activated rat neu oncogene (9) was tagged at its COOH terminus with CD8* (OT-I) and CD4* (OT-II) T-cell epitopes from ovalbumin. Transgenic C57BL/6 mice were generated that express neuOT-I/OT-II in mammary epithelium under the control of the MMTV promoter (ref. 9; Supplementary Data). neuOT-I/OT-II mice were bred with mice expressing a dominant-negative mutant of p53 (DNp53, R172H; ref. 18) under the control of the whey acid protein (WAP) promoter (Supplementary Data). TCR transgenic mice included the following: OT-I, recognizing ovalbumin residues 257 to 264 on MHC class I (19); OT-II mice, recognizing ovalbumin residues 323 to 339 on MHC class II (20); and P14 mice, recognizing gp33 from LCMV on MHC class I (21). OT-I Imice were kindly provided by Eric Butz (Immunex, Seattle, WA), whereas others were from The Jackson Laboratory. Genotyping was by PCR (Supplementary Data).

Adoptive transfer and flow cytometry. Single-cell lymphocyte suspensions were stained with 1.5 μmol/L CFSE (Molecular Probes) for 10 min at 37°C. Typically, 15 × 10^6 each of OT-I and/or OT-II lymphocyte preparations (equivalent to ~4.5 × 10^6 OT-I and/or OT-II T cells) were injected i.v. into tumor-bearing mice. Where indicated, some mice were immunized s.c. with 1 mg ovalbumin protein in PBS. To isolate tumor-infiltrating lymphocytes (TIL), tumors were pressed through a 40-μm membrane, and lymphocytes in the supernatant were stained with fluorescently labeled antibodies to CD4, CD8a, CD25, CD44, CD62L, CD69, membrane, and lymphocytes in the supernatant were stained with fluorescently labeled antibodies to CD4, CD8a, CD25, CD44, CD62L, CD69, and CD90.1 (Supplementary Data). Isotype-matched mAb served as negative controls.

Tumor measurement and outcomes. Tumor size (length × width) was measured with Vernier calipers. Responses were classified as complete response (CR; no measurable tumor), partial response (PR; >50% reduction), stable disease (SD; <50% reduction or <25% increase), or progressive disease (PD; >25% increase). In some cases, small tumors were discovered at necropsy and hence could not be classified with respect to outcome. Only mice with a total tumor burden <350 mm^2 and with no single tumor exceeding 180 mm^2 were used.

Cell lines. Mammary tumors were dissociated with a 100-μm cell strainer and grown in high-glucose DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1 x insulin, transferrin, and sodium selenite (11884, Sigma). Fibroblasts were removed by differential trypsinization. Cell lines were assessed by flow cytometry for H-2kb/H-2Db (28–86), I-Ab (KH74, BD Biosciences), c-Neu (Ab-4, Oncogene Research), or SIINFEKL/MHC class I (25-D1.15; kindly provided by Jonathan Bramson; ref. 22).

Tissue analysis. Tumor tissue was processed following standard methods and either stained with H&E or subjected to immunohistochemistry with antibodies to Neu (2242, Cell Signaling) or CD3 (C7930, Sigma; Supplementary Data). Lung metastases were evaluated by thorough histology with antibodies to Neu (2242, Cell Signaling) or CD3 (C7930, Sigma; Supplementary Data). Lung metastases were evaluated by thorough histology with antibodies to Neu (2242, Cell Signaling) or CD3 (C7930, Sigma; Supplementary Data).

Results

Development and characterization of the neuOT-I/OT-II transgenic mouse model. To investigate CD4* and CD8* T-cell responses to spontaneously arising mammary tumors, we tagged the activated allele of rat HER-2/neu at its COOH terminus with CD4* and CD8* T-cell epitopes from ovalbumin, generating the fusion protein NeuOT-I/OT-II (Fig. 1). We used epitopes that are recognized in the context of MHC class I and II by the TCR transgenic mouse strains OT-I (CD8*) and OT-II (CD4*), respectively (19, 20). The epitope-tagging strategy ensures that the OT-I and OT-II epitopes are present at 1:1 stoichiometry with Neu; therefore, the density of antigen mimics that of a physiologic membrane oncoprotein. Moreover, the strategy should theoretically constrain the ability of tumors to evade immune rejection through antigen loss. To test whether the OT-I and OT-II epitopes contained within NeuOT-I/OT-II could be processed and presented to T cells, we stably expressed NeuOT-I/OT-II in the murine tumor line ID8 (23). As expected, ID8 cells expressing NeuOT-I/OT-II induced potent proliferation of splenocyte cultures from both OT-I and OT-II TCR transgenic mice (data not shown).

We next created C57BL/6 transgenic mice that express NeuOT-I/OT-II in mammary epithelium under the control of the MMTV promoter/enhancer (9). Only ~50% of female transgenic mice expressing NeuOT-I/OT-II developed mammary tumors by 16 months of age. To accelerate tumor formation, a dominant-negative version of p53 (DNp53) was coexpressed under the control of the WAP promoter (24). In contrast to singly transgenic mice, 80% of mice transgenic for both NeuOT-I/OT-II and DNp53 developed mammary adenocarcinomas at 6 to 10 months of age (mean, 6.2 months). The majority of mice presented with one or two tumors, although mice with more than two primary tumors were also seen. Primary tumors did not form at sites other than mammary gland.

Consistent with previous reports for MMTV/neu–induced mammary adenocarcinomas (25), tumors were high grade and highly mitotic and showed a solid histologic subtype, with glandular differentiation in ~30% of cases (Fig. 2A). Most tumors (~90%) had minimal necrosis, consistent with their pronounced vasculature. Immunohistochemistry revealed a range of NeuOT-I/OT-II expression in tumors (Fig. 2B; Supplementary Fig. S1). In a blinded assessment of 40 tumors on tissue microarrays (TMA) using standard clinical immunohistochemistry scoring criteria (26), 3% were negative for NeuOT-I/OT-II (0), 20% were marginally positive (1+), and 77% were positive (2+ and 3+). By Western blot, high-level expression of NeuOT-I/OT-II was detected in mammary tumors, whereas all other tissues were negative except for low-level expression in ovary and lung (Fig. 2C). Tumors also expressed cytoherins, consistent with an epithelial origin (data not shown).
Expression of the OT-I epitope, MHC class I, and MHC class II was assessed by flow cytometry of 26 tumor cell lines derived by short-term culture of spontaneously arising tumors (Fig. 3; Supplementary Fig. S2). Similar to the immunohistochemistry results, 22 of 26 lines were positive for Neu\textsuperscript{OT-I/OT-II} by flow cytometry and the majority (n = 24 of 26) were also positive for MHC class I. About half of the cell lines (n = 14 of 26) stained positive with an antibody that recognizes the OT-I peptide in the context of MHC class I (22). Few lines (n = 4 of 26) were positive for MHC class II. After exposure to IFN-\gamma, a large majority of tumor lines expressed MHC class I (n = 26 of 26), the SIINFEKL/MHC class I complex (n = 22 of 26), and MHC class II (n = 22 of 26).

**In vivo T-cell proliferative responses to mammary tumors expressing neu\textsuperscript{OT-I/OT-II}.** Naive OT-I and/or OT-II T cells were labeled with CFSE and infused into mice bearing established tumors to achieve a circulating frequency of 1% to 2% of total CD8\textsuperscript{+} or CD4\textsuperscript{+} T cells, respectively. Adoptively transferred T cells were further demarcated by expression of the congeneric cell surface marker Thy1.1, which allows discrimination from Thy1.2\textsuperscript{+} host T cells. In the majority (83%) of mice, we failed to detect OT-II cell proliferation in the blood, even as late as 13 days after transfer (Fig. 4A and C). In contrast to blood, when lymph nodes were examined on day 6, 5% to 10% of the OT-II cells had undergone multiple rounds of division (8 of 8 mice) and were CD44\textsuperscript{+}, indicating that a small subset of OT-II cells did indeed respond (Fig. 4A). The limited extent of OT-II cell proliferation seen in most mice did not reflect a defect in the T cells themselves, as immunization of tumor-bearing mice with ovalbumin induced robust OT-II proliferation (Fig. 4A). Thus, exposure to antigen may be a limiting factor for the OT-II response *in vivo*. Indeed, OT-II cells are known to have a relatively high activation threshold (27).

Analogous studies of the CD8\textsuperscript{+} T-cell response yielded strikingly different results. Whether infused alone or together with OT-II T cells, OT-I cells consistently showed strong proliferation in response to neu\textsuperscript{OT-I/OT-II} × DNp53 tumors (Fig. 4B). OT-I cells began proliferating by day 3, and by days 4 to 7, they constituted anywhere from 2% to 43% of the total CD8\textsuperscript{+} T-cell population (Fig. 4C). This was invariably followed by a contraction period of several days, after which OT-I cells persisted at detectable levels (0.5–5%) for several weeks. Thus, OT-I cells followed a stereotyped course of expansion and contraction in response to spontaneous mammary tumors, similar to that seen with viral or bacterial challenges (28). The OT-I proliferative response was both antigen and tumor specific. Specifically, only negligible OT-I proliferation occurred in tumor-free neu\textsuperscript{OT-I/OT-II} × DNp53 mice, consistent with low expression of the neu\textsuperscript{OT-I/OT-II} transgene in nonmalignant tissues (Fig. 2C). Furthermore, CD8\textsuperscript{+} T cells expressing an irrelevant transgenic TCR (P14 T cells) failed to proliferate in tumor-bearing neu\textsuperscript{OT-I/OT-II} × DNp53 mice (data not shown).

**Mammary tumors show a range of responses to adoptively transferred T cells.** Remarkably, the adoptive transfer of naive OT-I + OT-II T cells lead to the complete response of 37% of tumors. Regressions started within 4 to 8 days of T-cell infusion and were complete by days 10 to 17. In other cases, PR, SD, or PD was
observed. In a study of 68 tumors from 28 mice given similar doses of OT-I + OT-II T cells, the response rates were as follows: CR, 37%; PR, 25%; SD, 15%; and PD, 24% (Fig. 5A). These antitumor responses were largely, but perhaps not entirely, attributable to the OT-I cells. Specifically, for 12 tumors (in four mice) treated with OT-I cells alone, the response rates were as follows: CR, 8%; PR, 25%; SD, 8%; and PD, 58% (Fig. 5A). Thus, adoptive transfer of OT-I cells alone can induce antitumor responses, but there is a trend toward more frequent regressions in the presence of OT-II cells. By contrast, when OT-II cells were infused alone or with P14 CD8+ T cells, 100% (16 of 16) of tumors grew progressively.

A representative neuOT-I/OT-II × DNp53 tumor cell line was grown for 48 h in the presence (heavy lines) or absence (thin lines) of 100 units/mL IFN-γ and analyzed by flow cytometry for expression of Neu (A), MHC class I (B), the SIINFEKL epitope from ovalbumin in the context of MHC class I (C), and MHC class II (D). Shaded histograms, staining with secondary antibody alone.

To determine if antigen loss might account for the apparent resistance of some tumors to T-cell infusion, expression of NeuOT-I/OT-II was assessed by immunohistochemistry of TMAs containing 40 untreated tumors and 27 tumors that had shown a nonregressing phenotype (i.e., PR, SD, or PD) after adoptive transfer of OT-I + OT-II cells (Supplementary Fig. S1). There was no significant difference in the proportion of negative-marginal tumors (i.e., immunohistochemistry scores of 0 or 1+) between the untreated group and the treated, nonregressing group (23% versus 12%; P = 0.26). Moreover, the mean immunohistochemistry scores for Neu expression were 1.98 and 2.29 for untreated and nonregressing tumors, respectively.

Antigen expression and presentation were also evaluated by flow cytometry. Similar to the untreated cell lines described previously, cell lines derived from treated, nonregressing tumors were typically positive for NeuOT-I/OT-II (n = 5 of 5) and MHC class I (n = 4 of 5) and showed more limited expression of the SIINFEKL/MHC class I complex (n = 1 of 5) and MHC class II (n = 0 of 5; Supplementary Fig. S1; data not shown). Following IFN-γ treatment, increased expression of MHC class I, the SIINFEKL/MHC class I complex, and MHC class II was observed in 5 of 5, 5 of 5, and 2 of 5 cases, respectively (Supplementary Fig. S1). Thus, 5 of 5 nonregressing tumors retained expression of Neu and presented the OT-I epitope on MHC class I after exposure to IFN-γ. Therefore, the majority of nonregressing tumors in this model have not lost antigen expression or presentation.

Fig. 3. Antigen expression and presentation by neuOT-I/OT-II × DNp53 tumor cells. A representative neuOT-I/OT-II × DNp53 tumor cell line was grown for 48 h in the presence (heavy lines) or absence (thin lines) of 100 units/mL IFN-γ and analyzed by flow cytometry for expression of Neu (A), MHC class I (B), the SIINFEKL epitope from ovalbumin in the context of MHC class I (C), and MHC class II (D). Shaded histograms, staining with secondary antibody alone.

Tumor-specific factors dictate the outcome of T-cell responses. Intriguingly, in mice bearing multiple primary tumors, combinations of CR, PR, SD, and PD were commonly observed in response to the same T-cell infusion (Supplementary Table S1). For example, Fig. 5C shows results for one mouse that presented with three primary tumors. After a single infusion of naive OT-I + OT-II cells, two tumors completely regressed, whereas the third progressed. Thus, factors in the local tumor environment, as opposed to systemic immunologic properties, seemed to dictate the outcome of T-cell responses.

To determine whether the different responses of tumors to T cells reflects an inherent, stable property of the tumor cells themselves, we derived several tumor cell lines, which were then implanted in host mice and challenged with adoptively transferred OT-I and OT-II cells. We selected cell lines that expressed NeuOT-I/OT-II, MHC class I, and the SIINFEKL/MHC class I complex by flow cytometry. In initial experiments, we attempted to implant the tumor lines into the mammary fat pad of wild-type C57Bl/6 mice, but the lines were invariably rejected. By contrast, when tumor-free neuOT-I/OT-II transgenic mice were used as hosts, the engraftment rate approached 100%. This presumably reflects tolerance of neuOT-I/OT-II transgenic mice to the NeuOT-I/OT-II protein, as has been shown for Neu in the conventional MMTV/neu transgenic model (29–31). Thus, young, tumor-free neuOT-I/OT-II transgenic mice were used as hosts for all subsequent experiments.

Tumor cell lines were implanted in the mammary fat pad, and when tumors reached 50 to 100 mm2, mice underwent adoptive transfer with naive OT-I + OT-II cells as before. As with spontaneous tumors, strong OT-I cell proliferation and weak OT-II cell proliferation was seen in virtually all cases within 3 to 6 days of adoptive transfer. Tumor cell lines showed a range of responses, but for each cell line, responses were remarkably consistent (Fig. 5D).
For example, the cell line NOP-21 underwent complete regression in 6 of 6 mice. By contrast, the cell lines NOP-12 and NOP-13 each showed a PR in 5 of 5 and 3 of 4 host mice, respectively. Finally, the cell lines NOP-6 and NOP-18 each showed PD in 3 of 3 mice. Furthermore, when the NOP-13 and NOP-18 cell lines were implanted on opposite sides of the same animal and treated with the same dose of T cells, they responded as if treated alone (data not shown). As with spontaneous tumors, the responses of tumor cell lines could not be predicted based on expression of NeuOT-I/OT-II, MHC class I, the SIINFEKL/MHC class I complex, or MHC class II (Supplementary Fig. S2). Thus, neuOT-I/OT-II × DNP53 tumors, whether they develop spontaneously or from implanted cell lines, differ in their intrinsic sensitivity to adoptively transferred T cells due to factors other than antigen expression or presentation.

**Many nonregressing tumors resist T-cell infiltration despite expressing antigen.** Having excluded antigen loss as a common mechanism of evasion in this model, we next evaluated the ability of OT-I and OT-II cells to traffic to and infiltrate spontaneous neuOT-I/OT-II × DNP53 tumors. Before adoptive transfer, tumors consistently showed minimal lymphocytic infiltrates by H&E staining and anti-CD3 immunohistochemistry (Fig. 6A). Similarly, minimal lymphocytic infiltrates were seen after adoptive transfer of OT-II cells alone or in combination with P14 cells (data not shown). In contrast, when OT-I and OT-II cells were coinfused, 80% (41 of 51) of tumors isolated on days 6 or 7 showed moderate to extensive infiltration by CD3+ T cells (Fig. 6B). Flow cytometry confirmed that OT-I was the predominant CD8+ T-cell clone in these infiltrates (75% of intratumoral CD8+ cells; range, 18–90%; Fig. 6D). Consistent with an activated phenotype, intratumoral OT-I cells displayed a range of molecular, cellular, and histologic features. The large majority of the PD cases.

**Discussion**

Using a novel transgenic mouse model, we have investigated for the first time the response of naive, adoptively transferred CD4+ and CD8+ T cells to spontaneous mammary tumors. The combination of a strong oncogene (neuOT-I/OT-II) and a disabled DNA repair mechanism (DNP53) resulted in mammary tumors displaying a range of molecular, cellular, and histologic features. After adoptive transfer, CD4+ OT-II cells generally mounted weak proliferative responses, whereas CD8+ OT-I cells proliferated vigorously. This was followed by the complete regression of tumors. However, these intratumoral OT-II cells showed elevated expression of CD44, CD25, and CD69 and down-regulation of CD62L, indicating an activated phenotype (Fig. 6D). The remaining ~20% of tumors were essentially devoid of CD3+ T-cell infiltrates on days 6 or 7 (Fig. 6C). In many cases, CD3+ T cells were present in the stroma, indicating that they had migrated to the tumor site but failed to cross from stroma to malignant epithelium. In other cases, even migration to the stroma had not occurred. Antigen expression was not an issue, as poorly infiltrated tumors showed moderate to strong expression of NeuOT-I/OT-II by immunohistochemistry in 90% of cases. Tumor size was also not an issue, as there was no significant difference in the average size of well-infiltrated and poorly infiltrated tumors (67.9 versus 71.1 mm^2, P = 0.85). Finally, failed T-cell activation or proliferation was not an issue, as virtually all mice exhibited robust proliferation of OT-I cells (and sometimes OT-II cells) in peripheral blood. Thus, a significant subset of tumors showed primary resistance to T-cell infiltration due to local barriers in the tumor environment, which likely accounts for a large majority of the PD cases.

**Figure 4.** Proliferation of adoptively transferred OT-I and OT-II T cells in mice bearing spontaneous neuOT-I/OT-II × DNP53 mammary tumors. A, left, OT-II cells assessed in peripheral blood on day 3. Middle, OT-II cells assessed in peripheral blood 8 d after adoptive transfer and 4 d after ovalbumin immunization, showing that weak proliferative responses to tumors can be overcome by immunization. B, OT-I cells assessed in peripheral blood on day 3, showing a typical robust proliferative response. C, OT-I and OT-II T cells (Thy1.1+) were enumerated by flow cytometry at serial time points after adoptive transfer into a tumor-bearing mouse. The relative number of donor OT-I or OT-II cells in peripheral blood is expressed as a percentage of total circulating CD8+ or CD4+ T cells, respectively. Results are from a single animal and are representative of 18 experiments.
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37% of tumors without the need for any other intervention. The remaining tumors showed partial/stable responses or PD, despite retaining antigen expression in most cases. In mice bearing multiple primary tumors, simultaneous regressions and non-regressions were often observed in response to the same dose of T cells. Thus, therapeutic responses were largely (if not entirely) dictated by local, inherent properties of the tumor rather than systemic immunologic effects.

Although there are numerous examples in which immune-based interventions can prevent or delay tumor development, or cure early-stage disease (13, 16, 17, 32), to our knowledge, this is the first demonstration of advanced, spontaneous tumors being permanently eradicated by adoptive T-cell transfer alone. The complete regressions we observed could be attributable to several factors, including the use of naive T cells, the use of a high-affinity T-cell epitope (SIINFEKL) linked to an essential oncogene, or the use of spontaneously arising tumors rather than implanted cell lines. Previous studies have assessed whether naive or activated CD8+ T cells have greater antitumor activity (33, 34). In general, the evidence favors the use of naive T cells, which have a greater proliferative and tumor killing capacity than previously activated T cells. However, in unpublished experiments, we found that activated OT-I cells showed equal or greater antitumor activity relative to naive OT-I cells when tested against implanted neuOT-I/OT-II × DNp53 tumor cell lines. Although preliminary, this suggests that naive T cells are not essential for antitumor responses in this model. Furthermore, several other groups have used naive, TCR transgenic CD8+ T cells and did not observe complete regressions unless other interventions, such as irradiation and vaccination, were also applied (35, 36). Thus, the use of naive T cells in our study does not fully explain the striking tumor responses we observed.

A second possibility may stem from our use of a high-affinity CD8+ T-cell epitope attached to a tumor-initiating oncogene. Expression of NeuOT-I/OT-II represents both a benefit to tumors, due to its oncogenic properties, and a liability, due to the increased immunogenicity conferred by the epitope tags. The majority of tumors seemed to resolve this dilemma by retaining expression of NeuOT-I/OT-II, often at high levels, while avoiding immune rejection by other mechanisms. One mechanism may simply be central and/or peripheral tolerance, which reduces the number of tumor-reactive T cells in circulation, as has been documented for CD4+ and CD8+ T-cell epitopes from Neu in conventional MMTV/neu transgenic mice (29–31). Importantly, tumors relying solely on this mechanism would be highly susceptible to adoptive transfer of T cells, as this would rapidly raise the number of tumor-reactive T cells, resulting in a large, unopposed immune response. This may

Figure 5. neuOT-I/OT-II × DNp53 tumors show a range of responses to adoptively transferred OT-I + OT-II T cells. A, mice bearing spontaneous tumors underwent adoptive transfer of either OT-I cells alone (white columns) or OT-I + OT-II cells (black columns), and tumor responses were scored as described in Materials and Methods. Numbers below each column are the absolute numbers of tumors in each group. B, a scar remaining at a regressed tumor site approximately 3 mo after adoptive transfer. C, simultaneous regression and progression of established spontaneous tumors in a single neuOT-I/OT-II × DNp53 mouse. After adoptive transfer of OT-I + OT-II T cells, two tumors (T1 and T3) regressed completely, whereas the third tumor (T3) grew progressively. D, cell lines derived from untreated neuOT-I/OT-II × DNp53 tumors and implanted into the mammary fat pads of recipient mice show reproducible responses to treatment with adoptively transferred OT-I + OT-II cells. Tumor cell line NOP-21 underwent a CR in 6 of 6 mice. By contrast, NOP-12 underwent a PR in 5 of 5 mice and NOP-6 grew progressively in 3 of 3 mice.
explain the large subset of tumors that underwent complete regression after adoptive transfer of OT-I + OT-II cells.

A third possible explanation for the complete regressions we observed may be biological differences between spontaneous tumors and implanted cell lines. A bolus of injected tumor cells may trigger inflammatory or immune responses that select for the outgrowth of immune-resistant subclones. By contrast, spontaneous tumors develop in a slow, progressive manner that may evoke less immune recognition and selection. If so, then immunotherapy may prove more effective against spontaneous tumors than implanted tumor cell lines. Indeed, fewer cell lines derived from neu$_{OT-I/OT-II}$ x DNp53 tumors show complete regressions after adoptive transfer (1 of 16; Supplementary Fig. S2) compared with the ~37% rate seen with spontaneous tumors.

Nonregressing tumors seemed to resist immune rejection by several mechanisms. About 10% of tumors expressed negligible levels of Neu$_{OT-I/OT-II}$ by immunohistochemistry, similar to the antigen-negative variants described in the conventional MMTV/neu

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**Figure 6.** Lymphocyte infiltration of untreated and treated neu$_{OT-I/OT-II}$ x DNp53 tumors. A to C, three different tumors were stained with H&E (top) or anti-CD3 antibody (bottom). A, an untreated tumor, showing minimal lymphocytic infiltration. B, a treated tumor harvested on day 6 after adoptive transfer of OT-I + OT-II cells, showing a heavily infiltrated phenotype. C, a treated tumor harvested on day 6 after adoptive transfer, showing a poorly infiltrated phenotype. Magnification, ×400.

D, flow cytometric analysis of TILs harvested on day 6 after adoptive transfer of OT-I + OT-II cells. Lymphocytes were gated by forward and side scatter and analyzed for expression of Thy1.1 (which marks all donor T cells), CD4 versus CD8, and the activation markers CD44, CD62L, CD25, and CD69 (black lines) versus isotype-matched controls (gray lines). Top, donor OT-I T cells (CD8+Thy1.1+ cells) were the predominant CD8+ T cell in tumor infiltrates and displayed an activated phenotype; bottom, donor OT-II cells (CD4+Thy1.1+ cells) were a minor component of tumor-infiltrating CD4+ T cells but nevertheless displayed an activated phenotype.
tumor model (11). A further 20% of tumors resisted T-cell infiltration, despite showing moderate to strong expression of NeuOT-1OT-II in the vast majority of cases. Poor lymphocytic infiltration has been reported in numerous other tumor models and has been attributed to diminished leukocyte-vessel wall interactions and adherence (37, 38). Angiogenic factors [vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)] and immunosuppressive cytokines (transforming growth factor-β and IL-10) can mediate this effect by down-regulating endothelial adhesion molecules, such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 (VCAM-1; ref. 39). Tumors from conventional MMTV/neu transgenic mice reportedly express both bFGF and VEGF but not VCAM-1 (16), which may account for the poor infiltration of some tumors in our model. Importantly, in animal models, several strategies have proven effective in overcoming infiltration barriers, including radiation, chemotherapy, administration of CpG oligonucleotides, CTLA-4 blockade, or infusion of cytokines, such as IL-12, granulocyte macrophage colony-stimulating factor, or LIGHT (40–45).

The remaining 70% to 80% of tumors expressed NeuOT-1OT-II and were permissive to T-cell infiltration, yet only about half completely regressed. This implies that other factors in the tumor environment may impair the OT-I and OT-II response, resulting in partial or stable response. Prior work in other models suggests several possibilities. In some tumors, the infiltrating OT-I cells may become anergic due to inadequate costimulation or the presence of local immunosuppressive factors (46). If tumor regression does not occur rapidly, OT-I cells could undergo clonal exhaustion or activation-induced cell death due to chronic antigen expression (47). Other cells in the tumor stroma or infiltrate could suppress the OT-I cells, including myeloid-derived suppressor cells or regulatory T cells (48–50). Indeed, we have observed infiltration of some tumors by host FoxP3+ T cells on days 6 and 7 after adoptive transfer (data not shown). The ability to generate new neuOT-1OT-II × DNP35 tumor cell lines with consistent immunologic properties will allow future investigation of the different immune evasion mechanisms at play in this model, as well as the most effective countermeasures.

It is noteworthy that T-cell–sensitive and T-cell–resistant tumors often arose at the same time in the same animals. This implies that evasion of the host immune response during early tumorigenesis occurs at a local level, with different tumors displaying different strategies. Alternatively, it may imply that host immune surveillance does not play a significant role in shaping tumor phenotypes and that immune resistance is instead a by-product of other selective pressures on the tumor.

The concept of predictive and personalized medicine is being applied more broadly in oncological practice, owing to our increasing recognition of the molecular heterogeneity of human cancer. However, with immunotherapy, this concept rarely extends beyond the typing of tumors for antigen and MHC class I expression. We have shown that even in a highly circumscribed experimental system in which the oncogene, antigen, T-cell dose, and genetic background are all uniform, spontaneous tumors show a range of inherent immunologic phenotypes. It seems likely that these different phenotypes will be sensitive to distinct immunologic interventions. With improved understanding of the different immunologic environments that develop in spontaneous cancers, it may be possible to prospectively identify the dominant immunologic barriers in individual tumors and counteract these with the most appropriate interventions, thereby optimizing both the cost and benefit of immunotherapy.

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References

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