Human T Cells Armed with Her2/neu Bispecific Antibodies Divide, Are Cytotoxic, and Secrete Cytokines with Repeated Stimulation

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Abstract

Purpose: Cancer immunotherapy has been limited by anergy of patient T cells, inadequate numbers of precursor tumor-specific CTL, and difficulty in producing therapeutic doses of CTL. To overcome these limitations, bispecific antibodies have been used to create artificial antibody receptors that direct polyclonal activated T cells (ATC) to target tumor antigens. Studies reported herein were designed to characterize bispecific antibody–armed ATC functions during multiple rounds of targeted cell stimulation.

Experimental Design: ATCs were generated from human peripheral blood mononuclear cells (PBMC) by culture with anti-CD3 and interleukin 2 for 14 days and armed with anti-CD3 × anti-Her2 bispecific antibody (Her2Bi). *In vitro*, Her2Bi-armed ATC were examined for a range of functions after repeated stimulation with the Her2/*neu*-expressing breast cancer cell line SK-BR-3. PBMC isolated from cancer patients treated with Her2Bi-armed ATC were tested *ex vivo* for cytotoxicity against SK-BR-3.

Results: *In vitro*, armed ATC divided, maintained surface Her2Bi, and expressed a range of activities for extended periods of time. Perforin-mediated cytotoxic activity by armed ATC continued for at least 336 hours, and cytokines and chemokines (i.e., IFN-γ and regulated on activation, normal T-cell expressed and secreted protein [RANTES]) were secreted during successive rounds of stimulation. Furthermore, PBMC isolated from patients over their courses of immunotherapy exhibited significant cytolytic activity against SK-BR-3 as a function of Her2Bi-armed ATC infusions.

Conclusions: These studies show that armed ATC are specific, durable, and highly functional T-cell populations *in vitro*. These previously unappreciated broad and long-term functions of armed ATC are encouraging for their therapeutic use in treating cancer.

Cancer immunotherapy using tumor-specific CTL has been limited in part by tumor-induced immunosuppression as well as the numbers of tumor-specific autologous CTL that can be generated for adoptive immunotherapy. Work done in this laboratory, taken together with that published by others, however, indicates that the practical limitations of specificity and magnitude of antitumor T-cell populations may be overcome for clinical exploitation. The use of bispecific antibodies in T-cell immunotherapy directly addresses these issues as polyclonal T-cell populations expanded and activated *ex vivo* can be armed to specifically target and kill tumor cells *in vivo*. Indeed, we have shown previously that anti-CD3 activated T cells (ATC) armed with either anti-CD3 × anti-Her2 (Her2Bi) or anti-CD3 × anti-CD20 (CD20Bi) bispecific antibodies mediate specific cytotoxicity and secrete cytokines on binding with cell lines expressing Her2/*neu* or CD20, respectively (1–3).

Interestingly, evidence has emerged showing that T-cell functions can be induced each time an activated T cell engages an appropriate antigen-presenting cell. For example, murine CTL clones recycle their lytic granules multiple times to repetitively mediate killing of allogeneic targets (4). In addition, recent studies have shown that human T cells targeted to lymphoma cells with anti-CD3 × anti-CD19 bispecific antibodies kill multiple times (5). The ability to repetitively stimulate certain T-cell functions also affects the production of certain cytokines that are reportedly secreted each time the TCR of virus-specific CTL engages infected target cells (6). Overall, the evidence showing that these T-cell activities are expressed with each antigen-presenting cell encounter suggests an underappreciated duration of T-cell function *in vivo* that cumulatively may increase the potential effect of T-cell immunotherapy.

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Each study highlighted above reports an individual aspect of repeated killing or cytokine production with multiple rounds of stimulation. A comprehensive study encompassing all of these functions, however, has yet to be carried out in a single population of effector cells. Studies here were undertaken to evaluate the duration of the functional capacity of armed ATC generated for purposes of human anticancer immunotherapy. We report herein that for up to 2 weeks after a single arming, *ex vivo* expanded ATC bearing anti-CD3 × Her2/*neu* bispecific antibodies (Her2Bi-armed ATC), cocultured with SK-BR-3 targets, increase in number, undergo multiple cell divisions, mediate specific cytotoxic activity, and secrete both cytokines and chemokines without undergoing Fas/FasL–induced apoptosis or activation-induced cell death. Data presented here are the first to show in a single system that one population of cells can be repeatedly stimulated to divide, kill, and secrete cytokines over extended periods of time. Moreover, we provide additional evidence that patient peripheral blood mononuclear cell (PBMC) populations, isolated during armed T-cell immunotherapy, exhibit augmented tumor cell–specific cytotoxic activity *ex vivo*. The implications of these findings for the use of bispecific antibody–armed T-cell immunotherapy in cancer treatment are discussed.

Materials and Methods

Preparation and culture of ATC. For the preclinical studies reported here, normal donor PBMC were isolated by Ficoll-Hypaque density gradient centrifugation, seeded into culture flasks, activated with 20 ng/mL OKT3 (Orthobiotech, Inc., Bridgewater, NJ), and expanded for 14 days in the presence of 100 IU/mL of interleukin 2 (Chiron, Emeryville, CA) in RPMI (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (BioWhittaker; refs. 7, 8).

Tumor lines, antibodies, and bispecific antibodies. SK-BR-3, a Her2/*neu*-positive breast adenocarcinoma, was maintained in Iscove's modified Dulbecco's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 2 mmol/L L-glutamine, HEPES buffer (Life Technologies), 10% fetal bovine serum, and antibiotics. Raji, a Her2/*neu*-negative Burkitt's lymphoma used here as a negative control, was maintained in RPMI medium (Life Technologies) supplemented with 2 mmol/L L-glutamine, 10% fetal bovine serum, and antibiotics. Her2Bi and CD20Bi, used here as irrelevant control bispecific antibodies, were produced as reported (1). Anti-CD3 [OKT3 (immunoglobulin (Ig) G2a); Orthobiotech] was chemically heteroconjugated with anti-Her2/*neu* (Herceptin, Genentech, San Francisco, CA) or anti-CD20 (Rituxan, Genentech), and ATC for both preclinical and phase I clinical studies were armed with 50 ng bispecific antibody per 10^6 T cells unless otherwise mentioned. FITC- or phycoerythrin-conjugated monoclonal antibodies (Becton Dickinson, San Diego, CA) were used to detect Ig G2a+, Fas+, FasL+, CD3+, CD4+, and CD8+ cells by flow cytometric analysis along with relevant isotype control antibodies.

Flow cytometry. Phenotyping and cell cycle analyses were done on a FACSCalibur flow cytometer (Becton Dickinson). Armed or unarmed ATC were labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma Aldrich, Milwaukee, WI) at a concentration of 1 mg/mL as described (9). The cells were then labeled with phycoerythrin-conjugated anti-CD4 or anti-CD8. Propidium iodide staining was used to confirm viability initially obtained by trypan blue staining. Fas and FasL on ATC and SK-BR-3 were detected using anti-Fas and anti-FasL antibodies followed by phycoerythrin-conjugated secondary antibodies.

Cell division after tumor engagement. Baseline samples (0 hour) of both populations were taken before they were exposed to SK-BR-3. At each time when the cells were repeatedly exposed to tumor targets, aliquots of armed and unarmed ATC were tested for fluorescent intensity of the CFSE-labeled T cells. The nonadherent cells were removed from the plates, stained with anti-CD4 or anti-CD8 phycoerythrin-conjugated monoclonal antibodies, and tested by flow cytometry. The cell populations were analyzed for CFSE and CD4 or CD8 positivity.

Preparation of patient armed ATC and PBMC. Five women with high-risk stage II/III or stage IV BrCa were treated with Her2Bi-armed ATC in accordance with two Roger Williams Medical Center Institutional Review Board–approved phase I clinical trials. Armed ATC for infusion into patients were produced on-site in Food and Drug Administration–regulated facilities according to Investigational New Drug–approved standard operating procedures. To reach the target doses of 80×10^9 to 160×10^9 armed ATC, anticipating an average 10-fold increase in cell yield, 8×10^9 to 20×10^9 PBMC were harvested by leukopheresis. Cells were maintained for up to 14 days in culture at densities of 1×10^6/mL to 3×10^6/mL in RPMI supplemented with 100 to 500 IU/mL interleukin 2 (Chiron), 10 to 20 ng/mL OKT3 (Orthobiotech), and 2% human serum (BioWhittaker). Cells were armed with Her2Bi as described above and cryopreserved until infusion. Patients were treated with eight infusions of Her2Bi-armed ATC; two infusions per week for 4 weeks, for a total of 80 billion ($n = 2$) or 160 billion ($n = 3$) armed ATC, given with low-dose interleukin 2 (Chiron). There were no armed-ATC dose-limiting toxicities observed in these patients. Patient PBMC were acquired from whole blood samples collected at the indicated time points over the course of treatment and follow-up in accordance with clinical protocols approved by the Roger Williams Medical Center Institutional Review Board. Samples taken at infusion times were drawn immediately before the indicated infusion number and at least 48 hours after the previous infusion. Patient PBMC were tested for cytotoxic activity against Her2-expressing SK-BR-3 cells and Her2-negative Raji cells in a standard cytotoxicity assay as described below.

Cytotoxicity assay. ^{51}Cr release assays were done in flat-bottomed microtiter plates as previously described (1) with some modifications. Briefly, armed ATC, unarmed ATC, or patient PBMC were plated in triplicate onto SK-BR-3 (4×10^4 per well) at effector/target ratios of 10:1, 5:1, and 2.5:1, and percent cytotoxicity was calculated as (experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm) × 100. For analysis of sequential cytotoxicity, baseline (0 hour), armed, or unarmed ATC were plated in 24-well plates containing SK-BR-3 (2×10^5 per well). At the designated time points, the armed or unarmed ATC were harvested, counted, plated in the specific cytotoxicity assay, and reseeded in 24-well plates containing fresh SK-BR-3 targets. Effectors were added at an effector/target ratio of 10:1 (2×10^6 cells per well) with six replicates. Aliquots of effectors were tested for specific cytotoxicity at each reseeding. Viability and cell counts were evaluated by trypan blue exclusion. Cells were split down to 1×10^6/mL when they exceeded 2×10^6/mL. A "reverse specific cytotoxicity assay" was designed to assess lysis of armed ATC by SK-BR-3 cells. Armed and unarmed ATC were labeled with ^{51}Cr (20 μCi/mL) for 6 hours at 37°C, washed, and added onto SK-BR-3 cells at an effector/target ratio of 10:1, 5:1, and 2.5:1. ^{51}Cr release was measured after 18 hours. To inhibit perforin/granzyme–mediated cytotoxicity, armed and unarmed ATCs were incubated for 2 hours with concanamycin A (100, 10, 1 and 0.1 nmol/L; ICN Biochemicals, Costa Mesa, CA) before coculturing them with SK-BR-3 targets for a standard ^{51}Cr release assay as described above.

Cytokine and chemokine assays. IFN-γ, tumor necrosis factor α, granulocyte macrophage colony-stimulating factor, macrophage inflammatory protein 1α, and regulated on activation, normal T-cell expressed and secreted (RANTES) were measured using Quantikine ELISA kits (R&D Systems, Minneapolis, MN) and are reported as pg/mL/10^6 cells cultured at indicated time intervals.

EliSPOTS for IFN-γ–secreting T cells. This procedure was adapted from previous publications (10, 11). Armed or unarmed ATC, previously stimulated with SK-BR-3 cells for 2 hours at 37°C at a 10:1 effector/target ratio, were plated onto multiscreen nitrocellulose

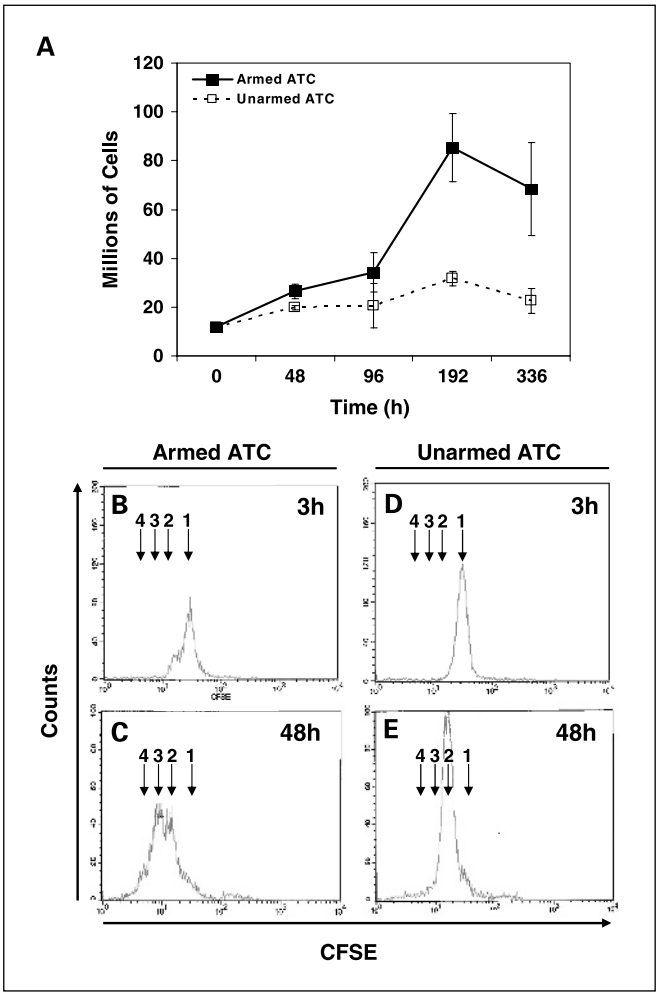

Fig. 1. Cellular expansion and division of armed versus unarmed ATC. *A,* the mean cell yields are shown for armed (■) versus unarmed (□) ATC after exposure to SK-BR-3 targets at 0, 48, 96, 213, and 336 hours from the same four experiments shown in Fig. 2. Cell counts and viability were taken each day using trypan blue during 2 weeks of culture. Points, mean of four experiments; bars, ±1 SD. Alternatively, armed (*B* and *C*) and unarmed ATC (*D* and *E*) were marked with CFSE dye and studied for cell division after binding to SK-BR-3 targets at 3 and 48 hours. Only the nonadherent cells were analyzed for CFSE fluorescence intensity and cell cycle status. *B* and *C*, cell division of armed ATC after 3 and 48 hours following binding to SK-BR-3, respectively. *D* and *E*, cell division of unarmed ATC after 3 and 48 hours following binding to SK-BR-3, respectively.

96-well plates in triplicate (1.2×10^4-5×10^4 per well). Unstimulated armed and unarmed ATC were used as controls. EliSPOTS were quantitated using a dissecting microscope (Leica, Wetzlar, Germany) connected to colony counting software (Bio-Rad, Hercules, CA).

Statistical analyses. All statistical analyses were done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Results from cytotoxicity assays, cytokine/chemokine assays, and EliSPOTs between the different groups were compared using two-way ANOVA followed by Bonferroni's posttests. Results from cytotoxicity assays done with patient PBMC were compared using the Kruskal-Wallis nonparametric test followed by Dunn's multiple comparisons test.

Results

Armed ATC are viable and divide with repetitive target stimulation. Duration of armed ATC responsiveness to serial stimulations with specific tumor target cells was determined by comparing survival and ability to divide between armed and unarmed ATC repeatedly exposed to SK-BR-3 cells over 336 hours of culture. At ~48-hour intervals, armed and unarmed ATC previously cocultured with SK-BR-3 were harvested for counting cell numbers and for reseeding on fresh SK-BR-3 cells. Over time, numbers of viable armed ATC significantly exceeded unarmed ATC in culture ($P < 0.001$ at 192 hours and $P < 0.001$ at 336 hours); after 336 hours of repeated stimulation, there were 6.84×10^7 armed ATCs (5.6-fold increase) and 2.26×10^7 unarmed ATC (2.7-fold increase; Fig. 1A). Although the numbers of armed ATC exceeded those of unarmed ATC, differences in mean viabilities were insignificant, ranging from 78% to 88% for armed ATC and from 75% to 90% for unarmed ATC.

To extend the finding that repeated SK-BR-3 stimulation resulted in increased cell yields of armed ATC as compared with their unarmed counterparts, unarmed ATC, ATC armed with Her2Bi, and ATC armed with CD20Bi as a negative control were loaded with CFSE dye and then cocultured with SK-BR-3 targets for assessment of cell division. Numbers of cell divisions within each population were determined after 3 and 48 hours by flow cytometric analysis of CFSE fluorescence intensity (Fig. 1B-E). After 3-hour stimulation, the majority of Her2Bi-armed (Fig. 1B), unarmed (Fig. 1D), and CD20Bi-armed (data not shown) ATC were clustered under peak 1 at maximal fluorescence intensity. In contrast, after 48 hours of SK-BR-3 stimulation, the armed ATC (Fig. 1C) had undergone an additional three divisions whereas unarmed ATC had undergone only one additional division (Fig. 1E). Of note, further analysis showed that both CD4 and CD8 subsets within the Her2Bi-armed population consistently underwent more rounds of division than unarmed CD4 and CD8 subsets (data not shown). Together, these data show that armed ATC are viable for at least 336 hours in culture and can be stimulated to undergo multiple rounds of cell division, resulting in an increase in armed ATC cell number over time.

Her2Bi-armed ATC mediate specific cytotoxicity through multiple cycles of stimulation. In preliminary studies not shown here, the specificity of Her2Bi-armed ATC for Her2-expressing target cells was first shown by (*a*) Her2Bi-armed ATC activity upon culture with the Her2+ cell lines PC3, DU145, and LNCaP; (*b*) lack of Her2Bi-armed ATC activity against the Her2− cell lines B9C and Raji; and (*c*) no increase in activity when unarmed ATC were incubated with free OKT3 and free Herceptin relative unarmed ATC alone.[6] Given the capacity of armed ATC to remain viable and increase in number over an extended period of stimulation, along with the observed duration of the bispecific antibody on armed ATC populations, experiments were next undertaken to evaluate the ability of Her2Bi-armed ATC to kill SK-BR-3 targets over this extended period of time. ATC derived from up to four normal subjects were either armed or unarmed, and seeded in 24-well plates containing SK-BR-3 targets. At the designated time points, armed and unarmed ATC were harvested either for assessment of cytotoxicity or for reseeding on fresh SK-BR-3 cells for continued culture (Fig. 2). At effector/target ratios of 10:1,

[6] Lum et al., unpublished results.

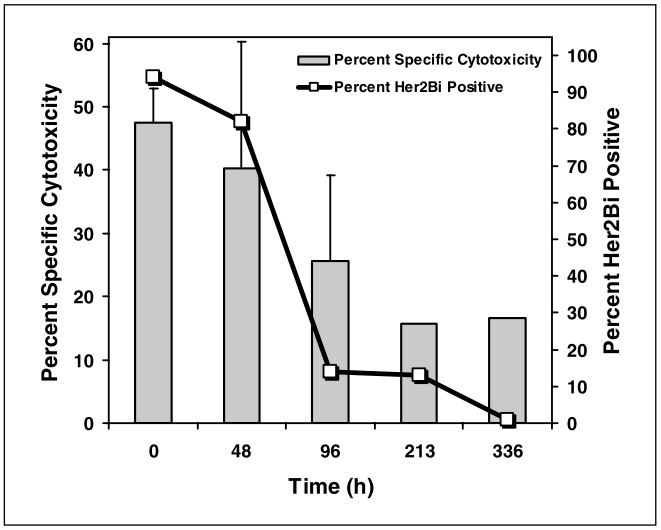

Fig. 2. Specific cytotoxicity by armed ATC and persistence of Her2Bi. Mean specific cytotoxicity ± SD (calculated as armed ATC – mediated cytotoxicity minus unarmed ATC – mediated cytotoxicity) was measured over the course of repeated exposures to SK-BR-3 tumor targets at an effector/target ratio of 10:1 in a ^{51}Cr release assay and graphed on the left axis for $n = 3$. Armed ATC were exposed to SK-BR-3 targets for 0, 48, 96, 213, and 336 hours and stained with a polyclonal goat anti-mouse Ig G2a phycoerythrin-conjugated antibody to detect the OKT3 portion of the Her2Bi. Percent Her2Bi-positive armed ATC are graphed on the right axis (□) for $n = 1$.

Her2Bi-armed ATC mediated a mean specific cytotoxicity of 47.55 ± 5.36%, 40.33 ± 19.88%, 25.67 ± 13.39%, 15.69%, and 16.53% after zero, one, two, three, or four rounds of stimulation, respectively, over a total of 336 hours.

Her2Bi persists on ATC. That numbers of armed ATC increased over 192 hours (Fig. 1A) and mediated target cell killing over 336 hours (Fig. 2) suggested that Her2Bi remained bound to the ATC and was functional for extended periods of time. Therefore, we sought to determine if Her2Bi persisted on the surface of armed ATC. By flow cytometric analysis (Fig. 2), the starting armed ATC population was 96% Her2Bi positive and decreased by only 13% after 48 hours of culture. At subsequent time points, proportions of Her2Bi-positive cells were substantially decreased: 15%, 14%, and 3% positive at 96, 213, and 336 hours, respectively. This experiment is consistent with an independent experiment in which cells were sampled at different times (0, 24, 48, and 72 hours). These data show a decline in Her2Bi-positive cells over time that closely parallels the cytotoxicity data plotted on the same graph (Fig. 2). Interestingly, the sudden decrease in Her2Bi on ATC observed by 96 hours was coincident with a period of increasing cell number (Fig. 1A), and thus may occur, in part, because of dilution of the bispecific antibody below its limit of flow detection due to multiple cell divisions. At all time points tested, background staining of unarmed ATC populations remained below the lower limit of detection (data not shown). Thus, Her2Bi persisted on >85% of ATC up to 48 hours and was still detectable, albeit on a small proportion of cells, for at least 336 hours.

Cytotoxic mechanims mediated by armed ATC. To explore whether the perforin/granzyme system plays a role in Her2Bi-mediated cytotoxicity, concanamycin A (0.1-100 nmol/L) was added to cocultures to inhibit specific cytotoxicity through the perforin pathway (12). A 75% inhibition of the cytotoxicity directed at SK-BR-3 in the presence of 100 nmol/L of concanamycin A was observed under these conditions (Fig. 3A). Specific cytotoxicities mediated by enriched CD4 and CD8 cells (separated by Miltenyi beads) armed with Her2Bi were inhibited >90% in the presence of 10 nmol/L of concanamycin A (data not shown).

Because Fas/FasL interactions are an alternative mechanism of target cell killing, we tested SK-BR-3 and armed ATC for Fas receptor (FasR) and FasL. Nearly all SK-BR-3 cells expressed FasL whereas a small proportion, ~9%, expressed FasR (Fig. 3B). These data indicate that SK-BR-3 cells are poor targets of FasR/FasL-mediated lysis and lend support to the data above that armed ATC, stimulated over extended lengths of time, kill SK-BR-3 cells by a predominately perforin-mediated mechanism. Interestingly, both armed and unarmed ATC populations were

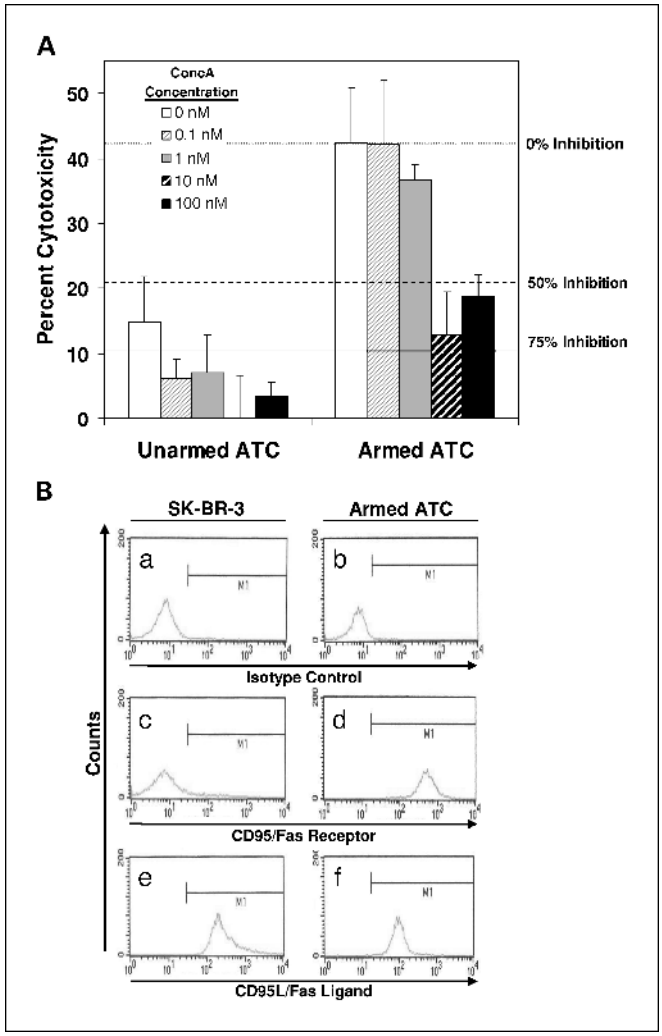

Fig. 3. Cytotoxic mechanisms mediated by armed ATC. *A*, concanamycin A (*ConcA*) was used to block perforin in both the armed and unarmed ATC. SK-BR-3 were used as targets and the effector/target was 10:1 for all concentrations of concanamycin A (0, 0.1, 1, 10, and 100 nmol/L). The specific cytotoxicity was measured in a ^{51}Cr release assay. Dashed lines, 0%, 50%, and 75% inhibition of the control (0 nmol/L of concanamycin A) specific cytotoxicity at an effector/target of 10:1. Columns, mean of triplicates; bars, ±1 SD. *B*, anti-FasR and anti-FasL antibodies were used to stain SK-BR-3 (*left*) and armed ATC (*right*) to determine surface expression of either the FasR (*middle*) or FasL (*bottom*) by flow cytometry. FasR and FasL were detected using a phycoerythrin-conjugated mouse anti-human FasR and FasL antibodies, respectively. The isotype-matched control is also shown (*top*).

strongly positive for both FasR and FasL, and there were no changes observed in FasR or FasL expression when either population was cultured with SK-BR-3, indicating that cell-surface expression of FasR/FasL on ATC is not a function of either the arming process or bispecific antibody–mediated stimulation (effector/target ratio = 10; Fig. 3B and data not shown). Furthermore, in a "reverse" CTL assay with ^{51}Cr-labeled armed ATC as targets, low cytotoxic activity indicated that armed ATC are generally resistant to Fas-mediated cytotoxicity as mediated by either SK-BR-3 cells or armed ATCs (data not shown).

Induction of cytokine/chemokine secretion. As binding of armed ATC to Her2/*neu* on SK-BR-3 cells triggered extended durations of expansion and cytotoxicity, we asked whether repeated stimulations of armed ATC by specific targets would elicit cytokine and chemokine secretion. In preliminary experiments, armed or unarmed ATC were stimulated with SK-BR-3 cells for 48 hours, at which time supernatants were harvested for measurement of IFN-γ, RANTES, tumor necrosis factor α, granulocyte macrophage colony-stimulating factor, and macrophage inflammatory protein 1α by ELISA. One cycle of stimulation was sufficient to observe significantly increased armed ATC production of IFN-γ, RANTES, tumor necrosis factor α, granulocyte macrophage colony-stimulating factor, and macrophage inflammatory protein 1α over unarmed control cultures ($P < 0.04$; data not shown). Next, these studies were extended to determine whether armed ATC could secrete cytokines and/or chemokines with serial stimulations extended over longer periods of time. Subsequent experiments focused on IFN-γ and RANTES because they were highly expressed in preliminary experiments. Armed and unarmed ATCs at effector/target ratios of 10:1 were serially cocultured four times with SK-BR-3 at 48-hour intervals. By ELISA, IFN-γ or RANTES detected in supernatants from armed ATC was significantly greater than that found in unarmed ATC supernatants at every time point ($P < 0.001$; Fig. 4). Interestingly, following the first 48 hours, IFN-γ production slowly declined (Fig. 4A). In contrast, the level of RANTES secretion at 48 hours was sustained through 96 hours but subsequently declined rapidly to near the lower limit of detection by 213 hours (Fig. 4B).

We next sought to determine if the extended kinetics were a function of IFN-γ production per cell or the number of ATC secreting IFN-γ over multiple rounds of SK-BR-3 stimulation. By EliSPOT (Fig. 5), baseline stimulation of armed ATC with SK-BR-3 produced more IFN-γ EliSPOTS (2,380 per 10^6 cells) than SK-BR-3-stimulated unarmed ATC (<50 per 10^6 cells). Strikingly, after multiple stimulations over 96 hours, plus a brief 2-hour SK-BR-3 restimulation, 72,000 IFN-γ secreting cells per 10^6 armed ATC were observed ($P < 0.001$ relative to baseline; Fig. 5). This is in stark contrast to armed ATC that were repeatedly stimulated over 96 hours but lacked the final 2-hour stimulation for EliSPOT (<1,000 IFN-γ spots per 10^6 cells). These data indicate that the number of IFN-γ-producing cells increases with successive rounds of target stimulation. Moreover, the number of IFN-γ-producing armed ATC that had been restimulated with SK-BR-3 cells thrice over 96 hours increased dramatically in response to a final 2-hour restimulation compared with the same population lacking the final 2-hour exposure. Taken together, these data show that armed ATC secrete IFN-γ in a specific response to multiple rounds of tumor cell exposure.

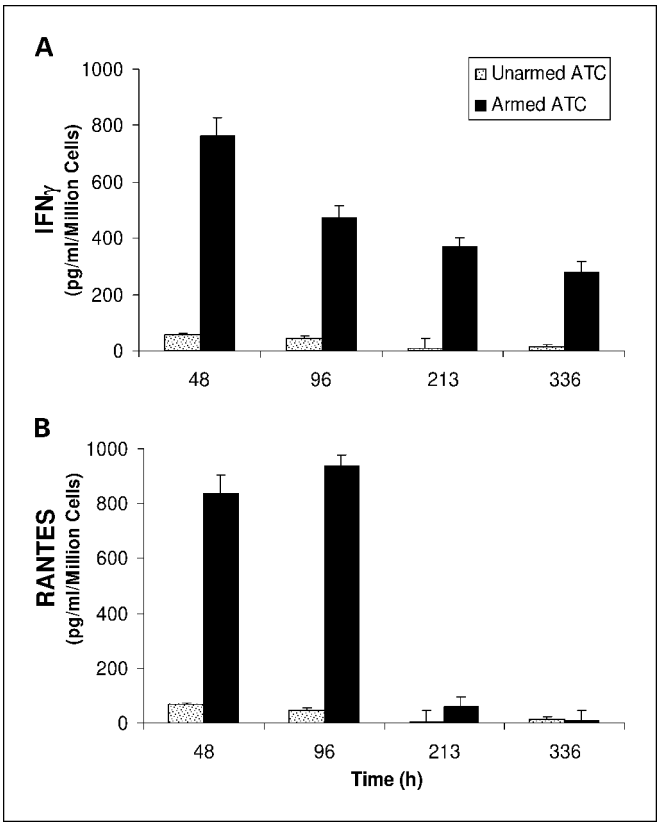

Fig. 4. IFN-γ and RANTES secreted after each cycle of sequential tumor engagement. Secretions of IFN-γ (*A*) and RANTES (*B*) by armed ATC after multiple exposures to SK-BR-3 targets. Armed (⚄) or unarmed (■) ATC (2×10^6) were cocultured with SK-BR-3 (2×10^5). Cocultures of armed or unarmed ATC with SK-BR-3 were harvested, counted, adjusted, and replated at 0, 48, 96, 213, 336, and 384 hours. Data expressed as pg/mL/10^6 cells/48 hours. Columns, mean of triplicates; bars, ±1 SD.

Specific cytotoxic activity observed in patient PBMC. The number and longevity of tumor-specific CTL and their functions are major obstacles to achieving *in vivo* efficacy with cancer immunotherapy. However, the results presented above show that polyclonal T-cell populations expanded, activated, and armed with Her2Bi can be specifically stimulated to kill tumor targets and produce high levels of cytokines/chemokines for at least 14 days *ex vivo*. Thus, the ability to generate large populations of Her2Bi-armed ATC, combined with their sustained antitumor functions for up to 2 weeks, prompted the question: can augmented tumor cell–specific cytotoxicity be observed in patients following infusions of armed ATC? To address this question, five patients undergoing Her2Bi-armed ATC immunotherapy were studied for evidence of antitumor cytotoxic activity before, during, and after treatment. PBMC were acquired from whole blood collected at various time points over the course of treatment. All patient PBMC samples were tested for cytotoxic activity against Her2-expressing SK-BR-3 cells and against Her2-negative Raji cells as a negative control (Fig. 6). A significant increase in specific cytotoxicity directed at SK-BR-3 cells by PBMC obtained from all patients was observed during the course of treatment, with peak levels per individual ranging from 14.15 ± 3.08% to 30.71 ± 0.54% cytotoxicity (Fig. 6). Although there were fluctuations between time points, specific cytotoxic activity

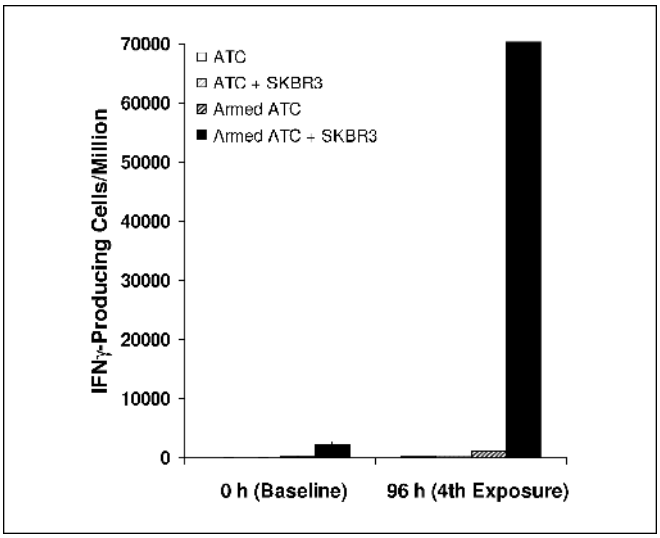

Fig. 5. Enrichment of IFN-γ-secreting T cells after multiple stimulations. EliSPOT assay was used to enumerate the numbers of IFN-γ-secreting T cells in armed ATC populations after multiple exposures to SK-BR-3 targets. The armed ATC were exposed to SK-BR-3 targets for three successive rounds at 0, 48, and 96 hours at an effector/target of 10:1; after which, they were exposed a fourth time (▨) to SK-BR-3 targets for 2 hours at an effector/target of 10:1 for the EliSPOT assay. Armed and unarmed ATC that were not previously exposed to SK-BR-3 before the EliSPOT assay served as the baseline control (■). Both armed and unarmed ATC that had been exposed to SK-BR-3 targets at 0, 48 and 96 hours, but not stimulated with SK-BR-3 in the 4th exposure, were studied as controls. Columns, mean of triplicates; bars, ±1 SD.

tended to increase over the course of treatment with four of five patients evaluated exhibiting maximal cytotoxicity just before or following infusion 8 (Fig. 6). Remarkably, 1 week after completion of all armed ATC infusions, three of four patients' PBMC maintained measurable specific cytotoxic activity, ranging from ~4.74 ± 2.03% to 17.42 ± 0.41% (Fig. 6). Cytotoxicity mediated by PBMC obtained 1-week post infusion 8 from two of three of these patients was significantly ($P < 0.05$) specific for SK-BR-3 cells compared with Raji cells. Thus, these data show that for up to 2 months following armed ATC infusions, increased immune activity targeted to Her2-bearing cells can be detected in patient PBMC. Furthermore, they suggest that armed ATC may persist and function *in vivo* such that they might provide a cumulative, clinically beneficial, immunologic effect.

Discussion

Individual T-cell functions can be elicited numerous times with repetitive stimulatory events, including repeated killing of allogeneic targets by cloned murine CTL, secretion of certain cytokines each time a specific TCR engaged the appropriate antigen/MHC class I complex, and multiple rounds of lymphoma cell–mediated killing by human anti-CD3 × anti-CD19 bispecific antibody–targeted T cells (4–6). Although each of these functions could, in itself, mediate a highly desirable anticancer response, individually none are sufficient to accomplish the therapeutic goals of tumor surveillance and eradication. In this current study, we provide novel insights into the breadth and duration of bispecific antibody–armed ATC functions over multiple rounds of targeted stimulation. Data presented here show that a single population of armed ATC provided with repeated antigen-specific stimulation can increase in number, kill, and secrete cytokines. To our knowledge, this study is the first to show that activated human T cells armed with bispecific antibodies display this broad and complementary array of functions over a series of stimulations spanning over 2 weeks.

When previously activated human T cells are triggered through the CD3/T-cell receptor complex without sufficient costimulation, a substantial number undergo activation-induced cell death (13–15). Interestingly, the armed ATC exhibit >75% viability after repeated *in vitro* stimulation in spite of their expression of both FasR and FasL (Fig. 3B). In fact, ^{51}Cr-labeled FasR-expressing armed ATC made very poor targets when cultured with FasL-positive SK-BR-3 effector cells in a reverse cytotoxicity assay. Our findings indicate that the immunobiology of armed ATC–target interactions is distinct from the activation-induced cell death paradigm and better resembles the behavior of endogenously elicited CTL.

Armed ATC, like conventional CTL, not only survive recurrent target interactions but also exhibit a full range of functions. The viability and functionality of armed ATC may be attributable to the Her2Bi complex on the T-cell membrane (Fig. 2). It may provide not only a cross-linking bridge between the TCR and Her2 on the tumor cells but it may also juxtapose and engage other coreceptors such as CD2, CD28, and LFA-1 with their respective ligands (i.e., LFA-3, B7, and intercellular adhesion molecule-1; refs. 16–19). The strength of the bispecific antibody–CD3 interaction, the number and/or proximity of CD3 molecules engaged in an armed ATC–target interaction, and the physical contact between the armed ATC and the target are all factors that may compensate for more biological costimulatory mechanisms promoting survival and activity (13, 14, 20).

Beyond the signals provided through direct cell-to-cell contact, immune functions are coordinated by cytokines and chemokines. Multiple representatives of both families (i.e., FN-γ, RANTES, tumor necrosis factor α, granulocyte macrophage colony-stimulating factor, and macrophage inflammatory protein 1α) were produced in armed ATC–target cell cocultures. IFN-γ and RANTES, shown to exert potent immunoregulatory properties of particular relevance here, were selected for further study. IFN-γ induces macrophage MHC class I and II expression and shapes T-cell responses by promoting T helper 1–type development (21–25). RANTES effects on T cells include stimulation of migration and degranulation, significant costimulation, and support of tumor-specific cytolytic activity (26, 27). The results provided here show that both IFN-γ and RANTES were produced with repeated target cell stimulation *in vitro* for at least 336 hours (Fig. 4A and B). Given the array and duration of functions displayed on interaction with specific targets, studies presented here suggest that at the interface of tumor and armed ATC interactions, multiple criteria may be met for initiating endogenous immune responses in patients treated with armed ATC immunotherapy. Sites at which armed ATC engage target cells should include a variety of antigens produced by the dying tumor cells, as well as T cell–produced cytokines and chemokines such as IFN-γ, RANTES, etc. These soluble factors can recruit antigen-presenting cells and naive T cells to the site, thus facilitating antigen presentation and endogenous T-cell activation (21–27). Accordingly, repeated exposures of armed ATC to Her2 on the tumor, as well as other

undefined tumor-derived antigens, may provide necessary stimuli for development of endogenous antigen-specific CTL (28, 29).

Discerning between armed ATC effects and host immune responses is an area of intense interest in our laboratory. To date, the ability to track armed ATC *in vivo* has been hampered by the detection limit of flow cytometric analysis for the cell-surface bispecific antibody over time: Her2Bi binding to T cells for the first 48 hours ranged between 83% and 96% and, after 336 hours, was 3% on the ATC (Fig. 2). It is therefore interesting that, although we reproducibly measure increased specific cytotoxicity in patient PBMC during armed ATC immunotherapy (Fig. 6), these data do not directly correlate with increased circulating Her2Bi-positive populations. The decreases observed are thought to be a function of cell division, and if so, the data might suggest that after a certain number of cell divisions, bispecific antibody may be present at densities sufficient to facilitate cytotoxicity but below the limit for detection by flow cytometry. An alternative explanation for finding specific cytotoxic activity without expected increases in Her2Bi-positive PBMC is that endogenous antitumor T-cell responses are elicited. Efforts are now under way to label armed ATC before infusion in such a way as to facilitate tracking of these cells in the patient and in *ex vivo* analyses.

As Her2Bi levels and cytotoxic activity wane (Fig. 2), numbers of IFN-γ-producing cells increase (Fig. 5). This observation correlates with published studies dissociating T-cell IFN-γ production from CTL activity as a function of the strength of the signal transmitted through the TCR (30). Here, lower levels of bispecific antibody on armed ATC that have undergone cell division may indeed be sufficient to trigger IFN-γ production while relatively inefficient for facilitating cytotoxicity. More intriguing to us is the hypothesis that armed ATC–target cell interactions stimulate "endogenous" activity of T cells, either unarmed and/or the progeny of armed ATC, in culture. Thus, we may have recapitulated a form of bystander activation; it has been shown that under certain *in vivo* conditions, cytokines elicited by an antigen-specific T-cell response induce local T-cell activity that is independent of MHC-TCR interactions (31). Efforts are under way to study patients enrolled in our phase I/II clinical trials receiving armed ATC infusions for evidence of endogenous immune activity.

The antigen dependence and longevity of individual T-cell functions have been a long-standing debate with critical implications in the fields of immunologic memory and vaccine design. Several groups have elegantly shown in murine models of immunity to infection that CD8 T-cell expansion primed by a transient antigen exposure will proceed through seven or eight rounds of cell division irrespective of further antigenic stimulation (32–34). Here, we report that despite decreasing levels of cell-surface Her2Bi, armed ATC are viable and increase in number over several days with target cell stimulation but without addition of exogenous growth factors or costimulatory signals (Fig. 1A and B). Whether the continuous presence of antigen is required for cell division in this context is beyond the scope of the present study. It is also possible that the potential to mediate a cytotoxic event or to produce a cytokine is initially primed in a target-dependent manner but may be maintained independent of target stimulation for extended periods of time. However, our evidence, along with that published by others, suggests that unlike proliferation, the cytotoxic event or cytokine secretion requires a contemporaneous interaction of effector T cells with antigen-bearing targets (4, 6, 35). We stress here our novel observation that armed ATC maintain the ability to function as target-specific CTL and to produce certain cytokines over an extended period of time, qualities that are of potential

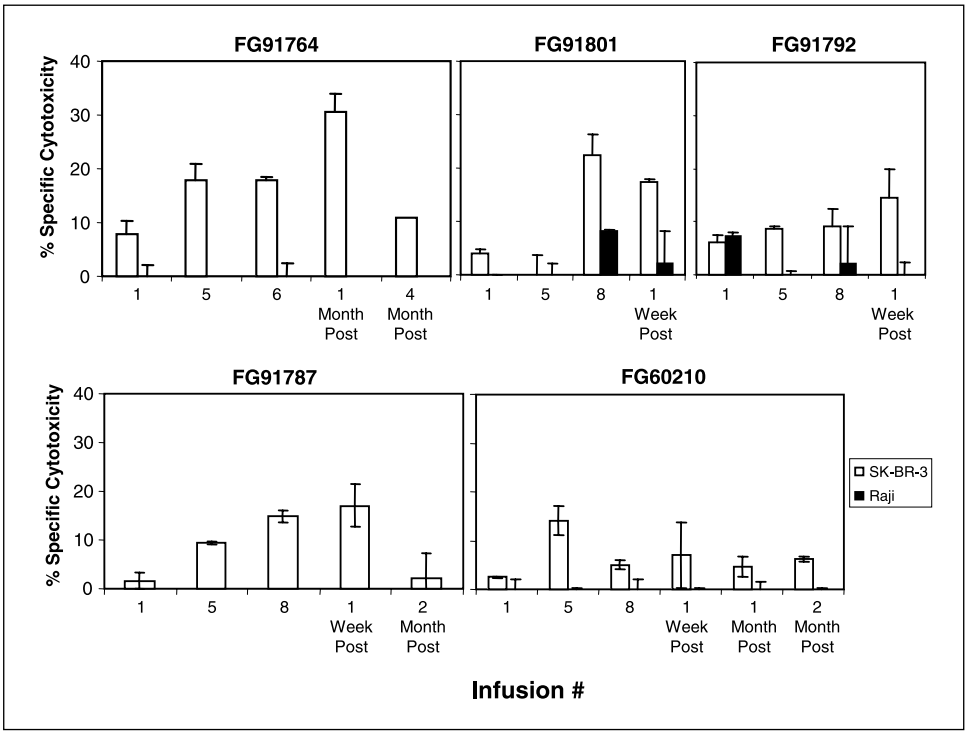

Fig. 6. Patient PBMC mediate increased cytotoxicity against SK-BR-3 targets during armed ATC immunotherapy. Patients were treated with Her2Bi-armed ATC twice weekly for 4 weeks. PBMC acquired from whole blood collected before the indicated infusion number and at least 48 hours after the previous infusion. All samples were exposed to labeled SK-BR-3 (□) and Raji (■) targets in a standard ^{51}Cr release assay. Specific cytotoxicity was calculated as percent cytotoxicity at the given time point minus percent cytotoxicity pretreatment. Individual patients are shown (± SD).

therapeutic benefit. Interestingly, it has been shown that transient TCR stimulation without cytokine support results in rapid cell death without proliferation (36–38). When taken together with our present data that armed ATC (*a*) yields increased over time, (*b*) viability remained high, and (*c*) required interaction with Her2-expressing cells to produce IFN-γ detected by ELISpot, one might speculate that there is some armed ATC–target cell interaction that enables armed ATC to meet their own requirements for survival over time in culture.

These studies indicate that the arming of ATC with bispecific antibodies engineered to bind CD3 on one end and a specific tumor antigen on the other end is a feasible approach for overcoming the practical limitations in cancer immunotherapy with regard to specificity and magnitude of antitumor T-cell populations. Armed ATC maintain a high proportion of viability and a wide range of antitumor and immunostimulatory functions that are elicited on repeated target cell stimulations. Moreover, it is interesting to speculate that not only will armed ATC effectively kill tumor cells *in vivo* but that these cytotoxic events may establish conditions favorable for priming endogenous tumor-specific immune responses. Her2Bi-armed ATC are currently under investigation in phase I/II clinical trials (39) and immune evaluation studies of these patients are ongoing in an effort to gain further insights into the immunostimulatory effects of this therapy.

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