Analysis of Immune Cells from Human Mammary Ductal Epithelial Organoids Reveals Vδ2+ T Cells That Efficiently Target Breast Carcinoma Cells in the Presence of Bisphosphonate

Nicholas A. Zumwalde1,2, Jill D. Haag2, Deepak Sharma2, Jennifer A. Mirrielees3, Lee G. Wilke3, Michael N. Gould2, and Jenny E. Gumperz1

Abstract

Developing strategies to enhance cancer prevention is a paramount goal, particularly given recent concerns about surgical treatment of preinvasive states such as ductal carcinoma in situ. Promoting effective immunosurveillance by leukocytes that scan for nascent neoplastic transformations represents a potential means to achieve this goal. Because most breast cancers arise within the ductal epithelium, enhancing protective immunosurveillance will likely necessitate targeting one or more of the distinctive lymphocyte types found in these sites under normal conditions. Here, we have characterized the intraepithelial lymphocyte compartment of non-cancerous human breast tissue and identified a subset of T lymphocytes that can be pharmacologically targeted to enhance their responses to breast cancer cells. Specifically, Vδ2+ γδ T cells were consistently present in preparations of mammary ductal epithelial organoids and they proliferated in response to zoledronic acid, an amino-bisphosphonate drug. Vδ2+ T cells from breast ductal organoids produced the antitumor cytokine IFNγ and efficiently killed bisphosphonate-pulsed breast carcinoma cells. These findings demonstrate the potential for exploiting the ability of Vδ2+ γδ T cells to respond to FDA-approved bisphosphonate drugs as a novel immunotherapeutic approach to inhibit the outgrowth of breast cancers. Cancer Prev Res; 9(4); 305–16. ©2016 AACR.

Introduction

Identifying genetic loci associated with reduced risk of breast cancer may provide novel targets for cancer prevention (1). Such loci may operate directly within mammary epithelial cells or may be mediated by the activities of non-mammary cells. We have recently reported that the rat Mcs5a locus acts via the immune system and that the resistant allele of Mcs5a is associated with increased frequency and functional activity of γδ T cells within spleen and mammary epithelium (2). These findings suggest that engaging key immune cell types to phenocopy the effects of the resistant Mcs5a allele may represent an effective breast cancer prevention strategy. However, given the substantial differences between humans and murine rodents in the molecular specificity of innate immune cells that mediate defense against incipient threats, an essential prerequisite to such an effort is identifying the immune subsets typically present in human mammary ductal epithelial tissues and determining how these can be medically targeted.

Cancerous cells are culled from tissues through the process of immunosurveillance, whereby several different types of leukocytes continuously scan for neoplastic transformed cells and eliminate them (3). Because breast cancers typically originate from the epithelial cells lining the mammary ducts and lobules (4), the immune cells responsible for immunosurveillance of transformed breast cells are likely to be those that patrol the ductal epithelium. Although recent studies have illustrated the presence of leukocytes in the human breast (5–8) and even in the epithelium (6, 7), the specific leukocyte subsets within this specialized tissue niche have remained poorly characterized. Moreover, a key unanswered question is whether immune cells are present that can be targeted to promote enhanced immunosurveillance of precancerous or cancerous cells.

Conserved T lymphocyte populations are particularly attractive for this type of approach because they recognize non-polymorphic antigen-presenting molecules and thus are present in all individuals regardless of human leukocyte antigen (HLA) type, and they can selectively be activated based on features of the T cell receptor (TCR). Some examples of conserved T lymphocytes are γδ T cells, mucosal-associated invariant T (MALT) cells, and invariant natural killer T (iNKT) cells. Based on their characteristic TCR chain usages, these types of T cells can be specifically targeted using monoclonal antibodies (mAbs), or in some cases by synthetic compounds.

1Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin. 2McArdle Laboratory for Cancer Research, Department of Oncology, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin. 3Department of Surgery, University of Wisconsin School of Medicine and Public Health, Madison, Wisconsin.

Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevention.aacrjournals.org/).

Current address for D. Sharma: Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Trombay Mumbai, India.

Corresponding Authors: Jenny E. Gumperz, 1550 Linden Drive Madison, WI 53706; Phone: 608-262-6902; Fax: 608-262-8418; E-mail: jegumperz@wisc.edu; and Michael N. Gould, E-mail: gould@oncology.wisc.edu
doi: 10.1158/1940-6207.CAPR-15-0370-T
©2016 American Association for Cancer Research.

www.aacrjournals.org
For example, human V82+ T cells are selectively activated by FDA-approved aminobisphosphonate (BP) drugs. These compounds act on V82+ T cells because they block the mevalonate biosynthetic pathway within target cells, which leads to the accumulation of a particular metabolite called isopentenylpyrophosphate (IPP). IPP associates with the cytoplasmic tail of a cell-surface protein called butyrophilin 3A1 (BTN3A1), causing a recognizable change in molecular features of the extracellular domain of BTN3A1 (9, 10). Target cells that express BTN3A1 and that have undergone an intracellular accumulation of IPP trigger TCR-dependent activation of V82+ T cells (11–13), causing them to proliferate, secrete cytokines such as IFNγ, and to kill the target cells (14). Thus, BPs may promote the antitumor functions of human γδ T cells in several ways, including (i) by expanding the numbers of V82+ T cells; (ii) by promoting their production of the antitumor cytokine IFNγ; and (iii) by promoting their killing of tumor cells. Indeed, administration of BPs to human cancer patients who had bone metastases (3 females with breast cancer and 6 males with prostate cancer) was associated with the expansion of an effector population of V82+ T cells in the blood, and with enhanced IFNγ production (15). These findings suggest that BP treatment may also provide an effective means to enhance the immunosurveillance functions of human V82+ T cells that play a critical role in eliminating nascent neoplastic cells before they can develop into tumors.

Administration of BP is likely to be particularly effective for cancers located in tissues patrolled by V82+ T cells (e.g., highly vascularized sites, such as bone). However, it is less clear whether this strategy represents a viable option for promoting the elimination of cancer cells located in epithelial tissues, because the γδ T cells in mucosal tissues mainly belong to other subtypes that do not respond to BPs. Some studies have shown lower prevalence of postmenopausal breast cancer in certain populations who have received BP treatment (16–20), although other studies found no evidence of decreased breast cancer risk in women who have taken BPs (21). Thus, the potential utility of BP administration for promoting immunological control of incipient breast cancer remains unclear. Here, we have addressed this question by investigating the presence and functionality of targetable T lymphocyte effector populations from primary human breast epithelial tissues.

Materials and Methods

Breast tissue acquisition and preparation

Non-cancerous breast tissue was obtained from the Cooperative Human Tissue Network (CHTN; funded by the National Cancer Institute) or provided by the University of Wisconsin’s Translational Science BioCore-BioBank from reduction mammoplasties or contralateral prophylactic mastectomies (Table 1). Acquisition and analysis of the breast tissue was approved by the University of Wisconsin Health Services Institutional Review Board.

Human breast organoids were isolated as previously published (22, 23). Briefly, breast tissue was minced and digested overnight in a 37°C shaker with 1X collagenase/hyaluronidase in Complete EpiCult B Human Media (Stem Cell Technologies) supplemented with 5% fetal bovine serum (FBS; Hyclone). After incubation, the digested tissue was spun for 1 minute or less at 80 to 100 × g to form a visible cell pellet enriched for epithelial ductal organoids. This cell pellet was washed and the breast organoids were collected on a 40-μm filter. In addition, a cell pellet containing stromal cells, red blood cells and small ductal epithelial fragments was also collected from the supernatant of the digested tissue. Organoids and stromal cell fractions were cryopreserved in 50% FBS/6% dimethyl sulfoxide, and stored in liquid nitrogen until needed. Single-cell suspensions from the organoids were prepared for all flow cytometric analyses and used for in vitro experiments by trypsinizing the organoids using 2 mL of ethylenediaminetetraacetic acid (EDTA)/trypsin solution for 1 to 2 minutes. EDTA (Thermo

Table 1. Human breast tissue donor information

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Age, sex, race</th>
<th>Elective procedure</th>
<th>Pathology report</th>
</tr>
</thead>
<tbody>
<tr>
<td>L625 A1</td>
<td>47 y, female, N/A</td>
<td>Prophylactic mastectomy</td>
<td>N/A</td>
</tr>
<tr>
<td>L625 B1</td>
<td>56 y, female, N/A</td>
<td>Prophylactic mastectomy</td>
<td>N/A</td>
</tr>
<tr>
<td>L625 C1</td>
<td>36 y, female, N/A</td>
<td>Reduction mammoplasty</td>
<td>N/A</td>
</tr>
<tr>
<td>L625 D1</td>
<td>28 y, female, N/A</td>
<td>Reduction mammoplasty</td>
<td>N/A</td>
</tr>
<tr>
<td>L625 G1</td>
<td>37 y, female, N/A</td>
<td>Prophylactic mastectomy</td>
<td>N/A</td>
</tr>
<tr>
<td>L625 H1</td>
<td>36 y, female, N/A</td>
<td>Reduction mammoplasty</td>
<td>N/A</td>
</tr>
<tr>
<td>L625 J1</td>
<td>18 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 K1</td>
<td>41 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>FPCL</td>
</tr>
<tr>
<td>L625 L1</td>
<td>23 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 M1</td>
<td>36 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Fibrosis; CLI; PASH</td>
</tr>
<tr>
<td>L625 N1</td>
<td>38 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 O1</td>
<td>46 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 P1</td>
<td>42 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 Q1</td>
<td>37 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 S1</td>
<td>50 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 T1</td>
<td>24 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 U1</td>
<td>51 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 V1</td>
<td>27 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>PASH</td>
</tr>
<tr>
<td>L625 W1</td>
<td>19 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Fibrosis</td>
</tr>
<tr>
<td>L625 X1</td>
<td>33 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>PASH</td>
</tr>
<tr>
<td>L625 Y1</td>
<td>21 y, female, BL</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 Z1</td>
<td>44 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
<tr>
<td>L625 A2</td>
<td>36 y, female, WH</td>
<td>Reduction mammoplasty (macromastia)</td>
<td>Normal</td>
</tr>
</tbody>
</table>

Abbreviations: CLI, chronic lobular inflammation; FPCL, focal periductal chronic inflammation; PASH, pseudoangiomatous stromal hyperplasia.
Fisher Scientific)/trypsin (Worthington Biochemical Corporation) solutions were made by adding 50 mg EDTA to 25 mL warm HBSS (Life Technologies) or PBS (Corning) without Ca²⁺/Mg²⁺; subsequently 5 mg trypsin was then added to 2 mL of EDTA solution and diluted 1:100 for usage.

Peripheral blood mononuclear cell isolation
Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors according to protocols approved by the UW Health Sciences and Minimal Risk IRBs. Written informed consent was obtained from all donors. Blood was processed using Ficoll-paque PLUS (GE Healthcare) and spun for 40 minutes without brake or acceleration at 400 RCF. The buffy coat was removed and washed with PBS for 15 minutes at 400 RCF. The supernatant was discarded and pellet resuspended in PBS and washed for 10 minutes at 300 RCF.

Flow cytometry and intracellular staining
For surface stains, cells were harvested, washed with PBS, blocked with 20% human AB serum (Fc block) for 15 minutes, stained with antibodies for 30 minutes at 4°C, washed, resuspended in PBS, and analyzed on a LSRII (BD Biosciences) with FlowJo analysis software (Version 9.3.1; Tree Star Inc.). Intracellular (IFN)-γ and IL17A staining was performed according to the manufacturer’s recommendations using the BD CytoFix/Cytoperm kit (BD Biosciences) in the presence of BD GolgiStop protein transport inhibitor (BD Biosciences). Intracellular FoxP3 staining was performed according to the manufacturer’s

Figure 1. Purified organoid fragments demonstrate epithelial enrichment compared with stromal fraction. A, light microscopic image of representative organoid fragments purified from human breast reduction tissue. B, flow cytometry analysis of the CD31⁻ (non-endothelial) and CD45⁻ (non-hematopoietic) cells shows that a higher percentage of cells express EpCAM (an epithelial marker) in the organoid fraction compared with the stromal fraction. C, quantification of ≥10 different patient reduction samples. **, P = 0.0003 (Mann-Whitney). D, quantification of the CD31⁺ or CD45⁺ cells from the organoids from ≥13 different patient reductions. Each symbol represents a different donor’s tissue sample.
A

Organoid:

PBMCs:

B

%CD3+ of CD45 gate

Organoids PBMCs

C

Organoid:

PBMCs:

D

%CD8α of CD3+ gate

Organoids PBMCs

E

%Tem of CD8α T cells

Organoids PBMCs

F

%Tem of CD8α T cells

Organoids PBMCs

G

Organoid:

PBMCs:

H

%CD103 of CD8α T cells

Organoids PBMCs
Biophosphonate activates human breast T cells

Bisphosphonate stimulation and V62 T cell expansion

PBMCs or single cells prepared from the breast organoids were exposed to 2.5 μmol/L zoledronate in combination with irradiated feeder PBMCs (exposed to 7 × 10⁴ to 8 × 10⁴ Rad) to stimulate and expand V62 y6 T cells. Specifically, 2 × 10⁵ isolated PBMCs were cultured with 5 × 10⁵ irradiated feeder cells in an end volume of 0.2 mL T cell media in 96-well plates. Single-cell preparations from organoids were plated with 5 × 10⁵ total irradiated feeder cells in an end volume of 0.2 mL T cell media in 96-well plates. Lymphocyte cultures were maintained in RPMI 1640 with L-glutamine (Corning) supplemented with 10% HI-BCS (Thermo Fisher Scientific) and 2% human AB serum (Atlanta Biologicals), 22°C to delineate effectors from targets. Samples were washed with PBS, spun, and resuspended in Annexin V binding buffer (BioLegend). Annexin V (5 μL/tube; BioLegend) and propidium iodide (10 μL/tube; BioLegend) were added to samples and incubated for 15 minutes in the dark at room temperature. Additional Annexin V binding buffer was added before flow cytometry analysis.

Statistical analysis

GraphPad Prism versions 4.0 and 4.0c software were used to construct data graphs and determine statistical significance using the Wilcoxon test for paired samples or the Student unpaired two-tailed t test or the Mann–Whitney. The P value cutoffs and notation were used as follows: *P < 0.05; **P < 0.01; ***P ≤ 0.0001.

Results

Preparation of human breast tissue yields highly enriched ductal epithelial organoids

Samples of breast tissue from human subjects who had undergone reduction mammoplasty or prophylactic mastectomy were collected for analysis (see Table 1). The samples were prepared using a protocol designed to separate tissue fragments representing ductal organoids from stromal layers. A representative image of the resulting tissue fragments showing ductal and alveolar structures is shown in Fig. 1A. To confirm that the fragment preparations were enriched for epithelial cells compared with the stromal fraction, we utilized multiparameter flow cytometry to assess relative frequencies of cells expressing epithelial, endothelial, or hematopoietic markers (Fig. 1B). Cells that were double negative for both the hematopoietic lineage marker CD45 and the endothelial marker platelet/endothelial cell adhesion molecule-1 (PECAM-1; CD31; ref. 24) were washed and resuspended at 3 × 10⁶ cells/1.0 mL media. Cells from primary organoids and cell suspensions containing BP expanded organoid- or blood-derived V62 T cells were each incubated with MDA-MB-468 cells for 4 to 6 hours at 37°C in a total of 0.2 mL in 96-well plates. After the incubation, V62 T cells were analyzed for cell-surface LAMP-1 and/or intracellular IFNγ expression by flow cytometry.

To assess γδ T cell killing of target cells, MDA-MB-468 cells were used as targets and day 7 BP expanded PBMC-derived V62 T cells were used as effectors. Briefly, targets were pulsed with or without 10 μmol/L zoledronate overnight. At the same time, day 7 stimulated V62 T cells were cultured with IL2 overnight. The following day, targets were trypsinized, washed and prepared at a concentration of 1 × 10⁵ to 2 × 10⁵ cells/0.1 mL T cell media without IL2. Effectors were prepared without IL2 and co-incubated with targets at different effector-to-target ratios in 96-well plates at 37°C for 4.5 hours. Additionally, 0.1 mLs of 0.1% trypsin was used for several minutes to recover adherent cells. The cells were washed with Fc block and stained with CD45 for 30 minutes at 4°C to delineate effectors from targets. Samples were washed with PBS, spun, and resuspended in Annexin V binding buffer (BioLegend). Annexin V (5 μL/tube; BioLegend) and propidium iodide (10 μL/tube; BioLegend) were added to samples and incubated for 15 minutes in the dark at room temperature. Additional Annexin V binding buffer was added before flow cytometry analysis.

Analysis of V62+ T cell responses to tumor cells

MDA-MB-468 breast carcinoma cells were obtained from ATCC as an authenticated cell line, and maintained in DMEM/F12 (Corning) supplemented with 10% HI-BCS (Thermo Fisher Scientific) and 1% P/S (Mediatech). The MDA-MB-468 cells were pulsed with or without 5 μmol/L zoledronate overnight, then
assessed for expression of the epithelial cell adhesion molecule (EpCAM; refs. 25, 26) to delineate epithelial content (Fig. 1B). Analysis of organoid and stromal fractions from at least 10 different breast tissue samples demonstrated that the organoids were significantly enriched for EpCAM+ cells compared with the stromal cell fractions, 43% and 9% respectively (Fig. 1C). EpCAM-expressing cells within the organoid preparations also expressed other markers associated with mammary gland-derived epithelial cells, including CD49dlow/high (25, 26), CD10low (25, 26), CD24+ (24), CD29+ (24), and Muc1int/high (ref. 25; Supplementary Fig. S1A–S1E). Organoid preparations contained an average of 24% CD45− cells (leukocytes), and 9% CD31+ cells (endothelial cells; ref. Fig. 1D). These results indicated that our breast tissue preparation method successfully enriches for ductal epithelial organoids, and thus the associated leukocytes are likely to be highly enriched for cells from the ductal epithelial tissue.

**Leukocyte populations associated with human breast organoids differ from those in peripheral blood**

We next examined the immune cell subsets within the CD45+ population of the breast organoid preparations. Typically, at least 90% (90.63%± 7.51%) of the CD45+ cells were CD3+, indicating that they are T lymphocytes (Fig. 2A and B). In contrast, CD3+ cells made up on average about 60% of the CD45+ cells in the peripheral blood (Fig. 2A and 2B). Of the CD3+ cells in the organoid preparations, about 75% (75.67%± 13.32%) were typically CD8+, whereas CD3+ cells in the blood typically contained less than 34% CD8+ cells (Fig. 2C and D). Essentially all of the organoid-derived CD8+ T cells co-expressed CD45RO, indicating that they are likely distinct from the CD8+ intra-epithelial lymphocytes that have been identified within intestinal epithelium (ref. 27; Supplementary Fig. S2A). Further analysis of the organoid CD8+ T cell population demonstrated that it consists almost exclusively of CD45RO+CD27- cells (Fig. 2E and F), a phenotype that is characteristic of effector memory T cells (28). In contrast, CD8+ T cells in the blood are composed of almost equal proportions of naïve T cells (CD27+CD45RO+; ref. 28), effector T cells (CD27−CD45RO+; ref. 29), central memory T cells (CD27+CD45RO−; ref. 28), and effector memory T cells (CD27−CD45RO−; ref. 28; Fig. 2E and F). Moreover, typically 80% (±12.94%) of the organoid preparation CD8+ T cells expressed the integrin α6 (CD103), which is a marker of intraepithelial lymphocytes (30), whereas less than 3% of the peripheral blood CD8+ T cells expressed this marker (Fig. 2G and H). Most of the CD8+ organoid T cells expressed intermediate levels of CD4 (Fig. 2C) and were essentially all CD45RO+CD27+ (Supplementary Fig. S2B). On average about 4% of the organoid CD4+ cells expressed FoxP3 (Supplementary Fig. S2C and S2D), which is characteristic of a regulatory T cell phenotype. Notably, in contrast to the CD8+ T cells, only a small fraction of organoid-derived CD4+ T cells expressed CD103 (Supplementary Fig. S2E). Together, these data demonstrate that the immune cell populations associated with our organoid preparations are clearly distinct from those of the blood, and are highly enriched for T cells with characteristics of IELs.

**Conserved T lymphocyte subsets are present in organoids and expand in response to BP**

We next wanted to determine whether we could detect lymphocyte subsets with conserved T cell receptors within the breast organoids that might be targetable in a chemoprevention strategy against breast cancer. We screened for two different subpopulations of γδ T cells (Vδ1 and Vδ2), MAIT cells, and iNKT cells. Both subsets of γδ T cells were clearly detectable in almost all of the samples analyzed (Fig. 3A and B), although they made up comparatively small percentages of the CD3+ cells (Vδ1 mean = 1.27%; Vδ2 mean = 0.42%); MAIT cells, which were identified using the Vα7.2 T cell receptor, were detectable as >3.1% of the CD3+ cells in organoids (Fig. 3A and B). iNKT cells were detected in some of the samples, but did not appear to be markedly enriched (data not shown). Thus, although Vδ2 cells are predominantly found within the blood, these results indicated that these potentially targetable T lymphocytes are typically present in the breast ductal epithelium.

We next investigated whether breast epithelial organoid Vδ2+ T cells are able to respond functionally to an FDA-approved BP. Preliminary studies confirmed that zoledronate more potently stimulated blood-derived Vδ2+ T cells compared with alendronate (Supplementary Fig. S3), consistent with prior studies by other groups (15, 31). Therefore, we cultured the total cells from organoid preparations with zoledronate or with medium alone for 2 to 3 weeks, and performed flow cytometric analysis to determine the relative frequencies within the culture of Vδ2+ T cells or MAIT cells as a control. Exposure to BP resulted in marked increases in the frequency of Vδ2+ cells, but not of MAIT cells, within the cultures (Fig. 3C). Increases in total Vδ2+ cell number were also observed, suggesting that the Vδ2+ T cells had proliferated (data not shown). Overall, exposure to BP induced clearly detectable Vδ2+ T cell expansion in approximately 45% of the organoid samples we tested (Fig. 3D). These data demonstrate that organoid-derived Vδ2+ T cells can respond to an FDA-approved BP drug.

**BP treatment facilitates IFNγ production by organoid-derived Vδ2+ T cells in response to triple-negative breast carcinoma cells**

Whereas γδ T cells typically have a T helper 1 (Th1) cytokine production phenotype that is characterized by the production of high levels of IFNγ, it has recently become clear that lymphoid cells in and around epithelial tissues are also responsible for the production of IL17, a cytokine that promotes epithelial integrity but that may also play a pathogenic role in tumorigenesis (32). Therefore, we tested cytokine production by primary T cells in organoid preparations by stimulating them with PMA and ionomycin, then performing intracellular cytokine staining for IFNγ and IL17. Detectable populations of cells expressing IL17 were observed in the non-Vδ2 CD8+ and CD8− T cell subsets; however, the Vδ2+ T cell population appeared heavily biased toward production of IFNγ with little or no evidence of IL17-producing cells (Supplementary Fig. S4). Based on these results, we focused our further analyses on IFNγ production.

We determined whether the organoid-derived Vδ2+ T cells produce cytokines in response to a human breast carcinoma cell line, MDA-MB-468, which is triple negative for estrogen receptor, progesterone receptor, and HER2/neu. Organoid cells were cultured with BP for 3 to 4 weeks, then exposed to MDA-MB-468 cells that were either pulsed with BP or mock-treated. Intracellular cytokine staining was performed to detect the frequency of γδ T cells expressing IFNγ. We observed robust
Bisphosphonate Activates Human Breast T Cells

Figure 3.
Breast organoids contain specifically targetable lymphocytes that respond to an FDA-approved BP drug. A, flow cytometry analysis of an organoid preparation from tissue sample L1 (see Table 1) for γδ T cells and MAIT cells (Vα7.2 T cell receptor). B, quantification of T cell subsets from organoid preparations from the indicated tissue samples (see Table 1 for tissue donor information). Symbols under the dashed line were below the limit of detection. C, example of flow cytometry results showing Vδ2 T cell expansion in the presence of 2.5 μmol/L BP compared with culture medium alone. Vα7.2 MAIT cells were used as a control for nonresponsiveness to BP. D, quantification of Vδ2 T cell frequency from organoids compared to PBMCs as a positive control after 2 to 3 weeks of culture in the presence of BP. Five of 11 samples (45.5%) displayed Vδ2 T cell expansion from the purified organoid fraction. Each symbol represents an independent expansion attempt using the indicated tissue samples. Dashed line indicates the threshold used to delineate expansion.

Figure 4.
Breast organoid-derived Vδ2 γδ T cells produce IFNγ in response to a triple-negative breast carcinoma cell line pulsed with BP. A, Vδ2 T cells were expanded in vitro from organoid preparations by exposure to BP. The T cells were coincubated with MDA-MB-468 breast carcinoma cells that were pulsed (right) or not pulsed (left) with BP, and intracellular IFNγ production was assessed by flow cytometry. Results shown are representative of two independent experiments. B, primary organoid-derived cells were coincubated with MDA-MB-468 cells that were pulsed (right) or not pulsed (left) with BP, and intracellular IFNγ production by Vδ2 and Vδ2 T cells was assessed by flow cytometric analysis. Numbers shown in the gates are the percentage of IFNγ-expressing cells from the Vδ2 or Vδ2 T cell populations. C, plot showing aggregated results for IFNγ production by primary Vδ2 T cells from the indicated breast tissue samples. Normalized mean fluorescent intensity (MFI) for IFNγ was determined by dividing the IFNγ MFI of the Vδ2 cells by the IFNγ MFI of the corresponding Vδ2 T cells in the same sample. Each symbol represents an independent experiment to assess IFNγ production by T cells from the indicated breast tissue samples. The dashed line represents a normalization ratio of 1. n.s., not significant; *, P = 0.0121 (Mann-Whitney test; plus BP to no BP); **, P = 0.0262 (Mann-Whitney test; plus BP to no BP).
Cytotoxic functions by breast-derived Vδ2 T cells in response to a triple-negative carcinoma cell line. A, Vδ2 T cells were expanded in vitro from breast tissue preparations by exposure to BP. The T cells were coincubated with MDA-MB-468 breast carcinoma cells that were pulsed (right) or not pulsed (left) with BP, and cell surface expression of CD107a (LAMP-1, a marker of recent cytotoxic activity) was assessed by flow cytometry after 4 hours. B, MDA-MB-468 cells were pulsed (black squares) or not pulsed (gray squares) with BP, and coincubated with in vitro-expanded Vδ2 T cells at the indicated effector:target ratios. The plot shows cytotoxicity of the target cells as assessed by cell-surface upregulation of Annexin V. Similar results were obtained in five independent experiments. C, primary organoid cells were coincubated with MDA-MB-468 cells that were pulsed (right) or not pulsed (left) with BP, and CD107a expression by T cells was assessed by flow cytometry.

IFNγ responses to BP-pulsed target cells, but little or no detectable IFNγ staining in response to mock-treated target cells (Fig. 4A). These results demonstrated that organoid-derived Vδ2 T cells that expand in response to BP treatment also produce cytokines in response to BP-pulsed breast cancer cells, but did not clarify whether a significant frequency of the primary γδ T cells within breast organoids can mount similar responses to transformed cells.

Therefore, we tested the ability of primary Vδ2 T cells within organoid cell preparations to produce cytokines in response to tumor cells directly \textit{ex vivo}. Indeed, when we exposed organoid-derived cells to BP-pulsed or mock-treated MDA-MB-468 breast carcinoma cells, we found that a detectable fraction of the Vδ2 cells that expanded in response to BP-pulsed MDA-MB-468 breast carcinoma cells than to mock-treated carcinoma cells (Fig. 4C).

These results demonstrate that primary γδ T cells from breast ductal epithelia are able to respond functionally to breast carcinoma cells that have been exposed to BP.

Organoid-derived Vδ2 T cells demonstrate cytotoxicity to BP-treated triple-negative breast carcinomas

To further investigate, we tested the cytotoxic responses of Vδ2 T cells to MDA-MB-468 breast carcinoma cells. We first analyzed surface expression of the LAMP-1 protein (CD107a), which becomes expressed at the cell surface as a result of vesicle fusion with the plasma membrane during a killing response. Vδ2 T cells within BP-expanded breast tissue cultures showed robust cell surface LAMP-1 expression after exposure to BP-pulsed MDA-MB-468 target cells, and a small percentage of the Vδ2 cells typically also showed cell-surface LAMP-1 expression in response to mock-treated target cells (Fig. 5A). To confirm that Vδ2 T cell surface LAMP-1 expression was correlated with target cell killing, we assessed the viability of the MDA-MB-468 carcinoma cells by Annexin V staining. BP-pulsed MDA-MB-468 carcinoma cells were efficiently killed upon exposure to \textit{in vitro}-expanded Vδ2 T cell cultures (greater than 75% target cell death within 4 hours; Fig. 5B). Notably, there was also significant killing of mock-treated MDA-MB-468 breast carcinoma cells, although this required much higher effector:target cell ratios (Fig. 5B). Analysis of T cells within primary organoid cell preparations revealed that a fraction of the Vδ2 cells typically showed cell-surface LAMP-1 expression after exposure to MDA-MB-468 cells (Fig. 5C). Exposure to BP-pulsed target cells elicited detectable cell-surface LAMP-1 expression on Vδ2 T cells nearly in 100% of the samples tested, while exposure to mock-treated MDA-MB-468 cells resulted in cell-surface LAMP-1 expression by Vδ2 cells in about
Figure 6.
Expression of tumor-associated ligands and receptors. A, flow cytometric analysis of MDA-MB-468 cells using antibodies specific for BTN3A1 (Vδ2+ T cell receptor ligand), or for the following NKG2D ligands: MICA and MICB, ULBP-1, ULBP-2 (antibody also cross-reacts with ULBP-5 and -6), ULBP-3, or ULBP-4. Filled histograms show staining with the specific mAb; dashed histograms show staining by an isotype control mAb. B, expression of NKG2D (an activating receptor that recognizes MICA/B) by Vδ2+ T cells, CD8α- T cells, and CD8α+ T cells from organoid preparations. Median fluorescence intensity (MFI) values for isotype control mAb staining are shown in italics, and specific mAb MFI values are shown in bold.
50% of the samples (Fig. 5D). These results demonstrate that BP treatment of breast carcinoma cells promotes highly efficient cytotoxic responses by Vβ2+ T cells, and indicate that Vβ2+ T cells also mediate less efficient cytotoxicity that is independent of BP exposure of the breast carcinoma cells.

The functional responses by breast Vβ2+ T cells may result from TCR-mediated recognition of BTN3A1 that has associated with IPP (33). Additionally, these responses could be mediated by the binding of NKG2D receptors on the γ8 T cells to a family of ligands, called MIC/A/B and ULBP1-6, that are upregulated during cellular stress and that are often expressed by tumor cells (34). Therefore, we investigated the expression of these key tumor control molecules. Flow cytometric analysis of the MDA-MB-468 cells revealed weakly positive staining for BTN3A1 (Fig. 6A). Staining for expression of NKG2D ligands showed clearly positive staining for MIC/A and at least one member of the ULBP family (Fig. 6A).

We also analyzed the organoid-derived lymphocytes for expression of the NKG2D receptor, and found that nearly all Vβ2+ and CD8+ T cells, and some of the CD8–T cells were NKG2D positive (Fig. 6B). Thus, most primary breast epithelial organoid associated T cells express receptors for recognition of stressed or neoplastic cells.

Discussion

It has recently become clear that the role of γ8 T cells in cancer can include both antitumorigenic and protumorigenic functions. Anti-neoplastic functions of γ8 T cells include their critical role in tumor immunosurveillance and as early responders to nascent transformations (35–37). Additionally, γ8 T cells can mediate rejection (killing) of established tumors (38, 39). Paradoxically, however, studies from murine models have indicated that γ8 T cells can also promote the outgrowth of carcinomas as a result of the expression of factors that promote epithelial cell growth (40). Moreover, a recent analysis of human breast cancer patients showed that the presence of regulatory γ8 T cells within the tumor was correlated with poor survival and high risks of relapse (41). These contrasting roles of γ8 T cells underscore that it is critical to understand the characteristics of the immune cells in ductal epithelial tissues in order to design effective immunopreventative strategies.

Prior analyses of immune populations associated with human breast have utilized unfractionated breast tissue that was not enriched specifically for the epithelial compartment. For example, Ruffell and colleagues analyzed tissue samples from prophylactic mastectomies and found that 60% to 70% of CD45+ cells were CD3+ T lymphocytes of several different varieties (5). The results presented here contrast with earlier studies of breast tissue and demonstrate that the human breast epithelial organoid represents a specialized immunological niche that is comprised mainly of T cells (>90% of the CD45+ cells). In contrast to the composition of T cells from blood, the breast epithelial T cell population was markedly skewed toward CD8+ cells, which is consistent with recent studies demonstrating that CD8 T cells are directly integrated within the breast epithelium (7). Moreover, our observation that both the CD4+ and CD8+ T cells from the breast organoids had a CD27– effector memory phenotype contrasts sharply with the dominant phenotypes of T cells from blood. Thus, our analysis clearly establishes that a distinct make-up of leukocyte subsets is associated with the breast epithelial organoids, and that blood contamination of the samples is not a major factor.

Given this, it was particularly noteworthy that we detected Vβ2+ T cells in 87% of organoids. This γ8 T cell subset is thought to localize mainly to the blood, while other types of γ8 T cells (e.g., Vβ1+ T cells) are thought to be characteristic of peripheral tissues (42). Because the organoid-associated Vβ2+ T cells were not uniformly positive for the epithelial-residency marker CD103 (data not shown), some of the Vβ2+ T cells we detected may have been in the process of transiently trafficking through the tissue rather than permanently residing there. Nevertheless, the presence of Vβ2+ cells as part of the steady-state surveillance population of breast epithelial organoids has important implications for immunotherapeutic enhancement of cancer prevention, because this population is highly targetable.

Vβ2+ T cells recognize the accumulation of specific molecules associated with the biosynthesis of isoprenoid lipids, and thus they detect changes to cellular metabolism that are associated with hyper-proliferation and neoplastic transformation (43). As a result of this, Vβ2+ T cells become activated by administration of FDA-approved BP drugs because these drugs block the farnesyl pyrophosphate synthase enzyme and cause accumulation of IPP within cells. Interestingly, it has recently been shown that human breast cancer cell lines vary in their endogenous accumulation of IPP after exposure to BP, and that the amount of IPP produced in response to BP treatment correlated with the ability of Vβ2+ T cells to infiltrate and control the growth of transplanted tumor cells in vivo (34). We found that breast-derived Vβ2+ T cells showed efficient cytotoxicity toward the MDA-MB-468 triple-negative breast carcinoma cell line after BP exposure, despite the comparatively low cell-surface expression of BTN3A1 on the tumor cells. Thus, these tumor cells may be particularly rich in their accumulation of IPP after BP exposure. Alternatively, they may elicit strong cytotoxic responses by γ8 T cells as a result of their high cell-surface expression of NKG2D ligands such as MIC/A. These results suggest that administering BPs or related compounds to breast epithelial tissues may be an efficient way to enhance the killing of neoplastic organoids by Vβ2+ T cells within the breast tissue. However, because we also found that the Vβ2+ T cells showed a less potent, but still significant cytotoxic response to the tumor cells that were not treated with BP (an effect that might be due to endogenous accumulation of IPP within the tumor cells, or simply to their upregulation of MIC/A/B molecules), it may be sufficient to use BP administration as a means to induce the proliferation of Vβ2+ T cells and thus to increase their frequency in vivo, without actually targeting breast epithelial cells for BP uptake.

In addition to Vβ2+ cells, we also detected Vβ1+ T cells in the breast epithelial organoid fraction. Vβ1 cells have been shown to be highly antitumorigenic to different tumors such as multiple myeloma (44), acute myeloid leukemia (45), and acute lymphoblastic leukemia (45). As a result of recent advances in understanding molecular interactions involved in activating Vβ1+ T cells, such as CD1d-mediated presentation of the cellular lipid sulfatide (46), it may soon be feasible to specifically target this subset. We also detected a subset of Vα2 TCR expressing lymphocytes that have been broadly characterized as MAIT cells (47). MAIT cells recognize vitamin metabolites that are presented by the non-classical antigen-presenting molecule MR-1 (47). Because many different microbes produce the chemical compounds recognized by MAIT cells, including Staphylococcus aureus and...
epidermis), which are the major causative agents for human mastitis, it is likely that there is an important interplay between the resident microbiota and the immune cells in the ductal networks of human breast.

Together, our findings demonstrate that the lymphocyte compartment associated with human breast ductal epithelial organeloids contains several conserved T cell populations that could be targeted, or in some cases possibly inhibited, to promote the immune-mediated clearance of nascent neoplastic cells. Such an approach may be particularly important for prophylactically treating women thought to be at high risk for breast cancer, and may also provide a novel non-invasive means to treat ductal carcinoma in situ.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: N.A. Zumwalde, J.D. Haag, D. Sharma, M.N. Gould, J.E. Gumperz

Development of methodology: N.A. Zumwalde, J.D. Haag, D. Sharma, L.G. Wilke

References


Analysis of Immune Cells from Human Mammary Ductal Epithelial Organoids Reveals V δ2+ T Cells That Efficiently Target Breast Carcinoma Cells in the Presence of Bisphosphonate

Nicholas A. Zumwalde, Jill D. Haag, Deepak Sharma, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1940-6207.CAPR-15-0370-T

Supplementary Material
Access the most recent supplemental material at:
http://cancerpreventionresearch.aacrjournals.org/content/suppl/2016/01/23/1940-6207.CAPR-15-0370-T.DC1

Cited articles
This article cites 47 articles, 20 of which you can access for free at:
hhttp://cancerpreventionresearch.aacrjournals.org/content/9/4/305.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
hhttp://cancerpreventionresearch.aacrjournals.org/content/9/4/305.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.