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# Functional T Cell Responses to Tumor Antigens in Breast Cancer Patients Have a Distinct Phenotype and Cytokine Signature<sup>1</sup>

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The overall prevalence with which endogenous tumor Ags induce host T cell responses is unclear. Even when such responses are detected, they do not usually result in spontaneous remission of the cancer. We hypothesized that this might be associated with a predominant phenotype and/or cytokine profile of tumor-specific responses that is different from protective T cell responses to other chronic Ags, such as CMV. We detected significant T cell responses to CEA, HER-2/*neu*, and/or MAGE-A3 in 17 of 21 breast cancer patients naive to immunotherapy. The pattern of T cell cytokines produced in response to tumor-associated Ags (TAAs) in breast cancer patients was significantly different from that produced in response to CMV or influenza in the same patients. Specifically, there was a higher proportion of IL-2-producing CD8<sup>+</sup> T cells, and a lower proportion of IFN- $\gamma$ -producing CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells responding to TAAs compared with CMV or influenza Ags. Finally, the phenotype of TAA-responsive CD8<sup>+</sup> T cells in breast cancer patients was almost completely CD28<sup>+</sup>CD45RA<sup>-</sup> (memory phenotype). CMV-responsive CD8<sup>+</sup> T cells in the same patients were broadly distributed among phenotypes, and contained a high proportion of terminal effector cells (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>) that were absent in the TAA responses. Taken together, these results suggest that TAA-responsive T cells are induced in breast cancer patients, but those T cells are phenotypically and functionally different from CMV- or influenza-responsive T cells. Immunotherapies directed against TAAs may need to alter these T cell signatures to be effective. *The Journal of Immunology*, 2007, 179: 2627–2633.

Pre-existing T cell responses to tumor-associated Ags (TAAs)<sup>3</sup> have been reported in patients with solid tumors, including melanomas, colorectal, lung, breast, ovarian, prostate, and pancreatic carcinomas (1–5). However, these responses usually involve a low frequency of T cells, and are not detected in the majority of patients, with the exception of melanoma (1, 5, 6). Results in breast cancer patients have been particularly conflicting with regard to the prevalence of pre-existing T cell responses (7–9). This might be due in part to a predominant focus on single epitope responses, and the use of a single cytokine (IFN- $\gamma$ ) to detect responses. As a result, the total prevalence of TAA-specific T cell responses in cancer patients is not clear.

When spontaneous TAA-specific T cell responses have been detected in cancer patients, those responses are not often correlated with clinical regression of the tumor (10). Thus, in most cases, these TAA-specific responses could be considered nonprotective. Data in mouse systems suggest that induction of a strong Th1-

dominated T cell response is associated with protective immunity to cancer (11). It is thus possible that spontaneous TAA exposure does not sufficiently prime Th1 immunity in most patients.

Protective T cell immunity to other chronic Ags is well studied, and is exemplified by the response to CMV in immunocompetent adults (12). Not only are CMV-specific T cells present at high frequencies in this subclinical infection, but they have a distinct pattern of phenotypes and produce characteristic cytokines (13–15). Specifically, the phenotype of CMV-specific T cells is heterogeneous, but dominated by CD8<sup>+</sup> terminal effector cells (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>). The cytokine profile of these cells is characterized by a high frequency of IFN- $\gamma$  production and a lower frequency of IL-2 production. This is consistent with a strongly Th1-biased response.

Influenza is another virus for which there are data on the cytokine composition and phenotype of responsive T cells (16, 17). Because influenza infection is acute rather than chronic, the response tends toward a central memory T cell phenotype, with a greater proportion of IL-2-producing T cells (17). Because the phenotype and cytokine signature of protective T cell responses differ between known pathogens like CMV and influenza, it is difficult to predict the exact nature of a protective immune response against a completely different agent such as cancer. However, given the chronic nature of tumors, it might be reasonable to expect that the successful response to CMV could provide a model for the type of response that we wish to elicit against cancer.

In this study, we compared spontaneous Ag-specific T cell responses to TAAs, CMV, and influenza in breast cancer patients. We found that these patients had significantly higher frequencies of TAA-responsive T cells than healthy adults, and that those T cells had phenotypes and cytokine signatures distinct from both

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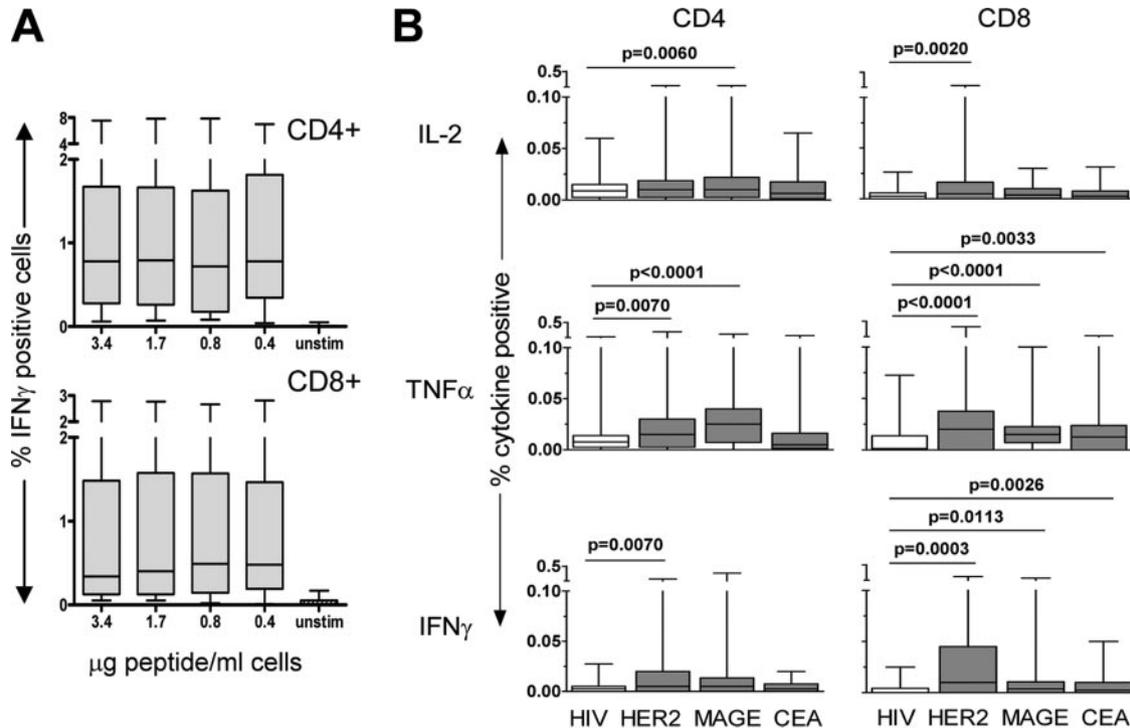
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<sup>3</sup> Abbreviations used in this paper: TAA, tumor-associated Ag; BFA, brefeldin A; ICS, intracellular staining.

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**FIGURE 1.** Validation of lyophilized plate ICS assay and TAA responses in healthy donors. *A*, Titration of CMV pp65 peptide mix in CMV-seropositive healthy donors ( $n = 21$ ), along with unstimulated background. CD4<sup>+</sup> T cell responses are shown in the *top panel*, and CD8<sup>+</sup> T cell responses in the *bottom panel*. Friedman ANOVA showed no titration effect over the range of 0.4–3.4  $\mu\text{g/ml}$ /peptide, for this and other peptide mixes used in this study. Therefore, all four titer points were averaged to achieve better precision for low-level TAA responses. *B*, Responses of 41 healthy HIV-seronegative donors to TAA (■) vs HIV (■) peptide mixes. Significant differences ( $p < 0.017$ ) vs HIV are marked, based on a Bonferroni correction. Boxes indicate the 25th to 75th percentile. The whiskers represent maximum and minimum, whereas the bars within the box represent the median. Data in *B* are shown after subtraction of unstimulated background.

CMV- and influenza-responsive T cells. Specifically, the patients' TAA responses showed a decreased proportion of IFN- $\gamma$ -producing CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells and an increased proportion of IL-2-producing CD8<sup>+</sup> T cells, as well as a lack of terminal effector differentiation.

## Materials and Methods

### Patients

Peripheral blood was collected after obtaining informed consent from 41 healthy volunteers (21 females) ranging in age from 29 to 63 years old, with a median age of 40 years. Peripheral blood samples were obtained after informed consent from 21 females with primary breast cancer. Patients enrolled in this study were within 5 years of diagnosis, not currently receiving chemotherapy, and with stable disease. All had received prior surgery and/or chemotherapy to reduce their initial tumor burden. There were 5 patients at stage I, 9 patients at stage II, 2 patients at stage III, 3 patients at stage IV, and 2 patients were of unknown stage. Patient age ranged between 31 and 79 years old, with a median age of 52 years.

### PBMCs

Cells from breast cancer patients collected by leukapheresis were washed with HBSS (Mediatech), and then PBMCs were isolated by density centrifugation on Ficoll-Paque (Amersham Biosciences) and washed twice with HBSS. PBMCs were then resuspended in RPMI 1640 + 10% heat-inactivated FBS (cRPMI-10) and counted. Blood samples from healthy donors were collected in CPT tubes (BD Preanalytical Systems) and processed according to product directions. PBMCs were washed in cRPMI-10 and resuspended at required densities.

### Peptides

Mixtures of peptides consisting of 15 aa residues, overlapping by 11 aa residues each (18), were designed to span the sequences of the proteins used in this study. These proteins (with GenBank accession numbers in parentheses) included the following: HIV p55 *gag* (AAB50258), CMV

pp65 (AAT68258), IE-1 (P13202) (19), CEA (CAE75559), MAGE-A3 (NP 005353), influenza A matrix protein (AAB03207), hemagglutinin (CAC86622), and the intracellular domain of HER-2/*neu* (P04626, p675–1255). All peptide mixes were obtained from SynPep at 80% purity, except IE-1 peptide mix, which was obtained from Jerini at 70% purity. All peptide and peptide mixes were reconstituted at  $>1$  mg/ml/peptide in DMSO and used at concentrations ranging from 3.4 to 0.4  $\mu\text{g/ml}$ /peptide. There was no significant titration effect (Fig. 1A); therefore, the results of the four titer points were averaged for all analyses. Staphylococcal enterotoxin B (Sigma-Aldrich) was used as a positive control for Ag stimulation. Brefeldin A (BFA; Sigma-Aldrich) was added at a final concentration of 10  $\mu\text{g/ml}$  with all peptides and peptide mixes. Negative controls included no addition, BFA, or DMSO + BFA.

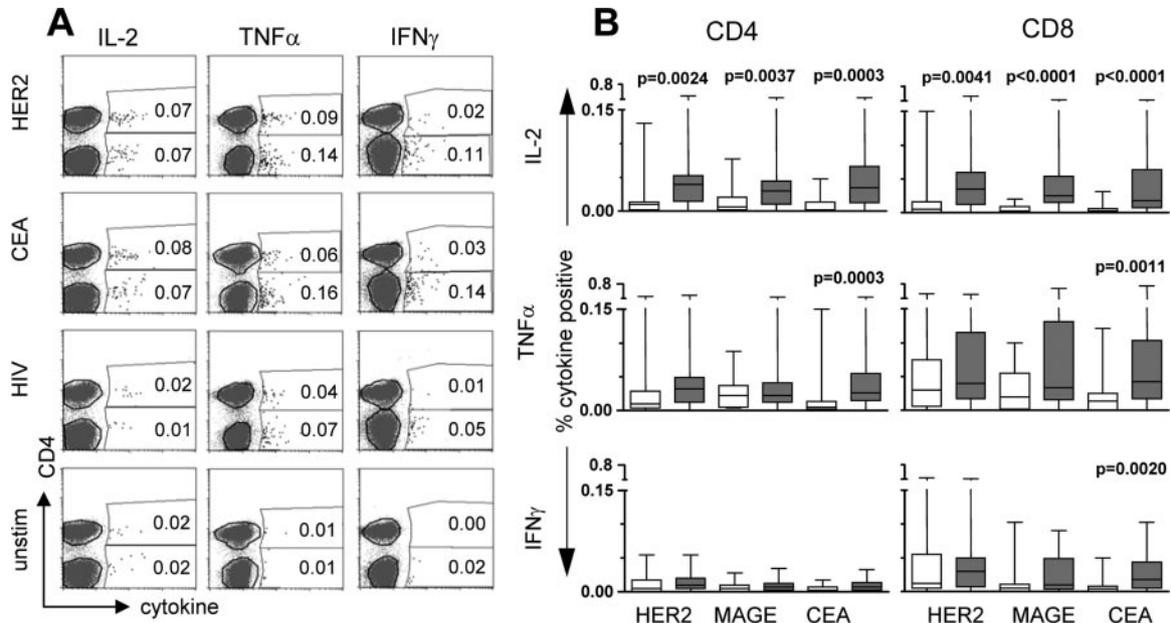
### Lyophilized stimulation and staining plates

We used a highly automated intracellular cytokine staining assay using preconfigured lyophilized stimulation and staining plates that had been previously validated (20–22). Clustering algorithms confirmed that seropositive donors clustered separately from seronegative donors based on cytokine responses to CMV peptide mixes pp65 and IE-1 (data not shown). This assay performed with similar sensitivity to published data (14), with a median CD8<sup>+</sup> T cell response to CMV pp65 peptide mix of 0.41% ( $n = 21$ ; see Fig. 1A), compared with 0.40% ( $n = 14$ ) in the previous study (14).

All Abs were obtained from BD Biosciences. Stimulation plates were formulated with peptide mixes and BFA, then lyophilized into polypropylene V-bottom 96-well plates. Staining plates were formulated with Ab mixtures in appropriate wells and lyophilized. Two Ab mixtures were used as follows: anti-IFN- $\gamma$  FITC, CD69 PE, CD4 PerCP Cy5.5, and CD3 allophycocyanin; and anti-TNF- $\alpha$  FITC, anti-IL-2 PE, CD4 PerCP Cy5.5, and CD3 allophycocyanin.

### Stimulation for intracellular staining (ICS) assays

Freshly isolated PBMCs from either healthy donors or breast cancer patients were added to each well of a lyophilized stimulation plate containing



**FIGURE 2.** Breast cancer patients make specific cytokine responses to TAAs. *A*, Representative flow cytometry dot plots of IFN- $\gamma$ , IL-2, and TNF- $\alpha$  responses to HER-2/*neu*, MAGE-A3, and irrelevant (HIV *gag*) peptide mixes in a 52-year-old stage IV breast cancer patient. Cells were first gated for small lymphocytes (using forward and side scatter), and for CD3<sup>+</sup> cells (using CD3 vs side scatter). Logical gates were then set to analyze cytokine responses. Detectable responses to the HIV mix were mostly limited to TNF- $\alpha$ , as seen in this example. *B*, To determine whether breast cancer patients naive to immunotherapy demonstrate pre-existent TAA-specific T cell responses, cytokine responses were compared between healthy donors ( $\square$ ) and breast cancer patients ( $\blacksquare$ ). Boxes indicate the 25th to 75th percentile. The whiskers represent maximum and minimum, whereas the bars within the box represent the median. Significant *p* values (*p* < 0.0060) are shown. All data are shown after subtraction of unstimulated background. Note that IL-2 was the only cytokine to show consistent significant differences between healthy donors and cancer patients, for all three TAAs.

peptides and controls with BFA, then mixed with a micropipettor to reconstitute the lyophilized pellet. The plate was incubated for 6 h at 37°C, after which the temperature was dropped to 18°C and held overnight.

#### Processing and staining for ICS assays

Activated cells were treated with 2 mM EDTA for 15 min at room temperature and mixed. The plates were centrifuged for 7 min at 250  $\times$  *g* to pellet cells. Supernatant was aspirated using a 12-channel manifold (V&P Scientific). FACS lysing solution (BD Biosciences) was added, and each well was mixed and incubated for 10 min at room temperature. Plates either continued with permeabilization and staining steps, or were stored frozen at -80°C before continuing with processing. Wash buffer (PBS + 0.1% NaN<sub>3</sub> + 0.5% BSA) was added, and plates were centrifuged 5 min at 500  $\times$  *g*. The supernatant was aspirated, and 1 $\times$  FACS Permeabilizing Solution 2 (BD Biosciences) was added. Cells were mixed and incubated for 10 min at room temperature. The plate was centrifuged 5 min at 500  $\times$  *g*, and the supernatant was aspirated. Wash buffer was added, the wells were mixed, and the plate was centrifuged for 5 min at 500  $\times$  *g*. While the wash was repeated, the wells of lyophilized stain plate were hydrated with wash buffer, then transferred to the appropriate well in the Ag plate, mixed, and incubated for 60 min at room temperature in the dark. Wash buffer was added before centrifuging 5 min at 500  $\times$  *g*. The supernatant was aspirated, and two additional washes were performed. The cells were then resuspended in PBS + 1% paraformaldehyde. All data from samples were acquired and analyzed using a FACSCalibur flow cytometer with HTS plate loader and CellQuest Pro software (BD Biosciences).

#### Data analysis

Gates were set for either CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD4<sup>-</sup> lymphocytes, and cells within these gates were examined for cytokine expression by applying dynamic gating using Snap-to regions tethered to negative regions. During analysis, samples were screened based on collection criteria. Data were omitted if gating and other technical errors were detected or a minimum of 10,000 CD3<sup>+</sup>CD4<sup>+</sup> events was not collected. We defined CD3<sup>+</sup>CD4<sup>+</sup> cells as the CD4<sup>+</sup> T cell subset and the CD3<sup>+</sup>CD4<sup>-</sup> cells as the CD8<sup>+</sup> T cell subset.

#### Immunophenotyping of cytokine-responsive cells

PBMC from three breast cancer patients with the highest overall TAA response levels were analyzed by eight-color immunophenotyping with

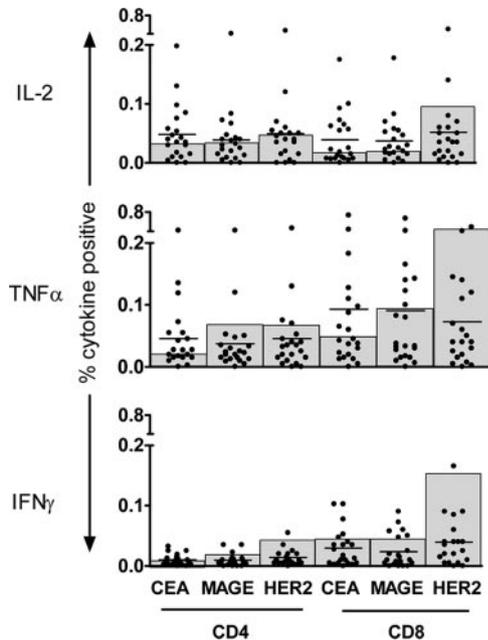
intracellular cytokine staining. Details of the protocol can be found elsewhere (Maecker Laboratory weblog (<http://maeckerlab.typepad.com>)). Briefly, frozen PBMCs were thawed and rested overnight, and stimulated with peptide mixes to CMV pp65 + IE-1, HER-2/*neu*, CEA, or MAGE-A3, as described above. Cells were stained for 60 min with CD28 PerCP Cy5.5, CD45RA PE Cy7, and CD27 allophycocyanin. Samples were treated with BD FACS lysing solution, followed by BD FACS Permeabilizing Solution 2, washed, then stained with anti-IFN- $\gamma$  FITC, anti-IL-2 PE, anti-TNF- $\alpha$  Alexa700, CD8 allophycocyanin Hilyte750, CD3 Pacific blue, and CD4 AmCyan (all from BD Biosciences), washed again, and held at 4°C until sample acquisition. Data from samples were acquired using a BD LSR II flow cytometer. Analysis was conducted, as previously described (15), with minor changes. Using BD FACSDiva software, "or" gates were used to define the cells responding to peptide mixes with the production of one or more cytokines. These Ag-responsive T cells were then phenotyped for memory/effector markers.

#### Statistical analysis

A two-tailed Mann-Whitney *U* test was conducted for comparisons of responses between healthy donors and breast cancer patients. A Friedman ANOVA by ranks was used to determine significance of titration effect for peptide mixes. A Bonferroni adjustment determined significant *p* values for all analyses. A Wilcoxon signed rank test was used to determine significant differences between normalized TAA and CMV responses of CMV<sup>+</sup> breast cancer patients. These data were normalized by range for each Ag in each donor, by calculating  $(x_i - x_{\min}) / (x_{\max} - x_{\min})$ , where  $x_i$  is each cytokine response for a particular donor for a particular Ag;  $x_{\min}$  is the smallest of these same responses; and  $x_{\max}$  is the largest of these responses.

#### Software

Data were acquired and analyzed using CellQuest Pro software (BD Biosciences) for ICS analysis. Data were managed using Multiwell Plate Manager software (BD Technologies). Immunophenotyping data were acquired and analyzed using BD FACSDiva software. Data analysis was performed using Spotfire DecisionSite 8.1. Statistical analyses were performed using GraphPad Prism (GraphPad).



**FIGURE 3.** More breast cancer patient TAA responses are positive for IL-2 than for TNF- $\alpha$  or IFN- $\gamma$ . Cancer patient responses are shown as scatterplots, with the 95th percentile of healthy donor responses shown as  $\square$ ,  $\blacksquare$ . Indicates the mean for the breast cancer population. Note that the highest proportion of responses was above the cutoff for IL-2 (41%), followed by TNF- $\alpha$  (28%) and IFN- $\gamma$  (20%). This was true for all three TAAs. All data are shown after subtraction of unstimulated background.

## Results

### Low-level T cell cytokine responses to TAAs in healthy females

As a baseline for comparison with breast cancer patients, we first assessed the level of T cell cytokine responses to HER-2/*neu*, MAGE-A3, and CEA peptide mixes in PBMC from 21 healthy females (Fig. 1B). These were compared with responses to an irrelevant peptide mix of similar complexity (HIV *gag*). Although

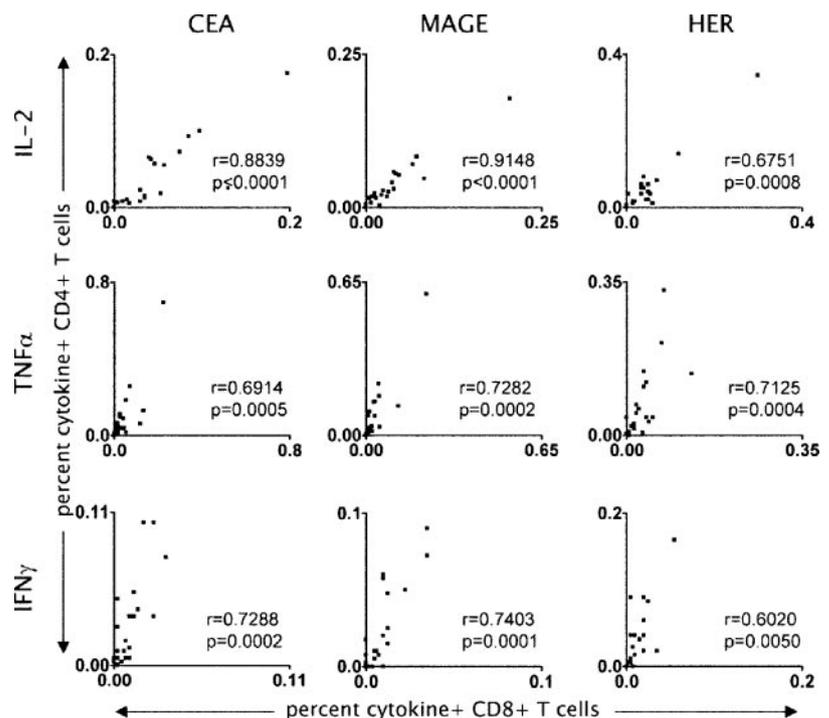
the TAA responses were generally very low (median <0.03%), some selected responses were significantly different from the irrelevant control. Most of these were TNF- $\alpha$  or IFN- $\gamma$  responses, with only one significant difference for IL-2 (CD8<sup>+</sup> response to HER-2/*neu*).

### Pre-existing Ag-specific cytokine responses to TAAs in breast cancer patients

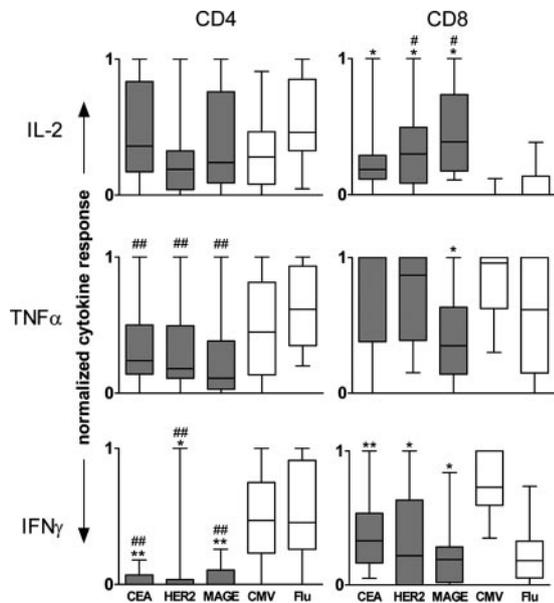
To assess natural immunity to TAAs in breast cancer patients naive to immunotherapy, T cell cytokine responses to HER-2/*neu*, MAGE-A3, and CEA were measured in 21 breast cancer patients, and compared with the healthy females (Fig. 2). Significantly higher responses in breast cancer patients were found for each TAA, but only for certain cytokines. Of note, IL-2<sup>+</sup> responses to all TAAs were consistently higher in cancer patients than healthy donors, among both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (median CD4<sup>+</sup> frequency in cancer patients = 0.03–0.04% vs healthy donors = 0.0002–0.010%; and median CD8<sup>+</sup> frequency in cancer patients = 0.018–0.035% vs healthy donors = 0.003–0.005%). For HER-2/*neu*, in which the lowest proportion of cancer patients showed positive responses, IL-2 was the only cytokine to show a significant difference between healthy donors and cancer patients for both CD4<sup>+</sup> ( $p = 0.0024$ ) and CD8<sup>+</sup> ( $p = 0.0041$ ) T cells.

To establish the number of patients with significant cytokine responses to TAAs, a positive response was defined as being above the 95th percentile of the healthy donor response range (Fig. 3). Seventeen of 21 patients had a positive response in at least one cytokine for CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells. Responses above the cutoff ranged from 6 to 75% of patients, depending upon the Ag and cytokine. The highest proportions of patients showed positive responses above the cutoff for IL-2, followed by TNF- $\alpha$  and IFN- $\gamma$ .

We also determined the correlation of CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in the same patients (Fig. 4). We found that these were significantly correlated ( $p < 0.005$ ) for all three TAAs and all three cytokines, further suggesting that the responses, although low level, were biologically induced and not random.



**FIGURE 4.** Correlation of TAA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in the same patients. Individual breast cancer patient cytokine responses are plotted for all TAAs. Significant correlations between CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were seen for each TAA and each cytokine. The coordinated presence or absence of CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses in these patients suggests that these responses, although low level, are not random, but may be biologically induced.



**FIGURE 5.** The pattern of T cell cytokine responses to TAAs is significantly different from that to CMV or influenza in the same CMV<sup>+</sup> breast cancer patients (*n* = 13). Data were normalized for each stimulus within each donor, to allow cytokine patterns to be compared statistically and visually. #, Denotes significant *p* values between TAA and influenza normalized responses. \*, Denotes significant *p* values between TAA and CMV pp65 normalized responses. \*\* or ##, Represents *p* < 0.001; \* or #, represents *p* = 0.001–0.017.

*The pattern of T cell cytokines differs between TAA and CMV or influenza responses*

To determine the pattern of cytokines in TAA-specific T cell responses, they were compared with CMV- and influenza-specific T cell responses in CMV-seropositive breast cancer patients. The magnitude of TAA-specific T cell responses was generally lower than the magnitude of CMV-specific T cell responses, so the data were normalized by range for each Ag for each donor (Fig. 5). This allowed cytokine profiles to be compared statistically and visually, regardless of the magnitude of response.

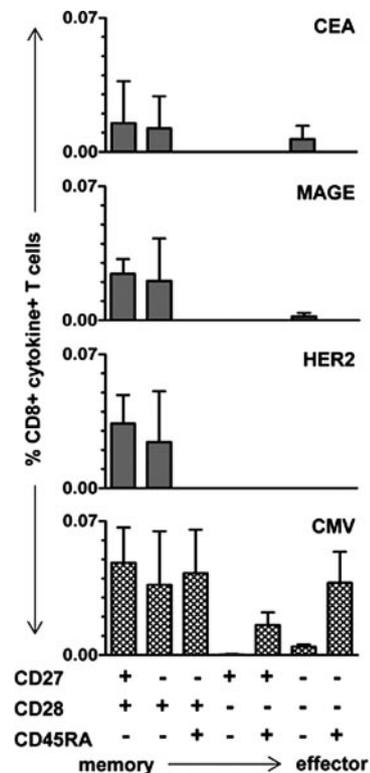
When compared with CMV, a significantly lower proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing IFN- $\gamma$  was found in all three TAA responses (*p* < 0.016). Also, significantly higher proportions of CD8<sup>+</sup> T cells expressing IL-2 were found in all three TAA responses, compared with the CMV response in the same donors (*p* < 0.014).

When compared with influenza, significantly lower proportions of CD4<sup>+</sup> T cells expressing IFN- $\gamma$  and TNF- $\alpha$  were found in all three TAA responses (*p* < 0.006). Conversely, a significantly higher proportion of CD8<sup>+</sup> T cells expressing IL-2 was detected in the HER-2/*neu* and MAGE-A3 responses (*p* < 0.02).

Together, these data show that all three TAA responses had a similar cytokine signature that was different from either CMV or influenza. Specifically, TAA responses consisted of proportionally more IL-2<sup>+</sup> and fewer IFN- $\gamma$ <sup>+</sup> T cells in CD4<sup>+</sup> and/or CD8<sup>+</sup> T cell subsets. Note that this became apparent only after normalization, as total cytokine-producing T cells were much fewer in all TAA responses compared with CMV, and somewhat fewer compared with influenza (data not shown).

*CD8<sup>+</sup> T cells responding to TAAs in breast cancer patients are predominantly a CD28<sup>+</sup>CD45RA<sup>-</sup> memory cell phenotype*

The distributions of effector and memory T cells responding to TAAs were determined in three breast cancer patients with the



**FIGURE 6.** The phenotypes of TAA and CMV responses in the same breast cancer patients are dramatically different. The distribution of effector and memory T cells of three breast cancer patients was determined by immunophenotyping the T cells responding to HER-2/*neu*, MAGE-A3, or CEA with expression of IFN- $\gamma$ , IL-2, and/or TNF- $\alpha$ . Each box represents the mean of three breast cancer patients; the error bars represent SD. T cell phenotypes are arranged with memory cells on the left and terminal effector cells on the right. Note the relative homogeneity of all three TAA responses, which were largely memory-like, compared with their CMV responses, which were more heterogeneous and included many terminal effector cells.

highest levels of responses. Although CD4<sup>+</sup> T cell responses were negligible, CD8<sup>+</sup> T cells of breast cancer patients responding to all three TAAs had a predominantly CD28<sup>+</sup>CD45RA<sup>-</sup> phenotype. In comparison, the CD8<sup>+</sup> CMV-responsive T cells in these three patients were heterogeneous in phenotype and included CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup> terminal effector cells that were lacking in the TAA responses (Fig. 6). Thus, both the breadth and the predominant phenotype of all three TAA responses differed from the breadth and the predominant phenotype of the CMV responses in these breast cancer patients.

**Discussion**

Induction of a strong and persistent tumor-specific T cell response is critical in the development of an effective cancer immunotherapy. In addition, it is becoming increasingly clear that the character of the tumor Ag-specific T cell response and not just the frequency of responding cells is important in conferring protection (23, 24). In the present study, we demonstrate that breast cancer patients often make significant T cell responses to TAAs, but the phenotype and cytokine signature of those responses are very different from that of CMV or influenza responses.

We found low-level TAA-specific T cell responses in some healthy donors, as others have described (2, 25, 26). We also found significant TAA responses in the majority of breast cancer patients, in contrast to some previous studies (7, 8). This might be due to the

use of fresh rather than cryopreserved cells (27), the use of overlapping peptide pools (18, 19) rather than single epitopes, and the analysis of multiple cytokines rather than just IFN- $\gamma$  (2, 25, 28, 29). To the latter point, IL-2 was the cytokine with the greatest number of positive responses in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells for all three TAAs, followed by TNF- $\alpha$ .

Coexpression of IL-2 and IFN- $\gamma$  by HIV-specific CD8<sup>+</sup> T cells has been correlated with protection (30). However, the majority of antiviral CD8<sup>+</sup> T cells make IFN- $\gamma$  and not IL-2, even in the protective responses of HIV nonprogressors (30) or of healthy donors to CMV (15). In this study, the TAA-specific cytokine responses observed in breast cancer patients had a much higher proportion of CD8<sup>+</sup> IL-2<sup>+</sup> cells than their responses to either CMV or influenza. Recent findings indicate that IL-2 has a role in the maintenance of self tolerance (31). It is thus conceivable that the prevalence of IL-2 in the CD8<sup>+</sup> TAA responses could be triggering regulatory T cells, resulting in suppression of effective responses.

In addition to an IL-2-dominated cytokine pattern, all three CD8<sup>+</sup> TAA responses were surprisingly similar in phenotype, consisting almost exclusively of CD28<sup>+</sup>CD45RA<sup>-</sup> cells. In contrast, the CMV response was heterogeneous and included many highly differentiated (CD27<sup>-</sup>CD28<sup>-</sup>CD45RA<sup>+</sup>) CD8<sup>+</sup> T cells, consistent with previously reported studies (15, 32). It is notable that the response to CMV in these cancer patients was similar to that in healthy donors (15), whereas the TAA responses were quite distinct. In melanomas, increased tumor burden has been found to correlate with the presence of tumor-specific CD8<sup>+</sup> T cells with a preterminally differentiated phenotype (33), similar to what has been reported for HIV-specific CD8<sup>+</sup> T cells (32). In contrast, differentiation to terminal effector cells is correlated with increased cytolytic potential of CD8<sup>+</sup> T cells (34, 35). Although terminal effector cells are certainly not the only cells that can confer protective immunity, there is reason to believe that the extent of CD8<sup>+</sup> effector T cell differentiation could be one correlate of an effective antitumor response.

The differentiation of T cells into terminal effector cells has been correlated with loss of IL-2 production in CMV-specific CD8<sup>+</sup> T cells (15). Therefore, the concurrent finding of IL-2-dominated and preterminally differentiated CD8<sup>+</sup> T cell responses to TAAs is, in itself, not unexpected. However, the restricted phenotype and consistently reduced CD8<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells in all the TAA responses (relative to CMV responses) strongly suggest an impaired protective capacity.

The patients enrolled in this study were not currently receiving chemotherapy, and had stable disease, although they had received prior therapy to reduce tumor burden. It is thus unknown whether their tumor-specific immune response, where present, had any protective component(s). Interestingly, immunization of patients such as these can lead to increased IFN- $\gamma$ -producing T cell responses, but not all of the patients with boosted immune responses show clinical benefit (1, 36, 37). In most cases, production of IL-2 by tumor-specific T cells has not been analyzed. It would thus be interesting to determine the overall phenotype and cytokine signature of tumor-specific T cells in these patients before and after vaccination, to see whether these patterns change, and whether they could predict clinical outcome.

When HIV-specific CD8<sup>+</sup> T cells have been analyzed for multiple functions, a clear distinction between progressors and non-progressors has emerged, in which the ability to produce multiple cytokines (including IFN- $\gamma$  and IL-2) is correlated with nonprogression (30). If truly nonprogressing cancer patients with TAA responses could be identified, one could determine whether such multifunctional T cell responses are also correlated with cancer nonprogression.

In conclusion, we detected positive Ag-specific cytokine responses in CD4<sup>+</sup> and CD8<sup>+</sup> T cells to CEA, HER-2/*neu*, and MAGE-A3 in the majority of breast cancer patients naive to immunotherapy. The phenotype and cytokine pattern of these TAA responses differed from that of CMV or influenza responses. Changes to the phenotype and cytokine signature of TAA responses might be necessary for cancer immunotherapies to be effective.

## Disclosures

Current employee (part- or full-time) or contractor Additional Comments: MI, CS, PH, DP, MT, MAS, SAG, DG, JFD, VCM, and HTM are employees of Becton Dickinson, which manufactures products used in this study.

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