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Activated NKT Cells and NK Cells Render T Cells Resistant to Myeloid-Derived Suppressor Cells and Result in an Effective Adoptive Cellular Therapy against Breast Cancer in the FVBN202 Transgenic Mouse

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Attempts to cure breast cancer by adoptive cellular therapy (ACT) have not been successful. This is primarily due to the presence of tumor-induced immune-suppressive mechanisms as well as the failure of tumor-reactive T cells to provide long-term memory responses in vivo. To address these clinically important challenges, we developed an ex vivo protocol for the expansion of tumor-reactive immune cells obtained from tumor-bearing animals prior to or after local radiation therapy. We used an Ag-free protocol that included bryostatin 1/ionomycin and sequential common γ-chain cytokines (IL-7/IL-15 + IL-2). The proposed protocol expanded tumor-reactive T cells as well as activated non-T cells, including NKT cells, NK cells, and IFN-γ-producing killer dendritic cells. Antitumor efficacy of T cells depended on the presence of non-T cells. The effector non-T cells also rendered T cells resistant to myeloid-derived suppressor cells. Radiation therapy altered phenotypic distribution and differentiation of T cells as well as their ability to generate central memory T cells. ACT by means of the expanded cells protected animals from tumor challenge and generated long-term memory responses against the tumor, provided that leukocytes were derived from tumor-bearing animals prior to radiation therapy. The ex vivo protocol was also able to expand HER-2/neu-specific T cells derived from the PBMC of a single patient with breast carcinoma. These data suggest that the proposed ACT protocol should be studied further in breast cancer patients. The Journal of Immunology, 2011, 187: 708–717.

The rationale for adoptive cellular therapy (ACT) for cancer is based on overcoming the low frequency of endogenous tumor-reactive T cells by ex vivo activation and expansion and to direct differentiation of T cells of interest toward the most effective phenotype(s). Several groups have shown that ACT directed against melanoma-associated Ags results in objective responses in animal models as well as in some melanoma patients (1, 2). To improve objective responses of ACT, a number of strategies have been developed which include using genetically modified lymphocytes (3), highly effective T cell phenotypes (4) and use of common γ-chain cytokines (5). However, unlike animal models, cancer patients usually receive ACT after conventional therapies, which could interfere with the efficacy of the donor T cells. No comparative analysis has been performed to determine whether previous radiation therapy reduces or enhances the antitumor efficacy of ACT. ACT has also been tested against breast cancer both in mouse models and breast cancer patients (6, 7). However, unlike melanoma, ACT has not produced complete protection against breast tumors. Barriers to success include difficulty in the ex vivo expansion of tumor-reactive T cells (8), uncertainty as to the most relevant Ags, a lack of consensus as to the appropriate origin of the T cells to be used for expansion as well as phenotypic distribution of the most effective T cells, presence of myeloid-derived suppressor cells (MDSC) in cancer patients and during premalignant carcinogenesis, which could abrogate antitumor efficacy of ACT (7, 9), and finally tumor stroma as a major barrier, which prevents penetration of T cells into the solid tumor (10).

In the current study, we addressed a number of key barriers listed above to produce objective responses against primary breast tumors and to generate long-term memory against recall tumor challenge. We took advantage of an Ag-free protocol for selective activation of tumor-primed immune cells by using bryostatin 1/ionomycin (B/I) as previously described by our group (7, 11). Bryostatin 1 activates protein kinase C and ionomycin increases intracellular calcium (12, 13). Taken together, B/I mimics signaling through the CD3/TCR complex and leads to activation and proliferation of tumor-primed T cells. Most recently, it was reported that bryostatin 1 can...
act as a TLR-4 ligand and activate innate immunity (14). They developed a sequential common γ-chain cytokine protocol for expansion of the B/I-activated tumor-primed immune cells. This ex vivo protocol induced expansion of tumor-reactive immune cells composed of central memory T cells (TCM) and effector T cells (TE) as well as cells of the innate immune system or non-T cells. The expanded cells were found to be resistant to MDSC and were capable of generating long-term memory responses against the tumor in the FVB/N202 transgenic mouse model of HER-2/neu overexpressing breast carcinoma. We also showed that HER-2/neu–specific T cells can be expanded from PBMC of a breast cancer patient by using B/I activation and addition of the common γ-chain cytokines ex vivo.

Materials and Methods

Mouse model

FVB/N202 transgenic female mice (Charles River Laboratories) were used between 8 and 12 wk of age throughout these experiments. These mice overexpress an unactivated rat neu transgene under the regulation of the mouse mammary tumor virus promoter (15). These mice develop premalignant mammary hyperplasia similar to ductal carcinoma in situ prior to the development of spontaneous carcinoma (16). Premalignant events in FVB/N202 mice include increased endogenous MDSC (16). These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Tumor cell lines

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from a spontaneous mammary tumor harvested from FVB/N202 mice as previously described by our group (17). Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS. Mice were challenged with 3–4 × 10⁶ MMC cells s.c. in the lower part of the mammary region close to the groin area.

Flow cytometry

Flow cytometry analyses were performed as previously described by our group (16, 18). Briefly, spleens were disrupted into a single cell suspension and 10⁷ cells were aliquoted into each sample tube. Non-specific binding of Abs to FcRs was blocked by incubating the cells with anti-CD16/32 Ab (BioLegend). Cells were stained with surface Abs toward various markers and incubated on ice in the dark for 20 min, then washed twice with cell staining buffer (PBS, 1% FBS, 0.1% sodium azide) and fixed with 1% paraformaldehyde.

For intracellular staining of perforin (Prf) and Fop3, we followed the Foxp3 staining protocol provided by the manufacturer (BioLegend). Cells stained for granzyme B (GrB) and IFN-γ were fixed with 2.5% paraformaldehyde for 10 min on ice, washed twice with 0.1% saponin cell staining buffer, and then stained with the indicated Abs. Cells were then washed twice with normal cell staining buffer and fixed with 1% paraformaldehyde. For Annexin V staining, cells were stained for respective surface markers, washed with cell staining buffer, and then washed with 1× Annexin V buffer (BD Pharmingen). The Annexin V staining protocol given in the product data sheet was then followed. Abs used for flow cytometry were purchased from BioLegend (FITC-, PE-, PE-Cy5-CD3, FITC-, PE-, PE-CY5-CD4, FITC-, PE-, PE-Cy5-Gr1, FITC-, PE-, PE-Cy5-Gr1, FITC-, PE-Cy5-CD4, FITC-, PE-Cy5-CD11b, PE-, PE-Cy5-Gr1, FITC-, PE-CD25, FITC-, PE-CD44, PE-CD62L, PE-CD49b, FITC-, PE-CD122, FITC-, PE-CD127, FITC-, PE-B220, PE-IFN-γ, and PE-Foxp3) or eBioscience (FITC-, allophycocyanin-, PE-Prf1, and PE-Cy5-Gr1). All Abs were used at the manufacturer’s recommended concentrations. Multicolor data acquisition was performed on a BD FACS Canto II and analyzed using BD FACSDiva software.

Cell sorting

To sort distinct cellular populations of splenocytes, 10⁷ cells were added to sample tubes in which FcRs were blocked and surface markers were stained with FITC-conjugated CD4 and PE-conjugated CD8 Abs. Cells were then washed with sterile PBS supplemented with 2% FBS. T cells and non-T cells were sorted into 100% FBS using a BD FACSAria III cell sorter. Purity of sorted cells was >98%.

Cytotoxicity assay

Freshly isolated tumor-primed splenocytes or the ex vivo-expanded cells were cultured with MMC at a 10:1 E:T ratio in 3 ml complete medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FBS, 10 mM l-glutamine, and 5 × 10⁻³ M 2-ME) with 20 U/ml IL-2 (PeproTech) in 6-well culture dishes. After 48 h, cells were harvested and stained for neu (anti–c-Erb2/c-Neu; Calbiochem), Annexin V, and propidium iodide (PI) according to the manufacturer’s protocol (BD Pharmingen). Flow cytometry was used to analyze the viability of neu+ cells.

IFN-γ ELISA

Freshly isolated tumor-primed splenocytes or the ex vivo-expanded cells were cultured in complete medium at a 10:1 ratio with irradiated MMC cells (14,000 c/s) for 24 h. Supernatants were then collected and stored at −80°C until assayed. IFN-γ was detected using a Mouse IFN-γ ELISA set (BD Pharmingen) according to the manufacturer’s protocol.

Expansion of effectors T cells from FVB/N202 mice

FVB/N202 transgenic mice were inoculated with 4 × 10⁶ MMC cells, and splenocytes were harvested after 21–25 d. Splenocytes (10⁸ cells/ml) were then stimulated in complete medium containing 15% FBS as well as bryostatin 1 (5 nM) and ionomycin (1 μM), and 80 U/ml IL-2 for 16 h, as previously described by our group (12). Cells were then washed three times and cultured at 10⁶ cells/ml in complete medium with 10–20 ng/ml each of IL-7 and IL-15 (PeproTech). After 24 h, 20 U/ml IL-2 was added to the culture. On the next day, cells were washed three times and cultured at 10⁶ cells/ml in complete medium with 40 U/ml IL-2. Cells were split and cultured at 10⁶ cells/ml in complete medium with 40 U/ml IL-2 every other day for a total of 6 d. After day 6, cells were then used for ACT or in vitro studies.

Adaptive cellular therapy

Twenty-four hours prior to ACT, FVB/N202 mice were injected i.p. with CYP (100 mg/kg) to induce lymphopenia. Mice were challenged with 3–4 × 10⁶ MMC cells and then received 70 × 10⁶ of the expanded cells by tail vein injection later the same day. Tumor growth was monitored by digital caliper, and tumor volumes were calculated by volume (v) = (L [length] × W [width])²/2.

Isolation of MDSC in vitro

The Gr1⁺ MDSC population was isolated using an EasySep-FITC selection kit from StemCell Technologies, as previously described by our group (7, 19). We have previously shown that endogenous MDSC isolated from bone marrow or secondary lymphoid tissues can inhibit T cell responsiveness to anti-CD3/anti-CD28 Abs (7, 19–22). Also, the Ly6Cl⁺Ly6G⁺ subset but not the Ly6G⁺Ly6C⁺ subset isolated from FVB/N202 mice was found to be suppressive (19). Because of the higher proportion of Ly6C⁺Ly6G⁺ subset in tumor-bearing mice compared with tumor-free mice, the suppressive effects of MDSC isolated from tumor-bearing mice was greater using an optimal 2:1 ratio of T cells to MDSC (19). Therefore, in this study, we used an optimal 2:1 ratio of splenic MDSC isolated from tumor-bearing FVB/N202 mice.

Expansion of HER-2/neu–specific T cells from PBMC

PBMC were harvested from a breast cancer patient under Institutional Review Board protocol H10920. PBMC were cultured at 37°C for 2 h; adherent cells were used for the generation of monocYTE-derived dendritic cells (DCs) in the presence of GM-CSF and IL-4, as previously described by our group (17). Floater cells were split into two groups. One group was maintained with IL-2 (40 U/ml/10⁷ cells) for 6–7 d until autologous DCs became available. Another group was activated with B/I and expanded with common γ-chain cytokines. The expanded cells or IL-2–maintained cells were cultured with autologous DCs (4:1) in the presence or absence of recombinant HER-2/neu (100 μg/ml) or PBS (10 μg/ml). After 24 h, supernatants and cells were collected and subjected to IFN-γ ELISA and flow cytometry analysis, respectively.

Recombinant HER-2/neu protein

Extracellular domain and intracellular domain of human HER-2/neu protein were expressed in Escherichia coli and purified by Ni-NTA–agarose (Qia-gen, Valencia, CA), as previously described by our group (23). Concentration of the recombinant proteins was determined using the Bradford assay.

Statistical analysis

Graphical data are presented as means with SEs. Statistical comparisons between groups were made using Student t test with p < 0.05 being statistically significant.
Results

Sequential common γ-chain cytokines expand B/I-activated T cells derived from tumor-bearing FVBN202 mice

We have previously shown that activation of splenocytes (7) or tumor-draining lymphocytes with B/I could mimic TCR signaling and selectively activate tumor-primed T cells (7, 20–22). Expansion of the B/I-activated T cells would then result in differential phenotype distribution depending on the cytokine formulation used during the expansion (7). We have previously tested different combinations of IL-2, IL-7, and IL-15 (7, 21, 22) and showed superiority of an alternating sequence of common γ-chain cytokines (IL-7 + IL-15 followed by IL-2 followed by IL-7 + IL-15). However, antitumor efficacy of cells grown in alternating cytokines was limited in the neu transgenic tumor model because of a high level of endogenous MDSC in the FVBN202 transgenic mice, which was further increased during tumor challenge (7, 16). Therefore, we sought to determine whether a sequential common γ-chain cytokine regimen (IL-7 + IL-15 followed by IL-2) would improve antitumor efficacy of the expanded cells in FVBN202 mice. We first examined the composition of cells that were expanded with the sequential common γ-chain cytokine formulation and showed a 3- to 4-fold expansion of CD8+ and CD4+ T cells during a 6-d culture ex vivo (Fig. 1A). Although expanded CD8+ T cells showed reduced viability during the ex vivo culture (Annexin V+CD8+ T cells, 13–38%; p = 0.047) (Fig. 1B), the proportion of CD25+, CD127+, and CD122+ T cells was significantly increased (6- to 8-fold increases of CD25+ cells [Fig. 1C]; 7- to 9-fold increases of CD127+ cells [Fig. 1D]; and 2- to 6-fold increases of CD122+ cells [Fig. 1E]). There were marginal increases in the number of CD4+CD25+Foxp3+ T cells, accounting for only 2% of the gated CD4+ T cells after a 6-d expansion (Supplemental Fig. 1).

T cells expanded with sequential common γ-chain cytokines are highly responsive to neu+ MMC cells

To determine whether the ex vivo expansion of T cells enriched tumor-reactive T cells, production of IFN-γ, Prf, GrB, as well as the expression of the CD69 early activation marker was determined in the presence or absence of irradiated neu+ MMC in vitro. Compared with freshly isolated splenocytes, the ex vivo-expanded T cells showed greater IFN-γ production upon stimulation with MMC (average, 1500 versus 4000 pg/ml; p = 0.042) (Fig. 2A, upper panel). T cells isolated from naive FVBN202 mice did not show IFN-γ production upon MMC stimulation in vitro (data not shown). Flow cytometry analysis of the ex vivo-expanded cells determined that CD8+ T cells were the source of tumor-specific IFN-γ production (Fig. 2A, lower panel). Although no significant increases of Prf+ T cells was detected upon MMC stimulation, the proportion of Prf+CD8+ T cells was greater among the ex vivo-expanded cells than freshly isolated splenocytes (average, 1 versus 7%; p = 0.028) (Fig. 2B). The presence of tumor-reactive T cells among freshly isolated splenocytes was further confirmed by detecting an increased proportion of CD69+ T cells upon MMC stimulation (Fig. 2C). The ex vivo expansion of CD8+ T cells resulted in an increased proportion of CD69+ early effector cells prior to MMC stimulation (average, 10 versus 81%; p = 0.011) (Fig. 2C) and after MMC stimulation (average, 28 versus 84%; p = 0.001) (Fig. 2C). Almost all T cells expressed GrB prior to and after a 6-d expansion ex vivo (Supplemental Fig. 2A).

Ex vivo-expanded T cells are enriched for CD44+CD62L+ Tem and CD44+CD62L− TEM phenotypes and provide complete protection against primary as well as recall tumors

CD44+CD62L− Tem and CD44+CD62L− Tem effector memory T cells (Tem) provide immediate protection against tumors, whereas CD44+CD62L+ Tem are important for generating long-term protection. Tem are particularly important during recall tumor challenge. Ideally, the presence of the both phenotypes can provide protection against primary and recall tumor challenges. Therefore, we sought to determine the phenotypic distribution of CD8+ and CD4+ T cells prior to and following the 6-d ex vivo expansion with sequential common γ-chain cytokines. Freshly isolated CD8+ T cells contained roughly equal proportions of Tem (30%), Tem (26%), and CD4+ CD62L− naive T cells (TN, 33%). Ex vivo-expanded CD8+ T cells were enriched for Tem (day 6,
55.6% versus day 0, 30%; \( p = 0.02 \) and TCM (day 6, 26% versus day 0, 7.2%; \( p = 0.008 \)) (Fig. 3A). Ex vivo-expanded CD4\(^+\) T cells showed an unchanged proportion of TE (day 6, 31.9% versus day 0, 26.3%) but were enriched for TCM (day 6, 61.3% versus day 0, 6.6%; \( p = 0.002 \)). TN phenotypes almost disappeared in the expanded CD8\(^+\) T cells (day 6, 1.8% versus day 0, 33.7%; \( p = 0.009 \)) and CD4\(^+\) T cells (day 6, 2% versus day 0, 14.1%; \( p = 0.003 \)). Such a phenotypic distribution toward CD8\(^+\) TE and TCM suggests the potential for immediate as well as long-term memory responses against the tumor. We then performed in vitro cytotoxicity assays and in vivo tests of tumor growth inhibition to determine the antitumor efficacy of the expanded cells. Freshly

**FIGURE 2.** Ex vivo-expanded, MMC-primed T cells respond to MMC cells in vitro. A, Tumor reactivity of freshly isolated (day [D] 0) and ex vivo-expanded (D 6) T cells was determined by a 24-hs culture of T cells in the presence or absence of irradiated MMC cells, followed by the detection of IFN-\( \gamma \) in the supernatant (**upper panel**). Medium alone and MMC alone were used as negative controls for IFN-\( \gamma \) production. The MMC-specific IFN-\( \gamma \) production in gated CD8\(^+\) and CD4\(^+\) T cells was determined by flow cytometry analysis (**lower panel**). The MMC-specific expression of Prf (B) and CD69 (C) were determined in gated T cells. Data represent five independent experiments.

**FIGURE 3.** Phenotypic distribution of tumor-reactive T cells and their antitumor efficacy in vitro and in vivo. A, Phenotypic distribution of freshly isolated (day [D] 0) and ex vivo-expanded (D 6) splenic T cells was determined by flow cytometry analysis of gated CD8\(^+\) or CD4\(^+\) T cells. Distribution of T cell phenotypes including CD44\(^+\)CD62L\(^-\) effector (E: T_E), CD44\(^+\)CD62L\(^{low}\) effector memory (EM: T_EM), CD44\(^+\)CD62L\(^{high}\) central memory (CM: T_CM), and CD44\(^-\)CD62L\(^+\) naive (N: T_N) was determined. B, Gated neu\(^+\) MMC cells were analyzed for apoptosis (Annexin V\(^+\)/PI\(^+\)) in the absence or presence of freshly isolated or ex vivo-expanded T cells. C, CYP-treated FVBN202 mice (n = 3) were inoculated with MMC cells and received no further treatment (**left panel**) or received ACT (**middle panel**). Animals that had rejected MMC following ACT were given rest for 2 mo and then were challenged with MMC cells on the contralateral side (**right panel**). Data represent five independent experiments.
isolated splenocytes or expanded T cells were cultured with viable neu+ MMC tumor cells in an E:T ratio of 10:1 for 2 d. Gated neu+ MMC cells were then analyzed for the detection of apoptosis as determined by Annexin V+/PI+ cells. As shown in Fig. 3B, freshly isolated T cells reduced viability of neu+ MMC from 87.5 to 50.79% (1.7-fold), whereas the ex vivo-expanded T cells displayed greater cytotoxic function, reducing the viability of MMC from 68.8 to 17.6% (3.9-fold).

To test in vivo efficacy of expanded T cells, we used FVBN202 mice, which harbor increased MDSC because of premalignant mammary hyperplasia preceding spontaneous mammary tumors. Endogenous MDSCs were further increased upon MMC tumor challenge (7, 16). In this study, we injected FVBN202 mice with cyclophosphamide (CYP) 1 d prior to ACT to generate a semilymphopenic condition. Animals were then challenged with MMC, followed by i.v. injection of the ex vivo-expanded cells 6–8 h after the MMC challenge. Recipients of ACT rejected the neu+ MMC (Fig. 3C), despite the presence of MDSC before and 7 d after MMC challenge (Supplemental Fig. 2B). All control mice that had received CYP alone developed tumors. To determine memory responses, ACT-treated mice were challenged on the contralateral side with MMC 2 mo after the rejection of primary MMC cells. During recall tumor challenge, animals received neither CYP nor ACT, yet all the mice rejected the recall tumors (Fig. 3C). To determine which T cell phenotypes were effective in vivo, we sorted T cells into CD62L2/low (TE/TEM) and CD62Lhigh (TCM) and performed ACT with the sorted cells. No protection was observed against the tumors (data not shown). These data suggest critical interactions among tumor-reactive T cell phenotypes, which require further investigation.

**The ex vivo-expanded T cells acquire resistance to inhibitory function of MDSC**

Because the ex vivo-expanded cells protected FVBN202 mice against primary and recall tumor challenges even in the absence of MDSC depletion (Fig. 3C), we sought to determine whether the ex vivo-expanded cells were resistant to MDSC in vitro. We have previously reported that MDSC isolated from bone marrow or spleens of tumor-bearing FVBN202 mice can inhibit T cell responsiveness to CD3/CD28 stimulation (7, 19). MDSC can also inhibit MMC tumor-specific IFN-γ production by T cells expanded with alternating common γ-chain cytokines (Supplemental Fig. 2C). Therefore, splenocytes expanded with sequential common γ-chain cytokines were cultured for 24 h in the presence or absence of irradiated MMC (10:1 ratio of expanded cells to MMC) to show their reactivity with MMC in the presence or absence of splenic MDSC (2:1 ratio of expanded cells to MDSC). Supernatants were collected and subjected to IFN-γ ELISA. T cells were also analyzed for the expression of IFN-γ, Prf, GrB, and CD69. As shown in Fig. 4A, upper panel, the ex vivo-expanded cells produced IFN-γ in the presence of MMC (p = 0.004) as expected. Importantly, addition of MDSC not only failed to suppress MMC-specific IFN-γ secretion by the expanded cells but also increased the IFN-γ response (p = 0.012). Flow cytometry analysis of the
expanded cells determined that CD8+ T cells were the main source of MMC-specific IFN-γ production (Fig. 4A, lower panel). Addition of MDSC increased MMC-induced production of IFN-γ by both CD8+ and CD4+ T cells. Increasing the dose of MDSC did not increase MMC-specific production of IFN-γ by the expanded T cells (data not shown). The presence of MDSC also resulted in an increased production Prf in CD8+ T cells upon MMC stimulation (Fig. 4B). There was no IFN-γ or Prf production by MNC as determined by flow cytometry analysis of gated neu+ MMC in the coculture (Supplemental Fig. 3A). In addition, MDSC did not suppress production of GrB or expression of the CD69 early activation marker in the expanded T cells (Supplemental Fig. 3B, 3C). PBMC from naive FVBN202 mice that was depleted of Gr1+ cells had no suppressive effect or supportive effect on tumor-reactive T cells (data not shown). These data suggest that MDSC did not inhibit antitumor responses of the T cells expanded with the sequential γ-chain cytokine regimen. We next determined whether MDSC could inhibit cytotoxicity of these T cells against MMC tumor cells in vitro. Expanded cells were cultured with viable neu+ MMC (10:1 ratio) in the presence or absence of MDSC (2:1 ratio). Control MMC cells were cultured with medium alone. As shown in Fig. 4C, expanded cells induced apoptosis in MMC cells in a 2-d culture, and the presence of MDSC did not alter the cytotoxic function of the tumor-reactive cells.

Presence of non-T cells in the ex vivo-expanded cells overcomes MDSC and enhances T cell responses to MMC

The ex vivo-expanded cells showed a significantly reduced proportion of CD4+CD8− cells compared with that of freshly isolated splenocytes (day 6, 20% versus day 0, 57%; p = 0.0001) (Fig. 5A). The expanded cells contained 17–20% CD4+CD8− cells. As shown in Fig. 5B, gated CD4+CD8− cells contained a significantly higher proportion of CD3+ cells in the expanded cells compared with freshly isolated cells (day 6, 60.8% versus day 0, 5.5%; p = 0.002). Then, we sought to determine the cellular composition of these CD4+CD8− cells. CD49b is a common marker for NK cells, NKT cells, and IFN-γ-producing killer DC (IKDC) (24). As shown in Fig. 5C, NKT cells (CD49b+CD3+) and NK cells (CD49b+CD3−) showed significant increases in the expanded CD4+CD8− cells. The proportion of IKDC (CD49b−CD3−B220+) in the gated CD3−CD11b+ cells was also significantly increased after the expansion (p = 0.001) (Fig. 5D). The CD3−CD49+ NKT cells and CD3+CD49b− NK cells showed higher expression of the activation marker CD25 after a 6-d expansion (day 6) compared with freshly isolated cells (day 0) (Supplemental Fig. 3D). The expanded CD3− non-T cells (NKT cells) showed higher viability compared with the expanded CD3+ non-T cells (NK cells and IKDC) (Fig. 5E: 71.9 versus 37.5% on day 6; p = 0.004).

To determine whether the presence of non-T cells renders the tumor-reactive T cells resistant to MDSC, in vitro and in vivo studies were performed on the sorted cells. The ex vivo-expanded cells were sorted into CD4+ plus CD8+ T cells and CD4+CD8− non-T cells. Sorted cells were then cultured for 24 h in the presence or absence of irradiated MMC (10:1 ratio of expanded cells to MMC) to show their reactivity with MMC in the presence or absence of MDSC (2:1 ratio of expanded cells to MDSC). Supemutants were collected and subjected to IFN-γ ELISA. As shown in Fig. 5F, MDSC induced secretion of IFN-γ by CD4+CD8− non-T cells in the presence of MMC but not in CD4+ plus CD8+ T cells (p = 0.031). Lower amounts of IFN-γ were secreted by sorted CD4+ plus CD8+ T cells compared with unsorted cells.

FIGURE 5. Ex vivo-expanded T cells include non-T cells (NKT cells, NK cells, and IKDC) that are responsible for rendering T cells resistant to MDSC. Freshly isolated (day [D] 0) and ex vivo-expanded (D 6) splenic cells were analyzed for the presence of CD4+CD8− non-T cells and CD4+ or CD8+ T cells (A). Expression of CD3 on non-T cells was determined (B). Non-T cells were also analyzed to determine the proportion of NKT cells and NK cells (C). Cells were gated on CD3−CD11b+ DC population and analyzed for the expression of CD49b and B220 as well as IFN-γ to identify IKDC population (D). Viability (Annexin V−/PI−) of CD3− and CD3+ non-T cells was determined (E). Ex vivo-expanded splenocytes from MMC-primed FVBN202 mice were sorted into non-T cells (CD4−CD8−) and T cells (CD4+ plus CD8+). The sorted cells were cultured with irradiated MMC in the presence or absence of MDSC for 24 h, and IFN-γ production was determined in the supernatants (F). The sorted cells were used for ACT (n = 3) at doses proportional to the unsorted cells, and tumor growth was determined (G). Data represent three independent experiments.
(107 pg/ml in Fig. 5F compared with 2642 pg/ml in Fig. 4A). This suggests that the presence of non-T cells boosts the tumor reactivity of T cells. In addition, the presence of MDSC significantly increased MMC-induced IFN-γ production by non-T cells (p = 0.006). However, CD4+ plus CD8+ T cells in the absence of non-T cells lost their ability to secrete MMC-specific IFN-γ while MDSC were present. This suggests that MDSC-stimulated, MMC-activated non-T cells render T cells resistant to MDSC. ACT with sorted T cells or non-T cells failed to protect FVBN202 mice from challenge with MMC cells (Fig. 5G). Because IL-12 induces the expression of IFN-γ by NK cells and T cells, we sought to determine whether CD4+CD8+CD49b+ cells produce IL-12 in the presence of MMC and MDSC, resulting in the induction of enhanced IFN-γ production by T cells. No IL-12 production was detected in non-T cells or T cells (data not shown).

Radiation therapy of tumor-bearing mice prior to the isolation of donor T cells results in failure of the expanded T cells to generate objective responses upon ACT despite sustained antitumor responses of the T cells in vitro

Cancer patients who participate in clinical trials of ACT have usually received conventional therapies, often including radiation therapy. Therefore, it is important to determine whether tumor-primed T cells that were isolated following radiation therapy can also be expanded and can generate objective responses against the tumors following ACT. To test this, FVBN202 mice were inoculated with MMC cells, and as soon as tumors reached 75–150 mm³, animals received three doses of local radiation therapy to the tumor site (5 Gy) in a 3-d interval. Animals were then sacrificed 1 wk after the last radiation treatment, and their splenocytes were subjected to B/I activation and a 6-d expansion with sequential common γ-chain cytokines. The frequency of freshly isolated T cells was significantly lower after radiation therapy (Fig. 6A) compared with that without radiation therapy (Fig. 1A) (CD8+ T cells, p = 0.002; CD4+ T cells, p = 0.0002). However, radiation therapy did not alter the ability of T cells to grow after B/I activation, such that after 6 d in culture, the cells showed similar rates of expansion compared with those from mice that did not receive any radiation (Figs. 1A, 6A). The frequency of apoptotic T cells did not increase during the ex vivo expansion (Supplemental Fig. 4A). However, expanded T cells from mice subjected to radiation failed to increase CD127 (Supplemental Fig. 4B), and increased expression of CD122 was evident only in CD8+ T cells (Supplemental Fig. 4C).

To determine the tumor reactivity of the expanded T cells from mice whose tumors had been irradiated, in vitro studies were performed. As shown in Fig. 6B, freshly isolated T cells (day 0) and ex vivo-expanded cells (day 6) failed to produce significant

**FIGURE 6.** In vitro and in vivo efficacy of the ex vivo-expanded and MMC-primed T cells harvested from FVBN202 mice after radiation therapy. A, Ex vivo expansion of splenocytes harvested from MMC-primed female FVBN202 mice that received three cycles of local radiation therapy (5 Gy) in 3-d intervals before and after activation with B/I and expansion with sequential common γ-chain cytokines as determined by trypan blue exclusion (left panel) or flow cytometry analysis of gated CD8+ or CD4+ T cells (right panel). B, Tumor reactivity of freshly isolated (day [D] 0) and ex vivo-expanded (D 6) T cells was determined by a 24-h culture of T cells in the presence or absence of irradiated MMC, followed by the detection of IFN-γ in the supernatant. Medium alone and MMC alone were used as negative controls for IFN-γ production. C, The MMC-specific IFN-γ production in gated CD8+ and CD4+ T cells derived from donor mice with no radiation treatment (NT) and radiation treatment (RAD) was determined by flow cytometry analysis. D, Gated neu+ MMC cells were analyzed for apoptosis (Annexin V+/PI+) in the absence or presence of freshly isolated or ex vivo-expanded T cells. E, Phenotypic distribution of freshly isolated (D 0) and ex vivo-expanded (D 6) splenic T cells was determined by flow cytometry analysis of gated CD4+ or CD8+ T cells. Data represent three independent experiments. F, CYP-treated FVBN202 mice (n = 3) were inoculated with MMC and received no further treatment (control) or received the expanded cells derived from donors after the local radiation therapy (ACT). Data represent three independent experiments.
amounts of IFN-γ upon MMC stimulation. However, the addition of MDSC resulted in the induction of IFN-γ by the expanded T cells (Fig. 6B). The expanded cells were also composed of 19.2% non-T cells (Supplemental Fig. 4D). Interestingly, the proportion of Prf1+ T cells in the expanded T cells (day 6) was markedly higher in this group that had received prior radiation therapy compared with T cells that were isolated from donors with no prior radiation therapy (Fig. 6C). Expanded CD8+ T cells were highly positive for the expression of CD69 and GrB (Supplemental Fig. 4E, 4F). Importantly, lack of IFN-γ production by the expanded T cells did not alter the ability of these cells to kill neu+ MMC cells in vitro such that viability (Annexin V−/PI−) of MMC was reduced from 64.64 to 12.35% in the presence of the expanded T cells (Fig. 6D). In addition, expanded T cells were able to kill neu+ MMC cells even in the presence of MDSC in vitro, as shown by a reduced viability from 64.64 to 12.35 and 8.46% (Fig. 6D).

Results of in vivo studies presented in Fig. 3 suggest a correlation between phenotypic distribution of T cells and objective response following ACT such that high proportions of TCM were associated with the rejection of primary and recall tumor challenge. Therefore, we performed phenotype analysis of post-radiation T cells before proceeding with ACT. As shown in Fig. 6E, phenotypic distribution of CD8+ and CD4+ T cells was different from those isolated from animals with no prior radiation therapy (Fig. 3A). Freshly isolated CD8+ T cells (D 0) were mainly of TCM and TN phenotypes, whereas CD4+ T cells contained TF and TEM (Fig. 6E). After 6 d of ex vivo expansion, CD8+ T cells were enriched for TEM, whereas CD4+ T cells contained TF and TEM phenotypes. After a 6-d expansion, CD8+ TCM and T N phenotypes had almost disappeared. An increased proportion of CD8+ TEM cells may account for the in vitro efficacy of the expanded T cells against MMC cells, although antitumor efficacy in vivo may require tumor-specific TCM. To test this possibility, ACT studies were performed as described above. As shown in Fig. 6F, ACT using the ex vivo-expanded cells from mice whose tumors had been irradiated showed minimal tumor inhibitory effects compared with the control group. The rate of tumor growth was the same in the two groups (p < 0.21), although the change from day to day was significant (p < 0.0001).

**HER-2/neu–specific T cells can be expanded from PBMC of a patient with breast cancer**

Splenocytes isolated from tumor-bearing mice with no radiation therapy were effective against the neu+ MMC cells despite the lack of splenic tumor metastasis. This suggests that tumor-reactive T cells may be present in the circulation. To test this, PBMC were collected from a breast cancer patient after Ficoll–Paque gradient centrifugation of blood and split into two fractions. Adherent cells were selected by 2-h culture and used for generating autologous DCs by a 6-d culture in the presence of GM-CSF and IL-4, as described elsewhere (25). Nonadherent cells were split into two fractions. One fraction was maintained in complete RPMI 1640 supplemented with 10% FBS and IL-2 (40 U/ml/10⁶ cells) at 37°C/5% CO₂ for 6 d (IL-2–maintained cells). The remaining cells were subjected to B/I activation and expansion with the common γ-chain cytokines (expanded cells with IL-7, IL-15, and IL-2). Viability of the T cells (Annexin V−) after a 6-d culture or ex vivo culture was >85% (data not shown). Cells were then cocultured with autologous DCs in the presence or absence of recombinant human HER-2/neu protein for 24 h. To rule out nonspecific IFN-γ production by CD4+ T cells as a result of low endotoxin levels in the HER-2/neu recombinant protein, we pulsclot wells with LPS. As shown in Fig. 7A and 7B, compared with IL-2–maintained T cells, the B/I-activated and expanded cells produced significantly higher amounts of IFN-γ when stimulated with HER-2/neu (average, 8,600 versus 32,500 pg/ml; p = 0.001). Flow cytometry analysis of IL-2–maintained and ex vivo-expanded T cells determined that both CD8+ T cells and CD4+ T cells were sources of HER-2/neu–stimulated IFN-γ production (Fig. 7C). It is yet to be determined whether consistently similar results can be obtained in a large group of breast cancer patients.

**Discussion**

Development of an ex vivo protocol that can expand highly efficient populations of tumor-reactive immune cells, which include cells of the adaptive and innate immune systems, may be the key to successful ACT in breast cancer model. Others have reported that rejection of mammary carcinoma in HER-2/neu transgenic mice depends on the stimulation of both innate and adaptive immunity (25). In addition, NKT cells have been shown to be involved in secondary antitumor T cell responses (26).

We demonstrated that activation of tumor-prime lymphoid cells with B/I, followed by ex vivo expansion with sequential common γ-chain cytokines, can activate and expand tumor-reactive T cells and non-T cells including NKT cells, NK cells, and IKDC. We
have tested a number of the cytokine combinations (7, 21, 22) and found that IL-7 + IL-15, followed by IL-2, was the best sequence for the expansion of the most effective cells. The presence of activated non-T cells in the expanded cells was critical not only for the in vivo antitumor efficacy of T cells but also for their resistance to MDSC. The absence of such activated non-T cells in freshly isolated splenocytes or depletion of these non-T cells in the expanded cells resulted in susceptibility to MDSC-induced suppression of tumor-reactive T cells. Neither tumor-reactive T cells nor these non-T cells alone were able to protect FVB202 mice against tumor challenge when they were used separately in ACT. Because the viability of NK cells was very low (37.5%) as opposed to the high viability (72%) of NKT cells, it is likely that NKT cells are the key component of the supportive cells. Our findings are consistent with recent reports showing that cells of the innate and adaptive immune system work together to produce objective responses against tumors (25, 26). Our results also showed that MDSC can further activate the ex vivo-expanded non-T cells, as shown by an increased CD25 expression, thereby enhancing the supportive function of non-T cells for tumor-reactive T cells. To our knowledge, this is the first report showing a cellular mechanism by which T cells may become resistant to MDSC. Because of the T cell inhibitory role of MDSC in a variety of cancers, the proposed protocol could be applicable to a variety of carcinomas.

Although the presence of T cells and cells of the innate immune system were critical for antitumor efficacy of the immune response, long-term protection against the tumor depended on the presence of TCM. Our data suggest that B/I activation and ex vivo expansion with sequential common γ-chain cytokines may have improved the quality of neu-specific cells for tumor rejection, and it was not just because of an increase in frequency of neu-specific T cells. For instance, freshly isolated T cells from tumor-bearing but not from tumor-free FVB202 mice produced IFN-γ upon stimulation with MMC in vitro; yet, such increased frequency of endogenous neu-specific T cells did not induce tumor rejection in donor mice. In addition, ACT with an increased number of freshly isolated T cells derived from tumor-bearing donors (2 x 10^7 cells/mouse) did not protect mice against tumor challenge (data not shown). These data suggest that an increase in neu-specific T cells without ex vivo expansion/differentiation using the proposed protocol cannot provide protection against the tumor. Whereas T cells from nonirradiated and postradiation donors showed comparable levels of antitumor efficacy in vitro (Fig. 6D), T cells obtained from nonirradiated donors provided long-term memory responses against recall tumor challenge in vivo, likely because of the phenotypic distribution of T cells toward TCM. Local radiation therapy of the tumors in donor mice altered the phenotypic distribution of freshly isolated T cells as well as the capacity of T cells to differentiate into TCM during the ex vivo expansion. These data suggest that CD8+ T cells and TCM may be more susceptible to radiation therapy than previously established TCM, as has been reported by others (27). Our data suggest that local radiation therapy could alter the differentiation of tumor-reactive CD8+ T cells and TCM toward TCM. However, we performed local radiation therapy of primary tumors, whereas breast cancer patients usually receive radiation therapy after surgery to destroy residual microscopic disease. Also, patients with advanced breast cancer have undergone multiple radiation treatments, followed by a period of recovery prior to ACT. These scenarios are somewhat different from the treatment protocol that we used in our study. For T cells to be effective for ACT, we had to isolate T cells from tumor-bearing animals. Therefore, we had to perform radiation therapy on primary tumors. Application of the proposed approach may be limited to patients with early-stage breast cancer (stages I–III), provided that PBMC are harvested and cryopreserved prior to radiation therapy for ACT in future. Feasibility of this strategy remains to be determined. The importance of TCM against cancer has also been reported by others (4). Our data showing a greater antitumor efficacy of ACT in association with the presence of TCM are consistent with other reports showing that effector cells derived from TCM rather than TEM possess greater ability to survive and establish immunologic memory following infusion (28). However, naive T cells have been reported to convey more antitumor activity than memory cells (28). Such contradictory results may be due to the use of a mouse model harboring a transgenic TCR for gp100 tumor Ag, which is different from the FVB202 mouse model of spontaneous breast carcinoma with no transgenic TcR against the tumor Ag.

IL-7 has been shown to support viability and homeostatic proliferation of T cells and enhance NK cell function (29). IL-15 supports differentiation of memory T cells and activation of quiescent NK cells more efficiently than IL-2 (30). IL-2 is T cell growth factor and is also involved in NK cell activation and proliferation. Therefore, culture of tumor-primed T cells initially with IL-7 + IL-15, followed by IL-2, can support differentiation of T cells as well as non-T cells. The presence of non-T cells in this model appears to be critical for rendering T cells resistant to MDSC, regardless of whether T cells were obtained before or after radiation therapy. A similar observation has been made in mice and humans with hematologic malignancies undergoing alloge

necic stem cell transplantation. In the animal model, depletion of donor NK cells abrogated antileukemia effects of donor T cells (31). In humans, early donor-derived NK cell recovery has been shown to be associated with a lower relapse risk in the non-

myeloablative setting in the recipients of T cell-replete allografts (32). Earlier observations in the same clinical model had demonstrated a significant impact of NK cell dose in the graft on day 28 T cell chimerism (33). Similarly, our group has shown a trend toward a higher level donor T cell chimerism at 12 wk post-

transplant, in patients with superior NK cell recovery at 4 wk (data not shown). Taken together, these data provide intriguing evidence of T cell–NK cell interactions in the clinical transplant setting and suggest interdependence between innate and adaptive immunity.

Altogether, these data suggest that lymph nodes (11) or PBMC of breast cancer patients may be explored as a source of tumor-reactive immune cells for ex vivo expansion and use in ACT, provided that immune cells are obtained prior to radiation therapy and expanded with the sequential common γ-chain cytokines. Expanded T cells and non-T cells obtained prior to radiation therapy may be able to be cryopreserved and used for experimental ACT protocols after the completion of conventional thera

pies in an attempt to eliminate residual disease and prevent tumor relapse. In fact, the majority of patients with breast cancer die of tumor metastases rather than from the primary cancer. Although tumor stroma in breast cancer patients makes regression of est

blished solid tumors difficult, regression of residual disease is possible (6, 10). Therefore, the proposed ACT protocol should be further explored in breast cancer patients. We have previously tested antitumor efficacy of ACT using different common γ-chain cytokine regimens (22). The efficacy of cells expanded with sequential common γ-chain cytokines remains to be determined in melanoma and other tumor models.

Acknowledgments

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References


Supplemental figure legends

**Figure S1: Presence of CD4+CD25+Foxp3+ T cells before and after the ex vivo expansion**

Freshly isolated (D 0) or ex vivo expanded (D 6) splenocytes harvested from MMC-primed FVBN202 mice. Red cells were lysed by using ACK lysis buffer and splenocytes were subjected to three-color staining to determine proportion of Tregs in CD4+ T cells. Data represent three independent experiments.

**Figure S2. T cell responses to MMC in the presence or absence of MDSC**

A) Freshly isolated and ex vivo expanded T cells were cultured in the presence or absence of irradiated MMC cells for 24 hs and subjected to three color staining to determine the expression of GrB. Data represent three independent experiments. B) Frequency of MDSC in the blood of FVBN202 mice before and 7 days after the challenge with MMC cells. C) T cells were harvested from MMC-primed FVBN202 mice and expanded with alternating common gamma-chain cytokines. Briefly, splenocytes (10^6 cells/mL) were stimulated in complete medium containing 15% FBS with bryostatin-1 (5 nM) and ionomycin (1 μM) along with 80 U/mL of IL-2 (Peprotech) for 16 hs. Cells were washed three times and cultured at 10^6 cells/mL in complete medium with 40 U/mL IL-2 and media was changed every other day for a total of 6 days. Cells expanded with alternating gamma-chain cytokines were cultured on day 1 with 10 ng/mL IL-7 and 10 ng/mL IL-15 (Peprotech). On day two, 40 U/mL of IL-2 was added. Medium was changed on days 3 and 5, each time culturing with 10 ng/mL each of IL-7 and IL-15 and injections were done on day 7. Production of IFN-γ by the expanded T cells was determined in the presence or absence of MMC or MMC+MDSC. Data represent two independent experiments.
**Figure S3.** Expanded T cells do not increase the expression of CD69 or GrB following stimulation with MMC cells *in vitro*, and include activated NK T cells and NK cells

*Ex vivo* expanded T cells were cultured in the presence or absence of irradiated MMC or MMC+MDSC. Expression of Prf was determined in gated neu positive MMC cells (A). Expression of CD69 (B) or GrB (C) was determined in gated CD8+ and CD4+ T cells. Data represent three independent experiments. D) Freshly isolated (D 0) or *ex vivo* expanded (D 6) splenocytes were gated on CD3+CD49b+ NK T cells or CD3-CD49b+ NK cells and expression of CD25 was determined. Data represent three independent experiments.

**Figure S4.** Analysis of the *ex vivo* expanded T cells derived from FVBN202 donor mice after local radiation therapy of the tumor

A) Viability of freshly isolated (D 0) and *ex vivo* expanded (D 6) T cells was determined by flow cytometry analysis of gated CD8+ or CD4+ T cells. Expression of CD127 (B) and CD122 (C) were determined on gated CD8+ or CD4+ T cells after a 6-day expansion. Data represent 3 independent experiments. D) Proportion of CD4-CD8- non-T cells and CD4+ or CD8+ T cells was determined by flow cytometry analysis of the *ex vivo* expanded T cells derived from donor mice after radiation therapy of the tumors. Freshly isolated and *ex vivo* expanded cells were cultured in the presence or absence of irradiated MMC cells and expression of CD69 (E) or GrB (F) was determined. Data represent three independent experiments.
Figure S1

Gated CD4⁺ T cells

D₀

D₆

CD25

CD4⁺

Gated CD4⁺ T cells

D₀

D₆

CD25

CD4⁺

Foxp3

0.2%

2.4%

54.8%

7.5%
Figure S2

**A**
- **Freshly isolated T cells**
  - Gated CD8+ T cells: medium + MMC
    - CD8: 98.12% + 96.40%
    - CD4: 98.55% + 96.84%
  - Gated CD4+ T cells: medium + MMC

- **Ex vivo expanded T cells**
  - Gated CD8+ T cells: medium + MMC
    - CD8: 84.10% + 90.88%
  - Gated CD4+ T cells: medium + MMC
    - CD4: 93.34% + 94.35%

**B**
- Day 0 vs Day 7
  - Gr1: 4.24% vs 17%
  - CD11B

**C**
- IFN-γ (pg/ml)
  - Expanded T cells: medium, MMC, MMC + MDMSC
Figure S3

A
Gated neu+ MMC
IFN-γ  Prf
0.39%  0.76%

B
Gated CD8+ T cells
medium  + MMC  + MMC+ MDSC
CD8
81.06%  84.41%  82.32%
Gated CD4+ T cells
medium  + MMC  + MMC+ MDSC
CD4
14.85%  18.87%  28.05%
CD69
82.32%  81.06%  84.41%
GrB
92.66%  84.1%  90.88%

C
Gated CD8+ T cells
medium  + MMC  + MMC+ MDSC
CD4
93.34%  94.35%  92.38%
GrB
84.9%

D
Gated CD3+CD49b+
CD49b
17.7%  84.9%
CD25
D0  D6
Gated CD3-CD49b+
CD49b
0.8%  33%
CD25
D0  D6
Figure S4