SV-BR-1-GM, a Clinically Effective GM-CSF-Secreting Breast Cancer Cell Line, Expresses an Immune Signature and Directly Activates CD4+ T Lymphocytes

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Targeted cancer immunotherapy with irradiated, granulocyte–macrophage colony-stimulating factor (GM-CSF)-secreting, allogeneic cancer cell lines has been an effective approach to reduce tumor burden in several patients. It is generally assumed that to be effective, these cell lines need to express immunogenic antigens coexpressed in patient tumor cells, and antigen-presenting cells need to take up such antigens then present them to patient T cells. We have previously reported that, in a phase I pilot study (ClinicalTrials.gov NCT00095862), a subject with stage IV breast cancer experienced substantial regression of breast, lung, and brain lesions following inoculation with clinical formulations of SV-BR-1-GM, a GM-CSF-secreting breast tumor cell line. To identify diagnostic features permitting the prospective identification of patients likely to benefit from SV-BR-1-GM, we conducted a molecular analysis of the SV-BR-1-GM cell line and of patient-derived blood, as well as a tumor specimen. Compared to normal human breast cells, SV-BR-1-GM cells overexpress genes encoding tumor-associated antigens (TAAs) such as PRAME, a cancer/testis antigen. Curiously, despite its presumptive breast epithelial origin, the cell line expresses major histocompatibility complex (MHC) class II genes (HLA-DRA, HLA-DRB3, HLA-DMA, HLA-DMB), in addition to several other factors known to play immunostimulatory roles. These factors include MHC class I components (B2M, HLA-A, HLA-B), ADA (encoding adenosine deaminase), ADGRE5 (CD97), CD58 (LFA3), CD74 (encoding invariant chain and CLIP), CD83, CXCL8 (IL8), CXCL16, HLA-F, IL6, IL18, and KITLG. Moreover, both SV-BR-1-GM cells and the responding study subject carried an HLA-DRB3*02:02 allele, raising the question of whether SV-BR-1-GM cells can directly present endogenous antigens to T cells, thereby inducing a tumor-directed immune response. In support of this, SV-BR-1-GM cells (which also carry the HLA-DRB3*01:01 allele) treated with yellow fever virus (YFV) envelope (Env) 43–59 peptides reactivated YFV-DRB3*01:01-specific CD4+ T cells. Thus, the partial HLA allele match between SV-BR-1-GM and the clinical responder...
might have enabled patient T lymphocytes to directly recognize SV-BR-1-GM TAAs as presented on SV-BR-1-GM MHCs. Taken together, our findings are consistent with a potentially unique mechanism of action by which SV-BR-1-GM cells can act as APCs for previously primed CD4+ T cells.

**Keywords:** SV-BR-1-GM, GVAX, targeted immunotherapy, whole-cell vaccine, therapeutic cancer vaccine, antigen-presenting cells

**INTRODUCTION**

In contrast to traditional chemo- or radiation therapies that kill fast-dividing cells irrespective of whether they are cancerous or normal, the goal of cancer immunotherapy is to eliminate malignant cells based on their antigenic makeup, their tumor-associated antigens (TAAs). There are several viable ways to induce an immune response against TAAs, in part determined by whether the antigens are localized intra- or extracellularly. For instance, although chimeric antigen receptors (1, 2) and bispecific antibodies crossinglinking cytotoxic T cells with cancer cells (3) rely on the antigens’ cell surface presence, ectopic T cell receptor (4), tumor-infiltrating lymphocyte (TIL) (5, 6), and vaccine-based approaches (7–18) require display of antigenic peptides by major histocompatibility complexes (MHCs), regardless of whether the peptides represent intra- or extracellular TAAs.

Whole-cell preparations with live but irradiated cancer cells express a very large number of antigens of which some may be coexpressed in the patient’s tumor(s) (19). Although a tumor shielded by an immune-suppressive microenvironment may not elicit an immune response, whole-cell preparations, if injected into immune-permissive sites, may allow development of otherwise inhibited antibody and cell-mediated immunity. However, even though an immune response induced by the injected cells may have a tumor-directed component, and even elicit tumor regression, the antigen(s) mediating this effect rarely are known.

For targeted immunotherapy studies with whole-cell preparations (also referred to as therapeutic cancer vaccines), both autologous and allogeneic cells have been applied. Autologous cancer cells, derived from the tumor of the patient to be treated, almost, per definition, are expected to express relevant antigens, including patient-specific neoepitopes. On the other hand, while allogeneic cell lines engineered to express granulocyte-macrophage colony-stimulating factor (GM-CSF) may induce strong immune responses by promoting antigen display on dendritic cells (DCs), they may lack key antigens (9, 15). With variable success, therapeutic cancer vaccines have been clinically tested against a variety of malignancies representing both hematologic and solid cancers, such as leukemia, melanoma, pancreatic, prostate, breast, lung, and colon cancers (7, 8, 10, 11, 14, 16, 18, 20–23).

Notably, in a mouse model, Ogawa et al. demonstrated that a similar approach may also be suitable to prevent tumor establishment (prophylactic treatment), i.e., that whole-cell preparations may prevent the development of tumors and not solely serve to reduce the tumor burden of already existing disease (24).

We previously established a cell line from a chest wall lesion of a metastatic breast cancer patient (17). The cell line, referred to as SV-BR-1, is estrogen receptor/progesterone receptor negative and very strongly HER2/neu (ERBB2) positive (17). To enhance the cells’ immune reactivity, SV-BR-1 cells were genetically engineered to stably overexpress GM-CSF, yielding the SV-BR-1-GM cell line. Several advanced-stage cancer patients, mostly with breast cancer, were treated with irradiated (200 Gy) SV-BR-1-GM cells (16). The study employed a pretreatment step with low-dose cyclophosphamide, which has similarly been used in other studies to blunt the activity of regulatory T cells (25). Additionally, 2 and 4 days after the administration of the SV-BR-1-GM cells, interferon-alpha 2b (IFN-α2b) was injected into each inoculation site to provide an additional “danger signal” (16, 26).

In our initial round of clinical assessment, four evaluable patients completed the SV-BR-1-GM program. One subject responded to the regimen with a near-complete regression of multiple breast lesions and a complete remission of a lung metastasis but relapsed 3 months after the sixth and last cycle, with lesions in the lung, soft tissue, breast, and brain. After obtaining Food and Drug Administration (FDA) permission, treatment resumed. Consequently, a systemic response was observed whereby tumors at multiple sites, including the brain, promptly regressed (16).

Here, we describe a molecular fingerprint of SV-BR-1-GM established with samples representing developmental intermediates including master cell banks (MCBs) and drug product. We present several lines of evidence suggesting that SV-BR-1-GM cells can act as antigen-presenting cells (APCs) and thereby mount an effective tumor-directed immune response. In particular, while likely other aspects such as cross-presentation by DCs contribute to SV-BR-1-GM’s mechanism of action (MoA), our observations are consistent with a role in which SV-BR-1-GM cells express, process, and display TAAs directly to T cells. However, SV-BR-1-GM cells do not express CD80 or CD86, encoding ligands for the costimulatory receptor CD28, and are thus likely to only activate previously primed, rather than naive, T cells.

**MATERIALS AND METHODS**

**Culturing of Cells**

SV-BR-1-GM lots were manufactured in RPMI-1640 supplemented with 10% FBS and l-glutamine or Gibco GlutaMAX.
the samples to be cross-compared were quantile-normalized using version 1 module. Expression levels of all Illumina (27). If applicable, datasets to be compared were merged using the [GenePattern](http://www.broadinstitute.org/cancer/software/genepattern/) using the public server portal below defined quality control (QC) criteria were analyzed with Studio intensities and detection-values were calculated using acquired on an iScan array scanner (Illumina). Average signal stained with Cy3-streptavidin. Fluorescent signal intensities were BeadChip arrays (Illumina, San Diego, CA, USA) and thereafter RNA was then hybridized onto HumanHT-12 v4 Expression to the manufacturer's instructions. The biotinylated antisense RNA was then hybridized onto HumanHT-12 v4 Expression BeadChip arrays (Illumina). This background cutoff definition coincides with the roughly 50% of genes expressed in a collection of human tissues at levels detectable by massively parallel signature sequencing in a study by Jongeneel et al. (29). However, since the tissues analyzed must have contained an unknown number of different cell types and unknown relative contributions of each cell type to the overall number of cells, this definition likely overestimates the extent of actual background. Nevertheless, consequently, it may reduce the probability of calling nonexpressed genes expressed.

**QC of Non-Normalized Data Sets**

The integrity of preamplified SV-BR-1-GM RNA was determined *via* Agilent's 4200 TapeStation system (Agilent, Santa Clara, CA, USA). Samples with an RNA integrity number equivalent (RIN) value of less than 7.5 were excluded from further analyses. Additionally, for SV-BR-1-GM as well as samples obtained *via* GEO (NCBI) and processed on HumanHT-12 v4 BeadChips, non-normalized data sets were assessed for gene expression variability. Except where stated otherwise, low-variability samples were excluded from further processing, with low-variability defined as a ratio between the expression value at the 95th and 5th, respectively, percentile of less than 10.

**Sample Representation**

For comparative gene expression analyses, individual genes were represented in the various SV-BR-1-GM sample types [MCB cryo, clinical product (CP) Lot IV culture, CP Lot IV 4p cryo, CP Lot IV 4p culture, CP Lot V cryo, CP Lot VIII cryo, CP Lot VIII culture 1d, CP Lot VIII culture 3d, and RES Lot II cryo] by their arithmetic means of their gene expression values. For calculations requiring one representative SV-BR-1-GM gene expression value, the median value among the arithmetic means was used. Representative gene expression levels for samples other than SV-BR-1-GM, obtained from GEO, were defined as follows: Breast cancer cell line samples from DataSet GSE48398 and human mammary epithelial cell samples (HMECs, "early proliferating" vs. "deep senescence", treated with siGLO siRNA) from DataSet GSE56718 (30) were represented by their arithmetic means. Normal breast sample types (ALDH NEG, ALDH POS, ERBB3 NEG, NCL, BASAL, STROMAL) from DataSet GSE35399 (31) were represented by their median expression values unless stated otherwise. For DataSet GSE48398, only expression profiles from cells cultured at 37°C were utilized. For the comparison between the breast cancer (DataSet GSE2943) and the normal tissues (DataSet GSE7307), the 95th percentile values among all breast cancer tissues (HER2_3+, HER2_2+, HER2_0-1+ of GSE2943) and the 95th percentile values among the *maximum* expression values of each group of normal tissue (Data Sheet S1 in Supplementary Material, GSE7307) were used as comparators. The 95th percentile rather than maximum (of the max.) expression values were chosen to accommodate potential "outliers."

Cancer/testis antigen (CTA) overexpression in SV-BR-1-GM cells was defined by the following criteria: The representative CTA transcript level in SV-BR-1-GM cells was defined by the following criteria: The representative CTA transcript level in SV-BR-1-GM cells was to be both >1.5 times the background cutoff value AND >1.5 times the max.

**Microarray Gene Expression Profiling**

SV-BR-1-GM cells, obtained directly from cryogenic vials following recovery from liquid nitrogen storage or harvested from cultures, were lysed in Buffer RLT (Qiagen, Valencia, CA, USA) with or without supplementation with β-mercaptoethanol. Total RNA was isolated from lysates *via* RNeasy Mini Kits (Qiagen) then subjected to microarray hybridization at the University of Minnesota Genomics Center (MN, USA). In short, RNA was amplified as antisense RNA and biotinylated using the Illumina™ TotalPrep™-96 RNA Amplification Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The biotinylated antisense RNA was then hybridized onto HumanHT-12 v4 Expression BeadChip arrays (Illumina). This background cutoff definition coincides with the roughly 50% of genes expressed in a collection of human tissues at levels detectable by massively parallel signature sequencing in a study by Jongeneel et al. (29). However, since the tissues analyzed must have contained an unknown number of different cell types and unknown relative contributions of each cell type to the overall number of cells, this definition likely overestimates the extent of actual background. Nevertheless, consequently, it may reduce the probability of calling nonexpressed genes expressed.
transcript level among the non-cultured (NC) normal breast cell types (SV-BR-1-GM/Max), AND the max. CTA transcript level among the NC normal breast cell types was to be <1.5 times the background cutoff value, whereby the max. NC transcript level was established among the representative values of each sample type.

### In Silico Identification of Putative SV-BR-1-GM TAAs

Quantile-normalized SV-BR-1-GM gene expression values were compared to those of normal human breast cells represented by the GEO DataSets GSE35399 (31), GSE56718 (30), and MCF10A from GSE48398. Genes for which the representative SV-BR-1-GM expression value was both >1.5 times the background cutoff value (defined above) and >1.5 times higher than the maximum representative value among all groups of normal breast cells were additionally subjected to the second, medium stringency, filtration step (expression level > 5 times the background cutoff value). Verification of the genes retained after medium stringency filtration was done via the quotients of the representative breast cancer samples in GSE2943 and those of the quantile-normalized and grouped normal tissues in GEO DataSet GSE7307 (high stringency filter). Groups of normal tissues are listed in Data Sheet S1 in Supplementary Material. As cutoff value for high stringency filter retention served the quotient (Breast Cancer/Normal Tissues) value for the ERBB2 Affymetrix probe 216836_s_at (quotient = 3.95): Genes of probes for which the quotient is ≥3.00 were defined as "verified."

To assess transcript expression levels of 279 confirmed or putative CTAs (Data Sheets S4 in Supplemental Material) in SV-BR-1-GM cells in comparison to several other breast cancer cell lines and normal breast cells, GEO DataSets of both cultured [GSE56718 (30) and GSE48398 (MCF10A)] and noncultured [GSE35399 (31)] normal breast cells were utilized. The CTA genes chosen for the analysis were selected from those described by Dobrynin et al., (2013) (32) and Chapman et al. (33), those listed in the CT database (34), and those represented by the nCounter Human PanCancer Immune Profiling Panel (NanoString Technologies, Seattle, WA, USA).

### Verification of Expression of Immune-Related Genes by Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), Transcript Counting (nCounter), ELISA, and Flow Cytometry

To confirm expression of immune-related genes, several methods were employed. For a subset of the genes, expression was assessed by qRT-PCR at the University of Minnesota Genomics Center (MN, USA) using commercially available TaqMan assays (Table S1 in Supplementary Data Sheet S2 in Supplementary Material) and the (nonirradiated) samples listed in Table S2 in Supplementary Data Sheet S2 in Supplementary Material, which were also assessed by microarray hybridization. Data were acquired on an ABI 7900HT real-time PCR instrument. To establish whether the immune-related genes are also expressed by irradiated SV-BR-1-GM cells from clinical formulations, cells were irradiated with 200 Gy then resuspended in Lactated Ringer's solution (LRS) and courier-transported under temperature controlled (2–8°C) conditions from the manufacturing site (UC Davis GMP facility, Sacramento, CA, USA) to the processing laboratory in Berkeley, CA, USA. The shipping containers (Crēdo Cube™ Series 4 parcel shippers; Pelican BioThermal, Plymouth, MN, USA) were opened, 4 h and 24 h after completion of the formulation process. Samples were immediately thereafter assessed for cell viability, seeded in 6-well plates, and cultured in full medium. Supernatants (SNs) and cells were harvested from the original clinical formulations, and after 1 and 3 days of culturing. RNA (extracted via RNeasy Mini Kit; Qiagen) was subjected to nCounter-based transcript counting (NanoString Technologies) at the University of Minnesota Genomics Center with the CodeSets (probes) listed in Table S3 in Supplementary Data Sheet S2 in Supplementary Material. Using nSolver Version 4.0 (NanoString), data was background-subtracted and normalized against the system positive controls and the reference genes (ADRM1, APTX, DGUOK, GNG5, PSMA4, RPL38, TMEM14C, and UBE3C), using the geometric means for both the positive control and reference gene sets. For the background subtraction, the max. values of the system negative controls were subtracted.

To assess protein expression levels of key immune-related factors, SNs from samples derived from the 4- and 24-h-old clinical formulations were subjected to ELISA using the Human GM-CSF, IL-6, IL-8, IL-10, IL-15, and KITLG (free SCF) ELISA MAX™ Deluxe kits (BioLegend, Inc., San Diego, CA, USA).

To establish whether and to what extent human leukocyte antigen (HLA)-DRβ3 (encoded by HLA-DRB3) is expressed on the surface of irradiated SV-BR-1-GM cells, cells were subjected to a modified formulation process whereby cells, after harvesting using TrypLE Express (Thermo Fisher Scientific) and irradiation (200 Gy), were cryopreserved in CryoStor 5 (CS5) cell freeze medium (BioLife Solutions, Inc., Bothell, WA, USA) then shipped overnight, on dry ice, to the processing laboratory. To assess HLA-DR cell surface expression by flow cytometry, irradiated SV-BR-1-GM cells cryopreserved in CS5 medium as well as nonirradiated SV-BR-1-GM and A549 cells, both freshly harvested using TrypLE Express, were treated with an Fc receptor blocking agent (Human TruStain FcX™, BioLegend; used 1:20 diluted) and stained with 20 µg/ml of a FITC-conjugated anti-human HLA-DR antibody (clone L243; BioLegend) or with 20 µg/ml of a FITC-conjugated mouse IgG2a, κ isotype control antibody (clone MOPC-173; BioLegend). Stained cells were subjected to flow cytometry, whereby the data was acquired on a BD LSRFortessa™ cell analyzer using FACSDiva software (BD Biosciences, San Jose, CA, USA) and analyzed using Flowing Software version 2.5.1 (Turku Bioimaging, Finland and Turku Centre for Biotechnology, Finland; software developer: Perttu Terho).

### SV-BR-1-GM Peptide Treatment and T Cell Activation

SV-BR-1-GM cells were either serum-starved for 24 h, harvested using trypsin and then irradiated (200 Gy) or cultured...
in full medium, harvested via trypsin and utilized without irradiation. Control human PBMCs (DRB3*01:01 and non-DRB3*01:01) were harvested from fresh whole blood. SV-BR-1-GM cells (serum-starved then irradiated or nonirradiated without serum-starvation) and control PBMCs (irradiated with 50 Gy) were treated with 1 μg/ml (final concentration) of the yellow fewer virus (YFV) Envelope (Env) 43–59 peptide (sequence: ISLETVAIDRPAEVVK) (35) or of a varicella zoster virus (VZV) open reading frame (ORF) 68 control peptide (Sequence: IWPRLYGFLENAHEHHGV) and cocultured with a T cell clone recognizing YFV-DRB3*01:01 peptide-loaded MHCs (pMHCs). No wash-out step of unbound peptide was employed. Prior to coculturing, T cells were rested for 4 days. For coculturing, 50 K SV-BR-1-GM cells or control PBMCs and 50 K rested CD4+ T cells per well in 96-well format were used. IFN-γ was assessed from SNs harvested after 72 h of coculturing by ELISA employing anti-human IFN-γ clones B27 and 4S-B3 as capture and detection, respectively, antibodies (both from BioLegend), and DELFIA Europalabe留意namidesteptavidin (PerkinElmer, Waltham, MA, USA) and DELFIA Enhancement Solution (PerkinElmer) for detection using a Perkin Elmer Wallac 1420 Victor2 Microplate Reader.

**HLA Typing and Immunohistochemistry (IHC)**

SV-BR-1-GM and peripheral blood cell samples were subjected to high-resolution HLA typing for HLA-A, HLA-B, and HLA-DRB3. HLA-DRβ3 expression on tumor specimens was assessed on paraffin-embedded tissues by IHC using a rabbit polyclonal antibody raised against an N-terminal region of human HLA-DRβ3 (product code ab196601; Abcam, Cambridge, MA, USA). Both HLA typing and IHC were conducted at the City of Hope (Duarte, CA, USA).

**Frequencies of HLA Allele Combinations**

From the allele frequencies (AFs) reported by Gragert et al. (36), estimated “phenotype frequencies” (PFs) were calculated indicating probabilities that an individual carries at least 1 of SV-BR-1-GMs expressed HLA-A, HLA-B, or HLA-DRB3 alleles (HLA-A*24:02, HLA-B*35:08, HLA-B*55:01, HLA-DRB3*01:01, HLA-DRB3*02:02) or allele groups (HLA-A*24, HLA-B*35, HLA-B*55, HLA-DRB3*01, HLA-DRB3*02). For the following definitions, alleles and allele groups are both referred to as “alleles,” and the sums of the individual SV-BR-1-GM HLA-A, -B, and -DRB3 AFs are referred to as ΣAFHLA-A, ΣAFHLA-B, and ΣAFHLA-DRB3, respectively. The PFs were calculated as follows: P(HLA-A) = 1 − (1 − ΣAFHLA-A)2, P(HLA-B) = 1 − (1 − ΣAFHLA-B)2, and P(HLA-DRB3) = 1 − (1 − ΣAFHLA-DRB3)2, whereby (1 − ΣAFHLA-A), (1 − ΣAFHLA-B), and (1 − ΣAFHLA-DRB3)2 are the probabilities that an individual does not carry at least 1 of SV-BR-1-GMs expressed HLA-A, -B, or -DRB3, respectively, alleles (exponent = 2 since diploid, i.e., 2n). AFs used for the calculations were obtained from Gragert et al’s supplementary data 5 and included frequencies of alleles with different designations but with amino acids identical in the antigen recognition site (Gragert et al’s supplementary data 1) (36).

**Ethics Approval and Consent to Participate**

The clinical aspect of this study was conducted with US FDA and St. Vincent Medical Center institutional review board approval, and written informed patient consent was obtained (16). The clinical trial was registered under ClinicalTrials.gov Identifier NCT00095862.

**RESULTS**

**SV-BR-1-GM Samples Used for This Study**

The SV-BR-1 cell line was established from a chest wall lesion of a female metastatic breast cancer patient. The polyclonal SV-BR-1-GM cell line was derived from SV-BR-1 cells following stable transfection with CSF2 (encoding human GM-CSF) and zeocin-selection (US7674456, Patent Application number: US 10/868,094) (16, 17) (Figure 1A). Even though additional parameters are expected to contribute to the potency of SV-BR-1-GM, we assume GM-CSF to be a major factor (15).

Granulocyte–macrophage colony-stimulating factor signaling involves GM-CSF binding to the α subunit of its receptor and recruitment of the receptor’s β subunit (37). Whereas a very restricted region in GM-CSF’s first α helix was suggested to interact with the receptor’s β subunit, several regions further downstream contact the α subunit (38). Compared to the NCBI Reference Sequence of GM-CSF (NP_000749.2), SV-BR-1-GM’s ectopic GM-CSF ORF contains some vector sequence and varies at positions 36 (Thr instead of Met) and 100 (Thr instead of Ile) of the mature GM-CSF protein sequence. However, neither Met36 nor Ile100 seems to be directly involved in receptor binding (38, 39) thus questioning whether these variations actually exert a biological effect, especially impair intracellular signaling. In agreement with signaling activity and thus GM-CSF bioactivity, cell culture SN from irradiated SV-BR-1-GM cells supported cell viability and proliferation of MUTZ-3 cells, a cell line reported to depend on cytokines such as GM-CSF (40), whereas SN from parental SV-BR-1 cells (not engineered to express GM-CSF) had at most a minimal effect (data not shown).

Since the excision of the original tumor specimen in 1999, several lots of both SV-BR-1 and SV-BR-1-GM have been manufactured. Figure 1A indicates SV-BR-1-GM samples for which gene expression profiles were generated and their genealogy. Whereas cell banks derived from the MCB (passage 8) and cryopreserved below or at around passage 20 were somewhat arbitrary designated “Clinical Product” (CP) lots, an SV-BR-1-GM sample cryopreserved at passage 30 (RES Lot II) is for this study considered a “research” sample. When used clinically following current practice, SV-BR-1 and SV-BR-1-GM cells are first serum-starved for 24 h in order to remove bovine antigens then irradiated (to abrogate cell proliferation). The serum starvation step was initially carried out prior to cryopreservation of the CP, which was irradiated upon thawing without a preceding culture step. More recently, cell banks have been cryopreserved without prior serum starvation. However, for clinical application, cells from such recent lots are thawed, short-term
The SV-BR-1-GM cell line was derived from SV-BR-1 breast cancer cells following stable transfection with CSF2 (encoding human granulocyte–macrophage colony-stimulating factor (GM-CSF)). The SV-BR-1 cell line itself was established from a chest wall lesion of a metastatic breast cancer patient (16, 17). The depicted developmental stages of SV-BR-1-GM represent samples used for this study rather than provide a comprehensive overview of all lots generated thus far. From an original master cell bank (MCB), several “clinical product” (CP) lots for actual or potential clinical use were established. “RES Lot II” refers to a research sample type and “ECB” to an engineering cell bank. RNA for gene expression analysis was extracted from cells taken directly from cryogenic vials (“cryo”) or following a culturing step (“culture”). “CP Lot VIII” was studied both as a presumptive static culture (“1d”) and as an expanded culture (“3d”), whereby for the former, samples were harvested on the first day ($t=0$), and for the latter, on the third day ($t=2$, i.e., 2 days later) of a time-course assessing GM-CSF secretion (outlined in Figure S5 in Supplementary Presentation S1 in Supplementary Material). Although no culture expansion took place from seeding to $t=0$ (“static culture”), cell numbers of cultures harvested at $t=2$ were 1.7–3.2 times the seeding cell numbers (“expanded culture”).

(A) The SV-BR-1-GM cell line was derived from SV-BR-1 breast cancer cells following stable transfection with CSF2 (encoding human granulocyte–macrophage colony-stimulating factor (GM-CSF)). The SV-BR-1 cell line itself was established from a chest wall lesion of a metastatic breast cancer patient (16, 17). The depicted developmental stages of SV-BR-1-GM represent samples used for this study rather than provide a comprehensive overview of all lots generated thus far. From an original master cell bank (MCB), several “clinical product” (CP) lots for actual or potential clinical use were established. “RES Lot II” refers to a research sample type and “ECB” to an engineering cell bank. RNA for gene expression analysis was extracted from cells taken directly from cryogenic vials (“cryo”) or following a culturing step (“culture”). “CP Lot VIII” was studied both as a presumptive static culture (“1d”) and as an expanded culture (“3d”), whereby for the former, samples were harvested on the first day ($t=0$), and for the latter, on the third day ($t=2$, i.e., 2 days later) of a time-course assessing GM-CSF secretion (outlined in Figure S5 in Supplementary Presentation S1 in Supplementary Material). Although no culture expansion took place from seeding to $t=0$ (“static culture”), cell numbers of cultures harvested at $t=2$ were 1.7–3.2 times the seeding cell numbers (“expanded culture”).

(B) Culture morphology of SV-BR-1-GM, as exemplified by 40× and 100× original magnifications of a culture derived from the SV-BR-1-GM Lot 11 bank following 24 h of serum starvation. Of note, SV-BR-1-GM cells may grow in monolayers but can also grow as minimally adherent, sphere-like, structures, especially when seeded at very low densities.

(C) Quality control (QC). (C) Hierarchical clustering of SV-BR-1-GM samples based on their microarray gene expression profiles. Normalized gene expression levels of samples belonging to the same sample type were averaged (arithmetic mean) prior to clustering. (D) Only samples with a RINe value of at least 7.5 were used for this study. Note that the CP Lot V cryo sample clustered separately and did not pass the minimal variability QC metric (see Materials and Methods) and was thus excluded from additional analyses.

FIGURE 1 | Development of SV-BR-1-GM. (A) The SV-BR-1-GM cell line was derived from SV-BR-1 breast cancer cells following stable transfection with CSF2 (encoding human granulocyte–macrophage colony-stimulating factor (GM-CSF)). The SV-BR-1 cell line itself was established from a chest wall lesion of a metastatic breast cancer patient (16, 17). The depicted developmental stages of SV-BR-1-GM represent samples used for this study rather than provide a comprehensive overview of all lots generated thus far. From an original master cell bank (MCB), several “clinical product” (CP) lots for actual or potential clinical use were established. “RES Lot II” refers to a research sample type and “ECB” to an engineering cell bank. RNA for gene expression analysis was extracted from cells taken directly from cryogenic vials (“cryo”) or following a culturing step (“culture”). “CP Lot VIII” was studied both as a presumptive static culture (“1d”) and as an expanded culture (“3d”), whereby for the former, samples were harvested on the first day ($t=0$), and for the latter, on the third day ($t=2$, i.e., 2 days later) of a time-course assessing GM-CSF secretion (outlined in Figure S5 in Supplementary Presentation S1 in Supplementary Material). Although no culture expansion took place from seeding to $t=0$ (“static culture”), cell numbers of cultures harvested at $t=2$ were 1.7–3.2 times the seeding cell numbers (“expanded culture”). (B) Culture morphology of SV-BR-1-GM, as exemplified by 40× and 100× original magnifications of a culture derived from the SV-BR-1-GM Lot 11 bank following 24 h of serum starvation. Of note, SV-BR-1-GM cells may grow in monolayers but can also grow as minimally adherent, sphere-like, structures, especially when seeded at very low densities. (C,D) Quality control (QC). (C) Hierarchical clustering of SV-BR-1-GM samples based on their microarray gene expression profiles. Normalized gene expression levels of samples belonging to the same sample type were averaged (arithmetic mean) prior to clustering. (D) Only samples with a RINe value of at least 7.5 were used for this study. Note that the CP Lot V cryo sample clustered separately and did not pass the minimal variability QC metric (see Materials and Methods) and was thus excluded from additional analyses.

SVC-BR-1-GM expresses a gene signature associated with immunostimulatory functions.

We discovered that SV-BR-1-GM cells expressed several genes with known immune system-associated roles, for example, MHC class II-based antigen presentation by professional APCs such as DCs. Among the latter category of genes are HLA-DMA,
HLA-DRA, and CD74, the latter of which giving rise to invariant chain (II) and class II-associated invariant chain peptide (CLIP).

To systematically address this observation, we generated a database from published reports (41–83) with 111 genes with known immunostimulatory roles (Data Sheet S3 in Supplementary Material). In particular, genes were included encoding (i) cell surface ligands for T cell costimulatory receptors or other cell surface-associated factors known to positively stimulate T cells (i.e., support T cell activation rather than inhibition), (ii) cytokines and other soluble (free) factors with positive T cell-stimulatory functions such as supporting activation, promoting survival, and/or inducing chemotaxis, (iii) factors promoting maturation, survival, chemotaxis, and/or in vitro generation of DCs, and (iv) factors promoting antigen presentation. Of the 111 genes, 22 had quantile-normalized expression levels in all SV-BR-1-GM samples of more than 1.5 times the background cutoff value (see Materials and Methods for definition), with 11 out of these 22 biomarkers expressed at levels more than five times the background cutoff as demonstrated by at least 1 Illumina probe (Figure 2; Figures S2 and S3 in Supplementary Presentation S1 in Supplementary Material). Of note, microarray-based expression levels of HLA-DRB3, even though apparently higher than 1.5 times the background cutoff value, are not shown as we did not consider the corresponding Illumina probe (ILMN_1717261) reliable since it suggested expression in more samples than expected considering HLA-DRB3 prevalence (data not shown).

Nevertheless, HLA-DRB3 does seem to be expressed in SV-BR-1-GM cells, as demonstrated by nCounter-based transcript counting (Figure 3).

Among the 22 immune-related biomarkers studied, one salient and unusual finding was the expression of both MHC class I and II components such as B2M, HLA-A, HLA-B, HLA-F, HLA-E, and HLA-H (MHC class I components) (see Figure S2 in Supplementary Presentation S1 in Supplementary Material), and HLA-DMA, HLA-DRA, and CD74 (MHC class II-associated factors) (see Figures S3 and S4 in Supplementary Presentation S1 in Supplementary Material). Of note, even though HLA-E and HLA-H were strongly expressed in SV-BR-1-GM cells, they do not seem to be clearly associated with immunostimulatory roles, and therefore are not considered factors likely contributing to the clinical efficacy of SV-BR-1-GM.

Intrigued by the possibility that SV-BR-1-GM may have direct immunostimulatory effects beyond those by GM-CSF, we sought to confirm expression of several of the immune-related genes by qRT-PCR. This gene set also included HLA-DRB3 and HLA-DMB, with the latter only barely expressed at more than 1.5 times the background cutoff value.
the background cutoff value but being functionally tied to HLA-DMA (84), another immunostimulatory biomarker expressed in SV-BR-1-GM cells (Figure 2). The confirmatory experiment was conducted on a subset of the SV-BR-1-GM samples used for Illumina microarray analysis and with RNA from MCF7 cells, a breast cancer cell line carrying the HLA-DRB3*02:02 allele.
Continued expression of the immune-related genes improves variable percentages of live cells, it is reasonable to speculate that the formulations' expiry time. The 4-h time point represented the actual protocol activities closely: The 4-h time point reflected the formation process, cells and SNs from the 4- and 24-h-old formulations were either analyzed directly (without culturing) or after a cell culturing period of 1 or 3 days. Whereas the original LRS and culture SNs were analyzed for presence and levels of secreted immune-related factors by ELISA, we used nCounter-based transcript counting to assess transcript levels of all 24 immune-related genes.

 Appreciable levels of RNA from all but three (IL10, TNFSF14, and endogenous CSF2) of the immune-related genes were observed in samples derived from the clinical formulations, both from formulated cells cultured prior to harvest and from cell aliquots of the formulations lysed without culturing (Figure 3 and Data Sheet S3 in Supplementary Material), consistent with continued transcription of most of these immune-related genes despite the irradiation step. The lack of more than 0.1% of the endogenous CSF2 (encoding GM-CSF) transcript levels is not concerning, since the nCounter CodeSet employed was not designed to detect transcripts from the ectopic (transfected) CSF2 ORF. However, in the case of HLA-A, only one of SV-BR-1-GM's alleles (HLA-A*24:02) seems to be expressed. Since the nCounter CodeSet (probe) for the other HLA-A*11:01, did yield a clear signal with another cell type, HMECs, we have no evidence suggesting lack of probe hybridization per se (Figures 3A,B), although we have not experimentally confirmed the hybridization efficiency of the CodeSet. Expression of HLA-B and HLA-DRB3 was apparent for each of SV-BR-1-GM's alleles: HLA-B*35:08, HLA-B*55:01, HLA-DRB3*01:01, and HLA-DRB3*02:02 (Figures 3C–F). Furthermore, substantial RNA expression levels were observed for IL6 and KITLG (Figures 3G,H). To compare expression levels of the 24 immune-related genes between parental SV-BR-1 cells and SV-BR-1-GM cells, also SV-BR-1 cells were analyzed by the nCounter approach. Almost identical expression profiles were obtained with 4-h-old formulations cultured for 1 day (Figure 3I). This is consistent with the assumption that the engineering of the SV-BR-1 cells to express GM-CSF did not affect the cells' ability to exert immunostimulatory functions. Furthermore, based on our microarray data, SV-BR-1-GM cells at most marginally express CSF2RB, encoding the β chain common for the GM-CSF, IL-3, and IL-5 receptors, suggesting lack of autocrine GM-CSF effects (data not shown) (37).

 Verification of Expression of the 24 Immune-Related Factors

To establish whether the 24 genes with immunostimulatory functions are also expressed in SV-BR-1-GM cells having undergone the clinical formulation process (which includes irradiation of the cells then resuspension in LRS), cells were formulated at the UC Davis GMP facility (Sacramento, CA, USA) then courier-transported under temperature controlled (2–8°C) conditions to the processing laboratory in Berkeley, CA, USA. The shipping containers were opened then samples processed, 4 h (4) and 24 h (24) after completion of the formulation process. This mirrored the actual protocol activities closely: The 4-h time point represents the approximate time from completion of the formulation process to inoculation for patients dosed at a clinical site in vicinity of the UC Davis GMP facility. The 24-h time point represents the formulations' expiry time.

Since in a clinical context, SV-BR-1-GM cells are inoculated as a replication-incompetent (irradiated) cell suspension with variable percentages of live cells, it is reasonable to speculate that continued expression of the immune-related genes improves SV-BR-1-GM's therapeutic efficacy. To assess whether these genes are indeed still expressed in cells having undergone the formulation process, cells and SNs from the 4- and 24-h-old formulations were either analyzed directly (without culturing) or after a cell culturing period of 1 or 3 days. Whereas the original LRS and culture SNs were analyzed for presence and levels of secreted immune-related factors by ELISA, we used nCounter-based transcript counting to assess transcript levels of all 24 immune-related genes.

 Appreciable levels of RNA from all but three (IL10, TNFSF14, and endogenous CSF2) of the immune-related genes were observed in samples derived from the clinical formulations, both from formulated cells cultured prior to harvest and from cell aliquots of the formulations lysed without culturing (Figure 3 and Data Sheet S3 in Supplementary Material), consistent with continued transcription of most of these immune-related genes despite the irradiation step. The lack of more than 0.1% of the endogenous CSF2 (encoding GM-CSF) transcript levels is not concerning, since the nCounter CodeSet employed was not designed to detect transcripts from the ectopic (transfected) CSF2 ORF. However, in the case of HLA-A, only one of SV-BR-1-GM's alleles (HLA-A*24:02) seems to be expressed. Since the nCounter CodeSet (probe) for the other, HLA-A*11:01, did yield a clear signal with another cell type, HMECs, we have no evidence suggesting lack of probe hybridization per se (Figures 3A,B), although we have not experimentally confirmed the hybridization efficiency of the CodeSet. Expression of HLA-B and HLA-DRB3 was apparent for each of SV-BR-1-GM's alleles: HLA-B*35:08, HLA-B*55:01, HLA-DRB3*01:01, and HLA-DRB3*02:02 (Figures 3C–F). Furthermore, substantial RNA expression levels were observed for IL6 and KITLG (Figures 3G,H). To compare expression levels of the 24 immune-related genes between parental SV-BR-1 cells and SV-BR-1-GM cells, also SV-BR-1 cells were analyzed by the nCounter approach. Almost identical expression profiles were obtained with 4-h-old formulations cultured for 1 day (Figure 3I). This is consistent with the assumption that the engineering of the SV-BR-1 cells to express GM-CSF did not affect the cells' ability to exert immunostimulatory functions. Furthermore, based on our microarray data, SV-BR-1-GM cells at most marginally express CSF2RB, encoding the β chain common for the GM-CSF, IL-3, and IL-5 receptors, suggesting lack of autocrine GM-CSF effects (data not shown) (37).
Also, we confirmed protein expression of several of the immune-related factors. GM-CSF, IL-6, IL-8, and KITLG (free SCF) were detected in SNs from cultured formulations (Figure 4A) and in the original 4- and 24-h formulations (Figure 4B). However, IL-10 and IL-15 were not detected in culture SNs (data not shown), but low levels were measured in the original LRS-based formulations (Figure 4B). Nevertheless, these data suggest only minimal, if any, contribution of IL-10 and IL-15 to the clinical activity of SV-BR-1-GM. In contrast to IL15, transcript levels of IL10 could not be verified (Data Sheet S3 in Supplementary Material), suggesting that indeed at most minute levels of IL-10 are expressed by SV-BR-1-GM cells. Therefore, IL-10 is not expected to contribute to SV-BR-1-GM’s MoA. Moreover, while IL-18 was detected by two different ELISA kits from two different vendors, the presumptive protein levels were highly discordant. Whereas high IL-18 levels were measured by one kit in (i) the original formulations (LRS), (ii) SN from cultured and irradiated SV-BR-1-GM cells, and (iii) SN from cultured and nonirradiated SV-BR-1-GM cells, IL-18 was only detected in LRS at the highest sample concentration tested (1:10 diluted) by the other kit (data not shown).

To address which of the immune-related factors are overexpressed in SV-BR-1-GM cells compared to normal breast cells, RNA and protein levels were measured from both nonirradiated SV-BR-1-GM and nonirradiated HMECs cultured in parallel. Similarly to the formulations, for SV-BR-1-GM cells, clear transcript expression was confirmed for all 24 genes except for IL10 and TNFSF14. Importantly, even though several genes were expressed at higher levels in HMECs than in SV-BR-1-GM cells, transcript levels of all MHC class II-associated genes (HLA-DMA, -DMB, -DRA, -DRB3, and CD74) were considerably higher in SV-BR-1-GM cells compared to HMECs (Figure 3; Data Sheet S3 in Supplementary Material). Furthermore, whereas GM-CSF, IL-6, IL-8, and KITLG were measured and detected in SV-BR-1-GM culture SNs, GM-CSF and IL-6 were not (or at most at minute levels) detected in HMEC SNs. On the other hand, free SCF (KITLG) levels were similar in SV-BR-1-GM and HMEC SNs, but IL-8 levels were >4 times higher in SNs from HMECs than from SV-BR-1-GM cells (Figure 4C).

To establish whether and to what extent HLA-DRβ3 (encoded by HLA-DRB3) is expressed on the surface of SV-BR-1-GM cells, cells were irradiated then cryopreserved in CS5 freeze medium. Both cryopreserved and freshly harvested, nonirradiated SV-BR-1-GM cells were subjected to flow cytometry employing a monoclonal anti-HLA-DR antibody (clone L243) recognizing a conformational epitope on HLA-DRα only present
when complexed with an HLA-DRβ chain. In the case of SV-BR-1-GM, the β chain is thought to be HLA-DRβ3 for the following reasons: (i), HLA-DRB1 is not (or at most marginally) expressed at the transcript level (Data Sheet S3 in Supplementary Material); (ii), SV-BR-1-GM’s diploid presence of HLA-DRB3 indicates absence of HLA-DRB4 and 5; and (iii), the remaining HLA-DRB genes (HLA-DRB2, 6, 7, 8, and 9) are pseudogenes (73, 85). As demonstrated in Figure 5, HLA-DR expression was heterogeneous, with about a third of the nonirradiated and some 15% of the irradiated SV-BR-1-GM cells staining positive for HLA-DR. Similarly, the per-cell signal intensity was higher in nonirradiated than in irradiated cells, suggesting that the irradiation process negatively affected HLA-DR gene expression and/or transport to the cell surface. A549 cells served as negative control and reference cell line (86, 87).

In collaboration with Creatv MicroTech (Potomac, MD, USA), in a preliminary study, nonirradiated SV-BR-1-GM cells were fixed and stained with a polyclonal anti-HLA-DR antibody (product code ab196601; Abcam) then analyzed in the context of an IHC-like process. Of note, given the high sequence similarity among HLA-DRβ chains, it seems likely that this antibody (which was also used to generate the data shown in Figure 6) can crossreact with HLA-DRβ chains other than β3. 15% of the cells were strongly positive for HLA-DRβ3, and another 21% had an intermediate signal (preliminary data not shown). However, while 0% of the negative control human umbilical vein endothelial cells (HUVECs) cells were strongly HLA-DRβ3 positive, 19% of the HUVECs had an intermediate signal (preliminary data not shown). Nevertheless, these findings also suggest that at least about 15 percent of SV-BR-1-GM cells express HLA-DR and that the β chain in these HLA-DR complexes is β3, since, based on our RNA and HLA typing data, SV-BR-1-GM cells do not express appreciable levels of another HLA-DRβ chain.

In preliminary flow cytometry experiments with nonirradiated SV-BR-1-GM cells, we also attempted to measure the cell surface levels of HLA-DRβ3 directly using a mouse polyclonal antibody raised against the full-length protein (MaxPab B02P; Abnova, Taipei, Taiwan). With this antibody, a very small (~3%) fraction of apparently HLA-DRβ3 (very weakly) positive SV-BR-1-GM cells was identified, with the negative control cell lines (A549 and T-47D) being negative (data not shown). However, given that the HLA-DRβ3 per-cell signal intensity was rather minuscule, the validity of this result is questioned, especially in light of the data both obtained with the anti-HLA-DRα antibody (Figure 5) and by Creatv MicroTech.

Taken together, we have confirmed expression of 22 out of the 24 immune-related genes considered relevant for SV-BR-1-GM’s MoA. The complete 22-gene Immune Signature is shown in Table 1.

**HLA Allele Matches Between SV-BR-1-GM and a Robust Clinical Responder**

By low-resolution HLA typing, we previously established that the robust clinical responder (here referred to as subject A002) and SV-BR-1-GM cells had similarities in their HLA phenotypes (16). To find out whether such similarities are further reflected at the allele level, peripheral blood cells from patients and SV-BR-1-GM cells were subjected to high-resolution HLA typing for HLA-A, -B, and -DRB3. Indeed, whereas the three clinical trial subjects who did not experience SV-BR-1-GM-induced tumor regression and a robust clinical responder (here referred to as subject A002) matched both at HLA-A (*A*11:01) and HLA-DRB3 (*B*02:02) (Table 2). However, since we cannot confirm expression of HLA-A*11:01 in SV-BR-1-GM cells with the nCounter CodeSet (probe) employed (Figure 3A), in this patient, the HLA-DRB3*02:02 match alone may have been clinically relevant. This finding agrees with an MoA in which tumor antigens displayed on HLA-DRβ3-based MHCs expressed on SV-BR-1-GM cells would contribute to the therapeutic efficacy of the cell line.

Since HLA typing was conducted using peripheral blood cells, and because targeted cancer immunotherapy requires cancer cell MHC expression, we sought to identify whether HLA-DRβ3 protein is present on a paraffin-embedded tumor specimen from clinical trial subject A002. As demonstrated in Figure 6, immunoreactivity was indeed apparent, thus further supporting the postulated role of MHC class II in the MoA of SV-BR-1-GM.

**CTAsExpressed in SV-BR-1-GM**

Cancer/testis antigens represent a class of antigens with physiological expression predominantly restricted to testicular or placental tissue, and, for a subset, brain tissue. However, CTAs may
become upregulated following malignant conversion of cells from a variety of organs. For many of such CTAs, immunostimulatory roles have been established (6, 32–34, 88–91).

Given such features, we assessed the mRNA expression levels of 279 confirmed or putative CTAs (Data Sheets S4 in Supplemental Material) in SV-BR-1-GM cells in comparison to several other breast cancer cell lines and normal breast cells. Following hierarchical clustering on both genes and samples, a group of CTA genes (KIF2C, OIP5, CEP55, PBK, KIF20B, TTK, CABYR, SPAG1, CCNA1, PLAC1, and PRAME) emerged with particularly good discrimination between SV-BR-1-GM and normal breast cells (Figure 7). However, expression of some of the genes in SV-BR-1-GM cells was weak and/or highly variable among the different SV-BR-1-GM samples. Nevertheless, PRAME, KIF2C, CEP55, and PBK were robustly expressed in SV-BR-1-GM cells (Figure 8) with PRAME exhibiting the highest fold-change ratio between the SV-BR-1-GM expression level and the maximum expression level among the normal breast samples (Table 3).

In contrast to PRAME (Figure 8A), KIF2C, CEP55, and PBK were also expressed in cultured HMECs. Interestingly, the expression values of the latter three genes are higher in “early proliferating” than in senescent HMECs (Figures 8B–D) (30), suggesting that perhaps also in vivo proliferating breast epithelial cells express these genes. A list of CTAs with transcript expression values greater in SV-BR-1-GM cells than in normal breast cell types is shown in Table 3.

Other Candidate Immunogens Expressed in SV-BR-1-GM Cells

Even though SV-BR-1-GM expresses an “immune signature” (Table 1), the latter alone is unlikely sufficient to induce a strong tumor-directed immune response as it does not provide cancer specificity. It is reasonable to hypothesize that for patients responding to whole-cell targeted cancer immunotherapies with tumor regression such missing directionality is provided by the cell line through overexpression of TAAs coexpressed in the

| Table 1 | 22-gene Immune Signature expressed in SV-BR-1-GM cells. |
|----------|-------------------------------------------------|
| Gene symbol | Official full name/description | Aliases |
| ADA | Adenosine deaminase | CD97, TM7LN1 |
| ADGRE5 | Adhesion G protein-coupled receptor E5 | |
| B2M | Beta-2-microglobulin | IMD43 |
| CAV1 | Caveolin 1 | BSLC3, CGL3, LCCNS, MSTP085, PPH3, VIP21 |
| CD58 | CD58 molecule | LFA-3, LFA3, ag3 |
| CD74 | CD74 molecule; invariant chain and CLIP | DHLAG, HLADG, II, la-GAMMA |
| CD83 | CD83 molecule | BL11, HB15 |
| CSF2 | Colony-stimulating factor 2 | GMCSF |
| CXCL8 | C-X-C motif chemokine ligand 8 | GCP-1, GCP1, IL8, LECT, LUCT, LYNAP, MDNCF, MONAP, NAF, NAP-1, NAP1 |
| CXCL16 | C-X-C motif chemokine ligand 16 | CXCLG16, SR-PSOX, SRPSOX |
| HLA-A | Major histocompatibility complex, class I, A | HLA |
| HLA-B | Major histocompatibility complex, class I, B | AS, B-4901, HLAB |
| HLA-DMA | Major histocompatibility complex, class I, DM alpha | D6S222E, DMA, HLADM, RING6 |
| HLA-DMB | Major histocompatibility complex, class II, DM beta | D6S221E, RING7 |
| HLA-DRA | Major histocompatibility complex, class II, DR alpha | HLA-DRA1, MLRW |
| HLA-DRB3 | Major histocompatibility complex, class II, DR beta 3 | HLA-DR1B, HLA-DR3B |
| HLA-F | Major histocompatibility complex, class I, F | CDA12, HLA-5.4, HLA-CDA12, HLA |
| ICAM3 | Intercellular adhesion molecule 3 | CD50, CDW50, ICAM-R |
| IL6 | Interleukin 6 | BSF-2, BSF2, CDF, HGF, HSF, IFN-beta-2, IFNB2, IL-6 |
| IL15 | Interleukin 15 | IL-15 |
| IL18 | Interleukin 18 | IGIF, IL-18, IL-1g, IL1F4 |
| KITLG | KIT ligand | DCUA, DFNA69, FPH2, FPHH, KL-1, Kit, MGF, SCF, SF, SHEP7 |

Genes with immunostimulatory roles expressed in SV-BR-1-GM cells. Gene symbols refer to the NCBI designations and HUGO Gene Nomenclature Committee (HGNC) recommendations. Gene symbols, official full names/descriptions, and aliases are indicated as shown on the respective NCBI Gene sites with or without additional information.

Other Candidate Immunogens Expressed in SV-BR-1-GM Cells

Even though SV-BR-1-GM expresses an “immune signature” (Table 1), the latter alone is unlikely sufficient to induce a strong tumor-directed immune response as it does not provide cancer specificity. It is reasonable to hypothesize that for patients responding to whole-cell targeted cancer immunotherapies with tumor regression such missing directionality is provided by the cell line through overexpression of TAAs coexpressed in the
tumors. Candidate TAAs for the SV-BR-1-GM cell line include the CTAs addressed above and illustrated in Figures 7 and 8.

To systematically search for SV-BR-1-GM antigens with potential to break immune tolerance by overexpression, we employed a 2-tier microarray-based approach. First, genes upregulated in SV-BR-1-GM cells relative to normal breast cells were identified. For this, we compared gene expression levels in SV-BR-1-GM cells to those of a variety of normal human breast cell types described by Shehata et al. [GEO DataSet GSE35399 (31)], Lowe et al. [GEO DataSet GSE56718 (30)], and MCF10A from GEO DataSet GSE48398. Two serial filters were applied to quantile-normalized gene expression values to enrich for genes likely differentiating SV-BR-1-GM from normal breast cells. After low-stringency filtration, 588 different genes (including some of non-coding

FIGURE 6 | Anti-HLA-DRβ3 antibody staining of a tumor specimen from the strong clinical responder. To assess whether the strong clinical responder (16), referred to as subject A002 in this article, presented with tumor expression of HLA-DRβ3, paraffin-embedded A002 tumor material was stained with a rabbit polyclonal antibody raised against an N-terminal region of human HLA-DRβ3. As demonstrated, immunoreactivity was apparent.

FIGURE 7 | Cancer/testis antigen (CTA) expression in SV-BR-1-GM cells. RNA expression levels of 279 confirmed or putative CTAs (Data Sheets S4 in Supplementary Material) were compared between SV-BR-1-GM, other established breast cancer cell lines, and several normal human breast cell types. Quantile-normalized and log2-transformed microarray-based RNA expression levels are displayed according to a global gradient color scheme. Red means a higher expression level than white, and white means a higher expression level than blue. Only CTAs with a maximum representative expression value among all samples >1.5 times the background cutoff value were included.
RNA) were retained, of which, after medium-stringency filtration, 353 remained (Figures S6 and S7 in Supplementary Presentation S1 in Supplementary Material; Data Sheet S5 in Supplementary Material).

Second, among the 353 genes retained after medium-stringency filtration, those not only upregulated relative to normal breast cells but also relative to tissues other than breast were considered verified immunogen candidates. This second
| **TABLE 3** | Cancer/testis antigen expression in SV-BR-1-GM cells. |
|----------------|---------------------------------|
| **Gene symbol (NCBI)** | **Probe identifier** | **SV-BR-1-GM** Max. expression values (among nonmalignant cells)** | **SV-BR-1-GM/Max C + NC** | **NC C + NC NC** |
| | | | | |
| **PRAE** | ILMN_1700031 | 869.1 | 142.8 | 114.7 | 6.1 | 7.6 |
| **PRAE** | ILMN_2306033 | 431.9 | 145.1 | 112.5 | 3.0 | 3.8 |
| **PBK** | ILMN_1673673 | 663.0 | 1465.5 | 107.2 | 0.5 | 6.2 |
| **CEP55** | ILMN_1747016 | 708.4 | 3756.0 | 126.2 | 0.2 | 5.6 |
| **KIF2C** | ILMN_1685916 | 688.0 | 484.9 | 130.8 | 1.4 | 5.1 |
| **PLAC1** | ILMN_1754207 | 415.4 | 150.1 | 144.9 | 2.8 | 2.9 |
| **OIP5** | ILMN_1759277 | 405.9 | 372.3 | 167.3 | 1.1 | 2.4 |
| **CABYR** | ILMN_2412139 | 309.6 | 503.8 | 179.0 | 0.7 | 2.1 |
| **SPAG1** | ILMN_1712773 | 289.1 | 203.7 | 181.6 | 1.4 | 1.6 |

Max refers to the maximum transcript level among the representative values of each non-cultured (NC) sample type. SV-BR-1-GM/Max refers to the quotients between the representative SV-BR-1-GM expression values and the Max values (see Materials and Methods for details). Note that for PBK, CEP55, and CABYR, SV-BR-1-GM/Max is >1 with the non-cultured (NC) cell types alone, but is <1 when including the cultured (C) breast cells (C + NC). This may suggest that culturing can upregulate expression of these genes. C: cultured normal cell types: MCF10A from GEO Data Set GSE48398, and early_proliferating and deep_senescence human mammary epithelial cells (hMEEC) from GSE56718 (30); NC: non-cultured normal cell types: ALDH NEG, ALDH POS, ERBB3 NEG, NCL, BASAL, STRONAL from GSE35399 (31). The background cutoff value is 141.16.

criterion is sought to enrich for genes with an “actual” potential to break immune tolerance since physiologically high levels of gene expression not only in breast but also in tissues of other organs may prevent breakage of tolerance. The high-stringency filter applied in this step compared GEO Data Set GSE29431 (breast cancer tissues) to a subset of samples represented by GEO Data Set GSE7307 (nonmalignant tissues) (Data Sheet S1 in Supplementary Material) and was conducted on 328 genes retained after medium-stringency filtration (no Affymetrix probes were found for 25 of the 353 genes retained after medium-stringency filtration). Of note, the filter cutoff criteria (see Materials and Methods) were selected to retain ERBB2 (HER2/neu), whose immunogenic properties are being explored in several clinical trials (13, 92). Thirty-one genes were verified as TAA candidates using this strategy (Table 4). Interestingly, four of them, ERBB2 (HER2/neu), MIEN1, PGAP3, and STARD3, mapped to 17q12, a region frequently amplified in breast cancer (Figure S8 in Supplementary Presentation S1 in Supplementary Material). In the context of breast cancer, ERBB2 (HER2) is clearly the most widely studied TAA among these four candidate immunogens. However, the apparent coregulation of the other three with ERBB2 suggests that HER2 positive tumors (likely also expressing one or several of the other antigens) may be effectively targeted by SV-BR-1-GM targeted immunotherapy by means of antigens beyond ERBB2.

**SV-BR-1-GM Cells Are APCs**

To establish whether the expression of the Immune Signature, especially the MHC class II components, translates into APC activity, SV-BR-1-GM cells were treated with yellow fever virus (YFV) Envelope (Env) 43–59 peptides (35) and cocultured with a CD4+ T cell clone known to recognize such YFV Env peptides when associated with HLA-DRB3*01:01-based HLA-DR complexes (Figure 9A). Indeed, IFN-γ secretion was significantly higher with SV-BR-1-GM cells treated with the YFV Env peptide compared to those obtained via an “irrelevant” peptide from VZV or via non-DRB3*01:01 PBMCs treated with the YFV peptide (Figures 9B,C). These results identify SV-BR-1-GM cells as APCs for CD4+ T cells. Of note, the T cells employed had been previously activated and were extensively expanded prior to their use in the study, thus not representing naïve T cells. Since SV-BR-1-GM cells do not express CD80 or CD86, encoding ligands for the costimulatory receptor CD28, SV-BR-1-GM is unlikely to activate naïve, i.e., nonprimed T cells. On the other hand, lack of CD80 (B7-1) may predict absence of NK cell-mediated destruction of SV-BR-1-GM (93).

**DISCUSSION**

With targeted immunotherapies using allogeneic whole-cell preparations, patients are inoculated with a wide variety of antigens of which some may be TAs coexpressed in patient tumors. However, whether or not an effective immune response is mounted against such TAs depends on numerous factors. In this report, we present four main lines of evidence suggesting that SV-BR-1-GM cells can act as APCs and thereby could potentially mount an effective tumor-directed immune response. First, despite their presumptive breast epithelial origin, SV-BR-1-GM cells express a set of genes including MHC class I and II components associated with immune cells rather than with epithelial cells. Second, in a pilot study, a robust clinical response occurred in a clinical trial subject with an HLA-DRB3 allele match to SV-BR-1-GM. This raises the possibility that in this patient, TAA-MHC complexes expressed on the surface of SV-BR-1-GM cells directly stimulated corresponding T cells. However, since in this pilot study both the number of patients (four evaluable patients), and the number of patients with an HLA-DRB3 allele match to SV-BR-1-GM (one, A002) are low, it is difficult to estimate the significance of such a match. Third, peptide-treated SV-BR-1-GM cells activated cocultured peptide-specific CD4+ T cells restricted to the SV-BR-1-GM expressed HLA-DRB3 *01:01 allele. Fourth, SV-BR-1-GM cells overexpress TAs including CTAs such as PRAE.

Among four evaluable clinical trial subjects in a pilot study, three with breast and one with ovarian cancer, objective tumor regression following SV-BR-1-GM inoculation was only seen in one patient (16), in this article referred to as A002. In contrast to the other three patients, subject A002 carried an MHC class II allele (HLA-DRB3*02:02) that was also present in SV-BR-1-GM (Table 2). Additionally, it is worth mentioning that in an ongoing phase I/IIa clinical trial (ClinicalTrials.gov identifier: NCT03066947) similarly conducted as the pilot study, the HLA matching hypothesis found continued support as demonstrated by the regression of multiple pulmonary nodules in a patient with metastatic breast cancer with a mixed clinical response (progression of metastatic disease in the liver) who matched at HLA-A*24:02 and HLA-DRB3*02:02 with SV-BR-1-GM (data not shown).
Together with a set of other genes with known immunostimulatory roles, SV-BR-1-GM cells express a 22-gene Immune Signature (Table 1). From a mechanistic perspective, these findings are consistent with a model in which TAAs are displayed on SV-BR-1-GM cell surface MHCs where they can directly and/or indirectly (upon “cross-dressing,” i.e., upon trogocytosis-based antigen display, as an additional contributor to SV-BR-1-GM's MoA. It even seems very reasonable to hypothesize that after antigen presentation to CD8+ T cells (97), in a mouse model, MHC class I-restricted tumor antigens were not detectably presented by the tumor itself (98), thus questioning whether MHC class I expression by SV-BR-1-GM cells indeed contributes to the cell line's therapeutic potential. Furthermore, it is important to emphasize that the data presented here does not exclude cross-presentation, the more classical mechanism for tumor antigen display, as an additional contributor to SV-BR-1-GM's MoA. It even seems very reasonable to hypothesize that after direct T cell stimulation by live, irradiated SV-BR-1-GM cells, antigens from apoptotic SV-BR-1-GM cells are taken up by DCs and used to activate patient T cells (Figure S9C in Supplementary Material). With such a mechanism, targeted immunotherapy with SV-BR-1-GM would both

### Table 1: In silico verified candidate TAAs.

| Gene symbol | Description/official full name | Location | Affymetrix probe ID | Score |
|-------------|--------------------------------|----------|---------------------|-------|
| ALO8        | Alpha-1,3-glucosyltransferase  | 11q14.1  | 203545_at           | 3.69  |
| ARPSL       | Actin-related protein 2/3 complex, subunit 5 like | 9p23.3 | 226914_at           | 3.96  |
| AZIN1       | Antizyme inhibitor 1           | 8q22.3   | 212481_at           | 3.28  |
| CBX2        | Chromobox 2                    | 17q25.3  | 226473_at           | 5.65  |
| CENPN       | Centromere protein N           | 16q23.2  | 222118_at           | 3.12  |
| COL8A1      | Collagen type VIII alpha 1 chain | 3q12.1 | 226237_at           | 10.44 |
| DCAF10      | DDB1 and CUL4-associated factor 10 | 9p13.2 | 230679_at           | 8.75  |
| EEE3H       | Eukaryotic translation initiation factor 3 subunit H | 8q23.3-2q4.11 | 230570_at | 6.87  |
| ERBB2       | erb-b2 receptor tyrosine kinase 2 | 17q12 | 234354_x_at          | 11.70 |
| HIST1H4H    | Histone cluster 1 H4 family member h | 6p22.2 | 216836_s_at           | 3.95  |
| IGFBP5      | Insulin-like growth factor binding protein 5 | 2q35 | 1555997_s_at          | 3.52  |
| INTS1       | Integrator complex subunit 7   | 1q22.3   | 218783_at           | 4.30  |
| KRT19       | Keratin 19                      | 17q21.2  | 228491_at           | 9.24  |
| KRT81       | Keratin 81                      | 12q13.13 | 213711_at           | 6.76  |
| MGAT4A      | Mannosyl(α-1,3-)glycoprotein   | 2q11.2   | 231283_at           | 5.85  |
| MHC II+ Cancer Cell Immunotherapy
| MEF2D       | Methyltetrahydrofolate dehydrogenase | 2p13.1 | 201761_at           | 3.01  |
| PAK1        | p21 (RAC1)-activated kinase 1   | 11q13.5-q14.1 | 230100_x_at | 3.18  |
| PDCD6       | Programmed cell death 6         | 5q15.33  | 222980_s_at          | 3.13  |
| PDRG1       | p53 and DNA damage regulated 1 | 20q11.21 | 225076_at           | 3.52  |
| PGAP3       | Post-GPI attachment to proteins 3 | 17q12 | 221811_at           | 5.92  |
| PGK         | Phosphatidylinositol glycan anchor biosynthesis class K | 1p31.1 | 209707_at           | 3.03  |
| RFC5        | Replication factor C subunit 5 | 12q24.23 | 203209_at           | 3.58  |
| RSF1        | Remodeling and spacing factor 1 | 11q14.1 | 222541_at           | 8.91  |
| SHB         | SH2 domain containing adaptor protein B | 9p13.1 | 1557458_s_at         | 4.11  |
| SLC35A2     | Solute carrier family 35 member A2 | Xp11.23 | 209702_at           | 5.47  |
| STAR0       | STAR-related lipid transfer domain containing 3 | 17q12 | 229891_at           | 3.50  |
| SYN1        | Synaptobrevin 1                 | 19q13.12 | 225153_at           | 4.00  |
| TNPO1       | Transportin 1                   | 5q13.2   | 1557278_s_at         | 3.71  |
| UBR5        | Ubiquitin protein ligase E3 component n-recognin 5 | 8q22.3 | 208682_at           | 3.09  |
| XOTP        | Exportin for RNA                | 12q14.2  | 212160_at           | 3.00  |

**Genes retained after high-stringency filtration, i.e., genes "verified" on breast cancer and normaltissue data sets.** "Score" refers to the quotient of the 95th percentile of the quantile-normalized breast cancer samples in GSE2943 and the 95th percentile of the quantile-normalized and grouped normal tissues in GEO DataSet GSE7307, whereby the maximum expression value across all samples per group was assigned to the group. An arbitrary score of 3.00 was selected as cutoff for the genes listed. The sample IDs of the different groups of normal tissues are shown in Data Sheet S1 in Supplementary Material. Gene symbols refer to the NCBI designations and HUGO Gene Nomenclature Committee (HGNC) recommendations. Official gene symbols, descriptions/official full names, and locations are indicated as shown on the respective NCBI Gene sites. 17q12, the location of B7-1 (CD80) positive tumor vaccines may result in some degree of direct antigen presentation to CD8+ T cells (97), in a mouse model, MHC class I-restricted tumor antigens were not detectably presented by the tumor itself (98), thus questioning whether MHC class I expression by SV-BR-1-GM cells indeed contributes to the cell line's therapeutic potential. Furthermore, it is important to emphasize that the data presented here does not exclude cross-presentation, the more classical mechanism for tumor antigen display, as an additional contributor to SV-BR-1-GM's MoA. It even seems very reasonable to hypothesize that after direct T cell stimulation by live, irradiated SV-BR-1-GM cells, antigens from apoptotic SV-BR-1-GM cells are taken up by DCs and used to activate patient T cells (Figure S9C in Supplementary Presentation S1 in Supplementary Material). With such a mechanism, targeted immunotherapy with SV-BR-1-GM would both
Fig. 9 | SV-BR-1-GM cells act as antigen-presenting cells (APCs). SV-BR-1-GM cells were cultured and serum-starved for 24 h then coincubated with yellow fever virus (YFV) Envelope (Env) 43–59 peptides (35) known to bind to HLA-DR complexes with an HLA-DRB3*01:01-based β chain and a YFV-DRB3*01:01-specific CD4+ T cell clone. (A) T cell clone after staining with YFV Env p8/DRB3*01:01 tetramers, as assessed by flow cytometry. Almost all T cells are both YFV Env p8/DRB3 and CD4 positive. (B) After 72 h of coculturing, T cell activation was assessed by determining the levels of secreted interferon (IFN)-γ. Values shown are arithmetic means from technical triplicates ± SDs, normalized to the mean IFN-γ level obtained from the YFV peptide-treated non-DRB3 PBMC reference wells. Background IFN-γ levels obtained from T cells treated with peptides in the absence of APCs (SV-BR-1-GM or PBMCs) were subtracted. (C) IFN-γ levels without background subtraction and normalization of a part of the experiment represented by panel (B). Values shown are arithmetic means of the Europium emission values at 615 nm from technical triplicates ± SDs. (B,C) one-tailed Student’s t-tests were employed to assess significance, with * referring to $0.01 \leq p < 0.05$, ** to $0.001 \leq p < 0.01$, and *** to $p < 0.001$.

Directly and indirectly stimulate patient T cells, with only the former requiring HLA matching between SV-BR-1-GM and the patient. Since the proposed MoA of the SV-BR-1-GM regimen (Figure S9A in Supplementary Presentation S1 in Supplementary Material) may similarly apply to other GVAX immunotherapies, one may wonder whether in such programs patients with MHC class I and/or II allele matches had better clinical responses to the vaccine than those without. Moreover, if HLA alleles indeed contribute to the efficacy of GVAX immunotherapies, high-resolution HLA typing should be considered as a companion diagnostic. Table 5 outlines estimated “phenotype frequencies” (PFs) of HLA-A, -B, and -DRB3 alleles expressed in SV-BR-1-GM for different subpopulations. As shown, combined HLA-DRB3*01:01 & *02:02 PFs range from 29.7 to 68.2%, i.e., the probability that a randomly selected individual carries at least one of SV-BR-1-GM’s expressed HLA-DRB3 alleles is 29.7–68.2% depending on the subpopulation. When loosening restrictions to only consider the allele groups, combination frequencies are only marginally higher (ranging from 29.8 to 68.6%) since SV-BR-1-GM’s HLA-DRB3 alleles (*01:01 and *02:02) are the most frequent alleles of the DRB3*01 and *02 allele groups (36).

To mitigate the risk for tumor development by the vaccine itself, SV-BR-1-GM cells are irradiated with 200 Gy (20,000 rad) prior to their clinical application (16). Interestingly, Sharma et al. demonstrated that ex vivo gamma-irradiation may
From the allele frequencies reported by Gragert et al. (36), estimated "phenotype frequencies" were calculated indicating probabilities that an individual carries at least 1 of SV-BR-1-GM’s expressed HLA-A, HLA-B, or HLA-DRB3 alleles (HLA-A*24:02, HLA-B*35:08, HLA-B*55:01, HLA-DRB3*01:01, HLA-DRB3*02:02). Shown are "phenotype frequencies" as percentage values. See Section "Materials and Methods" for details on the calculations. AAFA, African American; AFB, African; AINDI, South Asian Indian; AMIND, North American Indian; CARB, Caribbean black; CARHIS, Caribbean Hispanic; EURCAU, European Caucasian; FILII, Filipino; JAPI, Japanese; KORI, Korean; MENAFC, Middle Eastern or N. Coast of Africa; MSWHIS, Mexican or Chicano; NCHI, Chinese; SCAHIS, Hispanic—South or Central American; SCSEAI, Southeast Asian; VIET, Vietnamese. Since HLA-A*11:01 may not be expressed in SV-BR-1-GM cells (expressed at levels considered relevant (data not shown).

**TABLE 5** "Phenotype Frequencies" of HLA alleles expressed in SV-BR-1-GM cells.

| HLA allele matches with SV-BR-1-GM | Accuracy |
|-----------------------------------|----------|
| Allele group                      |          |
| HLA-A                             |          |
| HLA-B                             |          |
| HLA-DRB3                          |          |
| Allele                            |          |
| 1 HLA-A                           |          |
| 1 HLA-B                           |          |
| 1 HLA-DRB3                        |          |

*From the allele frequencies reported by Gragert et al. (36), estimated "phenotype frequencies" were calculated indicating probabilities that an individual carries at least 1 of SV-BR-1-GM’s expressed HLA-A, HLA-B, or HLA-DRB3 alleles (HLA-A*24:02, HLA-B*35:08, HLA-B*55:01, HLA-DRB3*01:01, HLA-DRB3*02:02). Shown are "phenotype frequencies" as percentage values. See Section "Materials and Methods" for details on the calculations. AAFA, African American; AFB, African; AINDI, South Asian Indian; AMIND, North American Indian; CARB, Caribbean black; CARHIS, Caribbean Hispanic; EURCAU, European Caucasian; FILII, Filipino; JAPI, Japanese; KORI, Korean; MENAFC, Middle Eastern or N. Coast of Africa; MSWHIS, Mexican or Chicano; NCHI, Chinese; SCAHIS, Hispanic—South or Central American; SCSEAI, Southeast Asian; VIET, Vietnamese. Since HLA-A*11:01 may not be expressed in SV-BR-1-GM cells (expressed at levels considered relevant (data not shown)."
organs. Since breakage of immune tolerance by overexpression may, at least in principle, only occur for genes with no/low physiological expression at every site permissive for immune surveillance, we reasoned that ideal candidate immunogens should be highly expressed in SV-BR-1-GM cells and breast cancer tissues but not, or only minimally, in normal tissues other than immune-privileged sites. The bioinformatics strategy applied to verify immunogen candidates reflects this theory. Thirty-one genes encoding candidate TAAs were considered more highly expressed in both SV-BR-1-GM cells and breast cancer tissues than in normal tissues (Table 4). Interestingly, among these thirty-one genes, six were located on chromosome 17 of which four mapped to 17q12, namely ERBB2 (HER2/neu), MIEN1 (C17orf37), PGAP3 (PERLD1), and STARD3 (Figure S8 in Supplementary Presentation S1 in Supplementary Material). This suggests that not only ERBB2 may be amplified in a subset of breast cancers but also other candidate TAAs located in vicinity of ERBB2 may be coamplified in the same cells (104, 105). Interestingly, in contrast to ERBB2, relatively little is known about MIEN1 (106–108) and PGAP3 (109), thus providing opportunities for further exploration. High StARD3 protein levels with a strong association with HER2 amplification was reported for approximately 10% of breast cancers in two Finnish nationwide patient cohorts (110). Cancer/testis antigens are a class of TAAs specifically expressed in cancer and germline tissues (6, 32–34, 88–91). The stringent filtering approach which yielded 31 in silico verified TAA candidates did not select for CTAs. However, when gene expression profiles of a set of 279 CTAs (Data Sheets S4 in Supplementary Material) were analyzed, several CTAs, most notably PRAME, were found to be selectively expressed in SV-BR-1-GM compared to normal breast cells (Figures 7 and 8; Table 3). Even though it was not found to be expressed in noncancerous tissues other than those of the testis and the endometrium, PRAME was missed in the stringent in silico screen because in the 54 breast cancer specimens analyzed, PRAME expression was restricted to only 11 (20%) samples of which only 2 (4%) demonstrated appreciable expression levels (data not shown). Furthermore, at least some of the CTAs may have low expression levels in SV-BR-1-GM cells (Figures 7 and 8). However, as demonstrated by Groeper et al., CTA-specific TILs could even be expanded from tumors with undetectable CTA levels (6). This may suggest that minuscule (below level of detection) CTA expression levels may suffice for CTA-specific T cell retention in the tumor or that such T cells only coincidentally happened to reside in the tumor tissue as it was resected. In agreement with the latter possibility, CTA-directed cytotoxicity of TILs from tumors with undetectable CTA expression was weak (6).

In summary, the study presented here supports a model in which SV-BR-1-GM’s tumor-directed effects reported previously (16) were mediated at least in part by the cell line's 22-gene "Immune Signature," which includes factors ranging from MHC class I and II components to ligands for T cell costimulatory receptors and chemokines known to promote attraction of immune cells, and by TAAs such as PRAME. Importantly, peptide-treated SV-BR-1-GM cells selectively activated pMHC-specific CD4+ T cells, which confirms that SV-BR-1-GM cells can act as APCs and suggests that this functionality is critical for SV-BR-1-GM’s MoA.

CONCLUSION

Unlike other established breast cancer cell lines, SV-BR-1-GM cells not only express known and putative TAAs; they also express a collection of factors with known roles in promoting immune responses. Most notably, in addition to MHC class I factors, also class II genes such as HLA-DMA, -DMB, -DRA, and -DRB3 are expressed. Since MHC class II components are associated with bone fide APCs such as DCs, their expression in SV-BR-1-GM cells is surprising and may point to a unique MoA. Since the patient who responded to the SV-BR-1-GM regimen with tumor regression (16) carried an MHC class II allele also expressed in SV-BR-1-GM cells, we hypothesize that patients coexpressing SV-BR-1-GM TAAs and expressing matching HLA alleles are more likely to develop a strong tumor-directed immune response than those without such characteristics.

DATA AVAILABILITY

Microarray data of the 22 samples passing QC (i.e., excluding CP Lot V cryo) discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (28) and are accessible through GEO Series accession number GSE112239 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE112239).

ETHICS STATEMENT

The clinical aspect of this study was conducted with US Food and Drug Administration (FDA) and St. Vincent Medical Center institutional review board (IRB) approval, and written informed patient consent was obtained (16). The clinical trial was registered under ClinicalTrials.gov Identifier NCT00095862.

AUTHOR CONTRIBUTIONS

ML conceived the study and interpreted data, conducted bioinformatics analyses and part of the wet laboratory experiments, and wrote the manuscript. GB lead the manufacturing of CP Lot VIII and oversaw technical procedures. BF and EF expanded MCB cells to CP Lot VIII and conducted experiments. TP and TH expanded cells. DC-B, SG, and YK conducted experiments. WK, JW, and WW provided intellectual input. CW oversaw the manufacturing of several SV-BR-1-GM lots and provided intellectual input. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00776/full#supplementary-material.

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Conflict of Interest Statement: BriaCell Therapeutics Corp. owns SV-BR-1-GM and the following SV-BR-1-GM-associated intellectual property: (1) US7674456 (issued US patent) and (2) PCT/US2017/019757 (international patent application). Furthermore, BriaCell Therapeutics Corp. acts as Sponsor on two current SV-BR-1-GM clinical trials (ClinicalTrials.gov Identifiers NCT03066894 and NCT03328026). ML, SG, CW, and WW have equity in BriaCell Therapeutics Corp. The other authors declare that they have no competing interests.

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