Visualization of Effective Tumor Targeting by CD8+ Natural Killer T Cells Redirected with Bispecific Antibody F(ab’)_2HER2xCD3

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ABSTRACT

HER2 is an attractive immunotherapeutic target for neoplastic disease because this cell surface molecule is overexpressed on a large fraction of malignant tumor cells. To directly assess therapeutic responses to targeted therapy by noninvasive in vivo imaging in small animals, human HER2-expressing ovarian carcinoma cells were genetically modified with a firefly luciferase gene, and light emission was used for visualization of tumor growth and response to therapy. This imaging approach was able to demonstrate in real-time tumor regression in a HER2 xenograft mouse model by adoptive transfer of in vitro induced and expanded cytotoxic CD8+ natural killer T (NKT) cells retargeted with a humanized bispecific antibody F(ab’)_2HER2xCD3. Immunotherapy with effector cells alone or a humanized monoclonal antibody anti-p185HER2 (4D5-8) resulted in significant but slower reduction in tumor burden. Long-term survival of tumor xenografts correlated inversely with visible residual tumor burden. In vitro, F(ab’)_2HER2xCD3 substantially augmented cytotoxic activity of CD8+ NKT cells. By flow-sorting, CD8+ NKT cells coexpressing CD56 were found to have the highest redirected killing ability. Treatment with concanamycin A or EGTA abrogated CD8+ NKT cytotoxicity indicating that perforin is a major pathway of tumor cell lysis. In contrast, when CD8+ NKT cell were cross-linked with F(ab’)_2HER2xCD3 neither the immunosuppressants cyclosporine A and FK506, nor the increase of intracellular cyclic AMP by dibutyryl cyclic AMP were able to inhibit cytotoxicity demonstrating that signaling via the CD3 antigen changes the biological activity of non-MHC-restricted effector cells. These studies have demonstrated that CD8+ NKT cells can be successfully redirected to tumor cells using bispecific antibodies and offer a promising strategy for adoptive immunotherapy of neoplastic diseases.

INTRODUCTION

The HER2 gene encodes a M, 185,000 transmembrane glycoprotein with tyrosine-specific kinase activity (1). It is a self-antigen with very low expression on the surface of normal epithelial cells and a TAA with high expression on malignant epithelial cells, which makes it ideal for antigen-specific immunotherapies (2, 3). Currently the most promising approach to target HER2 is the Food and Drug Administration-approved humanized MAb anti-HER2 (Herceptin), which is now a well-established treatment for women with metastatic breast cancer (4, 5).

The identification of human epithelial cancer antigens has also facilitated the in vitro generation of HER2-reactive CTL (6). These antigen-specific effector cells recognize HER2-derived immunogenic peptides presented in the context of MHC class-I molecules. Although the in vitro induction of tumor-reactive CTL is a procedure that can be performed on a clinical scale there are still some concerns about their applications in terms of effective cancer immunotherapy. For example, HER2-specific CTLs can be detected in breast cancer patients but in most cases do not prevent disease progression (7). A possible explanation of this observation may be that HER2 as a self-antigen induces active tolerance resulting in a low avidity TCR (8). Another concern is whether peptide-induced CTLs generated in vitro will lyse targets endogenously expressing the antigen (9).

Novel approaches in cellular immunotherapy are BsAb that bind to TAA on a malignant cell and so-called trigger-antigens on an immune effector cell (10). BsAb can recruit the effector cell to a tumor cell that would otherwise not be recognized. This redirection of effector cells to tumor cells has the potential to bypass tumor escape by unfolding its own trigger mechanism for destructive immune response (11). After the promising preclinical results BsAb are now being tested in a number of clinical studies targeting the extracellular domain of the HER2 protein (12–14).

We have shown previously that cytotoxic CD8+ NKT cells are readily expanded in vitro in large quantities suitable for adoptive immunotherapy by mitogenic stimulation with MAb anti-CD3 against the TCR complex and IL-2. These cells have the capacity of lysing a variety of tumor cell lines, some of which are resistant to NK cells, whereas normal hematopoiesis is exempted (15–20). CD8+ NKT cells represent a subpopulation of T lymphocytes, which share functional and phenotypic properties of both cytotoxic T cells and NK cells, i.e., the ability of killing class I-negative targets in a non-MHC-restricted fashion. These activated effector cells have significant in vivo cytotoxic activity and can be used for adoptive immunotherapy with limited toxicity. Recently, the potential clinical efficacy was demonstrated in a murine model across major MHC barriers where adoptive transferred CD8+ NKT cells protected animals from an otherwise lethal tumor challenge without clinically significant graft-versus-host disease (21). However, some tumor cells have shown resistance, presumably because of lack of sufficient avidity for these targets.

In the present study, we used in vitro expanded human CD8+ NKT lymphocytes redirected with a humanized BsAb F(ab’)_2HER2xCD3 as a novel approach to cellular immunotherapy of malignant diseases. We demonstrate that CD8+ NKT cells can overcome their natural specificity for target cells by using a recombinant BsAb containing a target arm against the HER2 tumor-antigen and an effector arm against CD3. The potent antitumor efficacy of this therapeutic approach was investigated in a scid xenograft model using bioluminescent real-time imaging (22, 23). This methodology allowed us to assess the effectiveness and rapidity of tumor immunotherapy at multiple time points. In vitro studies revealed that the therapeutic effect of redirected CD8+ NKT cells was dependent on perforin as a
major pathway of cytotoxicity and could not be inhibited when pretreated with immunosuppressants CsA and FK506 or through the increase of intracellular cAMP by pretreatment with db-cAMP. Redirected cytotoxicity also remained intact after blocking adhesion molecules of effector cells (LFA-1) or of target cells (ICAM-1).

MATERIALS AND METHODS

Expansion of CD8+ NKT Cells. Human peripheral blood mononuclear cells obtained from buffy coats of healthy donors were used for the generation of CD8+ NKT cells by culturing in RPMI 1640 containing 10% FCS (HyClone, Logan, UT), penicillin 100 units/ml, streptomycin 100 mg/ml, l-glutamine 2 mM/liter, and 2-mercaptoethanol 50 uM/liter. On day 1, IFN-γ (Genentech, South San Francisco, CA) was added to a final concentration of 1000 units/ml. On day 2, IL-2 (Chiron, Inc., Emeryville, CA) was added to a final concentration of 300 units/ml and a MAb anti-CD3 (OKT-3; Ortho/Pharmacia, Raritan, NJ) at 25 ng/ml were added. Cells were maintained for 3 weeks by subculturing every 3 days with fresh medium and IL-2 (300 units/ml). Starting from a mixed population of CD4+ and CD8+ T cells, after 21 days in culture >95% of cells were CD3+, αβ TCR+ with 30–50% of cells coexpressing the adhesion molecule CD56.

Animals and Cell Lines. All of the animal experiments were performed under protocols approved by the institutional animal care and use committee at Stanford University. Female CB.17scid mice were used at the age of 6–12 weeks (Department of Comparative Medicine, Stanford, CA). Starting 1 day before tumor implantation, mice received whole body irradiation (200 cGy) to limit endogenous NK cell activity (24). The human ovarian carcinoma cell line (SK-OV-3) was obtained from American Type Culture Collection (Manassas, VA). The human B-lymphoma cell line (OCI-Ly8) was kindly provided by Ronald Levy (Stanford University).

Reporter Gene Marking of Tumor Cells. The luciferase gene from pSP-luc+ vector (Promega, Madison, WI) was cloned into an LNCK vector by using Clal and HindII restriction enzymes. Human ovarian cancer cells (SK-OV-3) expressing the HER2 antigen were cultured with 80% confluency and transfected by incubating with a complex of 3 μg DNA and 30 μl lipofection reagent (GenePorter; Gene Therapy Systems, San Diego, CA). The optimal DNA delivery was identified by adding luciferin (0.15 mg/ml; Biosynth, Naperville, IL) to the cell culture. Stable transfectants (referred to as SK-OV-3luc) were selected in geneticin (200 μg/ml; Invitrogen, Carlsbad, CA) and bioluminescence was used to screen cultures for luciferase gene expression using an ICCD camera (C2400-32; Hamamatsu Photonics, Hamamatsu City, Japan).

Antibodies. For in vitro and in vivo assays, the following fully humanized antibodies were provided by Genentech; MAb anti-p185HER2 (HuMabD5-8) against the HER2 antigen (referred to as 4D5), BsAb F(ab’2)HER2xCD3 (anti-p185HER2 Fab’; HuMabD5-8; anti-CD3 Fab’; HuMabUCHT1-9), and a control BsAb F(ab’2)CD18xCD3 (anti-CD18 Fab’; HuMabH52AA; anti-CD3 Fab’; HuMabUCHT1-9), which does not bind detectably to either human or murine CD18 or any known human or murine antigen (25–27).

Immunophenotyping of Effector and Target Cells. For flow-cytometric analysis the following human MAbs, conjugated either with fluorescein or phycoerythrin, were used: CD3, CD4, CD5, CD8, CD28, CD56, CD80, CD86, TCR-α/β, anti-HER2, and isotype-matched controls (Becton Dickinson, San Diego, CA). Three-color analysis was performed on the FACScan (Becton Dickinson, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Inc., San Carlos, CA). Effector subsets (CD5+CD56+, CD4+CD56−, CD8+CD56+) of cultured CD8+ NKT cells were sorted on a dual laser Vantage sortor (Becton Dickinson).

Cell-mediated Cytotoxicity Assay. Tumor cell lysis by effector cells was quantitated in a 4-h 31Cr-release assay (19). Target cells (1 x 10⁵) were labeled with 200 μCi sodium chromate 51 (DuPont-NEN, Boston, MA) and plated at 1 x 10⁵ cells/well. Effector cells were added at various E:T cell ratios, and after 4 h of incubation, the supernatant was removed and radioactivity measured in a gamma counter. The percent cytotoxicity was determined as follows: % cytotoxicity = ([sample release − spontaneous release]/[maximum release − spontaneous release]) x 100. Maximum release of target cells was measured following treatment with 2% detergent IGEPAL CA-630 (Sigma, St. Louis, MO). For redirected cytotoxicity assays a BsAb concentration of 0.1 μg/ml was used based on an antibody-dose titration assay, which showed maximum cytotoxic activity of CD8+ NKT cells at this concentration. For selective inhibition of perforin CD8+ NKT cells were pretreated for 2 h with increasing concentrations of CMA (0.1–1000 nm) or EGTA (0.1–2.5 mM) and then assayed for cytotoxicity. For inhibition of effector function CD8+ NKT cells were pretreated for 18 h with CsA (100 ng/ml), FK506 (2 ng/ml), or db-cAMP (2 mM; Sigma) and then assayed for cytotoxicity. In blocking experiments using antiadhesion molecule-specific MAb LFA-1 (α chain CD11a) and ICAM-1 (CD54; Pharmingen, San Diego, CA) a concentration of 1 μg/ml was used.

In Vivo Imaging of Tumor Therapy. In vivo bioluminescence imaging experiments were performed on scid mice bearing tumor cells constitutively expressing the HER2 receptor and the luciferase gene. HER2 tumor xenografts were established by i.p. injection of 1 x 10⁶ SK-OV-3Luc cells. Before in vivo imaging animals were anesthetized with pentobarbital (50 μg/kg, i.p.). An aqueous solution of the substrate luciferin (150 mg/kg; BioSynth) was then injected i.p., and mice were placed in the light-tight chamber of a low light imaging system equipped with an ICCD camera (Hamamatsu Photonics) fitted with a 50-mm F1.2 Nikkor lens (Nikon, Tokyo, Japan; 22). A gray-scale surface image was collected under weak illumination. Next, a pseudocolor image representing light intensity (blue is least and red is most intense) was generated using LivingImage software (Xenogen, Crop., Alameda, CA) as an overlay on the Igor image analysis package (WaveMetrics, Lake Oswego, OR). Gray-scale references and pseudocolor images were superimposed and the relative light intensity from the whole animal quantified using the image analysis capabilities of the LivingImage software. Tumor xenografts received two treatment courses with a total volume of 200 μl by i.p. injection at 24 and 72 h after tumor implantation. Adoptive transfer of cellular immunotherapy consisted of CD8+ NKT cells (2 x 10⁶ cells) given alone or redirected with F(ab’2)CD18xCD3 or F(ab’2)HER2xCD3 (0.1 μg/ml). In control experiments, saline or HER2-targeting antibodies [F(ab’2)HER2xCD3; MAB 4D5] were given at a dose of 0.1 mg antibody/kg bodyweight.

Statistical Analysis. For comparison between experimental groups in imaging living animals, the tumor signal measure (pixel) was used, comparing the geometric means at 28 days. ANOVA was first performed to demonstrate statistically significant differences among experimental groups at the 0.05 level. The t test was then used to analyze the difference between each experimental group and the control group, and their nominal Ps are reported. To adjust for multiple comparisons using the same control in each of the experiments, the Dunnett’s test was used (28).

For comparison between experimental groups in tumor survival the log-rank test Ps between each of the overall survival of the animals at 21 weeks were used. Ps < 0.05 were considered to be significantly different (Bonferroni Inequality).

RESULTS

Redirected Cytotoxicity of CD8+ NKT Cells. The luciferase gene was inserted into the HER2-expressing ovarian carcinoma cell line SK-OV-3. Luciferase gene transfer slightly impaired HER2 antigen expression of SK-OV-3 tumor cells. This is likely related to the selection of a subclone after transfection of the parental SK-OV-3 tumor cell line with slightly different expression of HER2 (Fig. 1). However, cytotoxic efficacy of CD8+ NKT cells in combination with F(ab’2)HER2xCD3 was not impaired when tested in chromium release assays. CD8+ NKT cells alone were found to have a high resistance against SK-OV-3 tumor targets where minimal cytotoxic activity was detectable at an E:T ratio of 100:1 (7% range: 5–10). Addition of F(ab’2)HER2xCD3 to effector cells resulted in significant enhancement of cytotoxicity of SK-OV-3 target cells between the concentration of 10–1,000 ng/ml (Fig. 2A). Addition of CD8+ NKT cells with F(ab’2)HER2xCD3 resulted in 39% (range: 9–63) specific lysis at an E:T ratio of 10:1 and 73% (range: 49–93) at an E:T ratio of 100:1 (Fig. 2B). Redirected cytotoxicity was in part dependent on stimulation of the invariant CD3 portion of the TCR complex (TCR/CD3). As a result, stimulation with the control F(ab’2)CD18xCD3 contributed to minimal nonspecific cytotoxicity.
(26%; range 17–35) at an E:T ratio of 100:1. Costimulation with MAb anti-CD28 did not augment lytic activity indicating that redirected cytotoxicity of CD8\(^+\) NKT cells is not dependent on secondary signals (data not shown). In contrast, freshly isolated peripheral blood mononuclear cells redirected with F(ab\(^\prime\))\(_2\) HER2xCD3 exhibited no significant cytotoxic activity demonstrating their dependency on costimulatory secondary signals for effector cell function (data not shown).

To determine target cell specificity of CD8\(^+\) NKT cells in the presence of F(ab\(^\prime\))\(_2\) HER2xCD3, the same experiments were carried out against a human B-lymphoma cell line, OCI-Ly8, which is known to be sensitive to CD8\(^+\) NKT cells but does not express the HER2 antigen. At different E:T ratios, there were no differences in tumor cell killing whether we used CD8\(^+\) NKT cells alone, or redirected with F(ab\(^\prime\))\(_2\) HER2xCD3 or F(ab\(^\prime\))\(_2\) CD18xCD3 (Fig. 2C).

To better define the population of cells with cytotoxic activity, CD8\(^+\) NKT cells at day 21 in culture were flow-sorted into CD5\(^+\) NKT cells at day 21 in culture were flow-sorted into CD5\(^-\)/CD4\(^+\) and CD4\(^-\)/CD8\(^+\) subsets. Purity of subsets was \(\geq 95\%\), and cytotoxicity assays were performed against SK-OV-3 target cells. In the presence of F(ab\(^\prime\))\(_2\) HER2xCD3, the highest redirected cytotoxic activity was found in the CD5\(^+\) subset of CD8\(^+\) NKT cells (48%, E:T ratio 10:1), compared with CD8\(^+\)/CD4\(^+\) and CD4\(^+\)/CD8\(^-\) T cells (36% and 11% respectively; Fig. 2D). In control experiments without the addition of F(ab\(^\prime\))\(_2\) HER2xCD3, no significant lysis of SK-OV-3 tumor cells was observed in any cellular subsets (data not shown).

Perforin Is a Major Cytotoxic Pathway of CD8\(^+\) NKT Cells. CMA (Sigma), an inhibitor of vacuolar type H\(^+\)-ATPase and EGTA, a calcium complexing agent, were used as selective inhibitors of perforin-mediated cytotoxicity (29, 30). Direct cytotoxicity of CD8\(^+\) NKT cells against OCI-Ly8 targets and redirected cytotoxicity with F(ab\(^\prime\))\(_2\) HER2xCD3 against SK-OV-3 targets were almost completely abrogated by pretreatment of effector cells with CMA at a concentration \(\geq 100 \text{ nM}\) (Fig. 3A) or with EGTA at a concentration \(\geq 1 \text{ mM}\) (Fig. 3B). Viability of effector cells was simultaneously determined with trypan-blue exclusion assays. These results demonstrate that perforin is a major cytotoxic pathway for both the direct and redirected non-MHC-restricted cytotoxicity of CD8\(^+\) NKT cells.
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Redirected Cytotoxicity Remains Intact in the Presence of Immunosuppressants or after Blocking of Adhesion Molecules. To gain more insight into gene activation and transcriptional pathways of redirected non-MHC-restricted cytotoxicity, CsA and FK506, immunophilin-binding drugs, which inhibit the calcium-dependent phosphatase activity of calcineurin, and db-cAMP, which elevates intracellular cyclic AMP, were used to inhibit immune effector function (Refs. 31–33; Fig. 4). CD8+ NKT cells without BsAb (control) had a specific lysis of 41% against OCI-Ly8 tumor cells (E:T ratio 20:1). Treatment with CsA and FK506 modestly suppressed direct cytotoxicity of CD8+ NKT cells with 32% and 29% of specific lysis, respectively. A substantial reduction of tumor cell lysis occurred after treatment with db-cAMP (7%) or by blocking the adhesion molecules LFA-1 (16%) on the effector cell and its counter receptor ICAM-1 (12%) on the target cell. In contrast, redirected cytotoxicity using F(ab′)2 HER2xCD3 against SK-OV-3 targets was consistently functional when pretreated with CsA (52%), FK506 (48%), db-cAMP (50%), or MAB against LFA-1 (45%) and ICAM-1 (48%; specific lysis of the control: 56%; E:T ratio 10:1). These results indicate that the signaling pathway of non-MHC-restricted cytotoxicity is different after cross-linking with F(ab′)2 HER2xCD3.

Visualization of Tumor Burden and Correlation with Survival Time. The in vivo cytotoxicity of CD8+ NKT cells was studied in a xenograft tumor mouse model. Genetically modified SK-OV-3 tumor cells expressing the luciferase gene were injected i.p. into scid mice, and the bioluminescent signal from labeled tumor cells was measured. Using this sensitive assay system tumor cell growth in living animals could be quantitated and localized. Light emission was detectable immediately after tumor cell injection and was followed over 28 days. Tumor xenografts received two treatment courses of adoptive transferred CD8+ NKT cells (E:T ratio 20:1) alone or redirected with F(ab′)2,CD18xCD3 or F(ab′)2,HER2xCD3 (Fig. 5A). Control experiments consisted of treatment with saline or HER2-targeting antibodies [F(ab′)2HER2xCD3; MAB 4D5] alone (Fig. 5B).
At day 28, a substantial reduction of bioluminescence was observed in animals treated with CD8+ NKT cells redirected with F(ab')2 HER2xCD3 (median 1.93 log-units). Significant tumor regression occurred as early as 48 h after initiation of therapy compared with the saline treatment group (P < 0.0001). In vivo, treatment with CD8+ NKT cells alone or after nonspecific stimulation with the control F(ab')2 CD18xCD3 resulted in a modest reduction of light intensity but only within the first 96 h (median at day 28: 0.38 and 0.83 log-units, respectively). The application of F(ab')2 HER2xCD3 alone had no effect on tumor proliferation, and light intensity increased over time accordingly, similar to the saline treatment group (P = 0.09). Targeting HER2-expressing tumors with MAb 4D5 resulted in a gradual and significant reduction of light intensity over time compared with the control group treated with saline (day 28: 1.22 log-units, P < 0.0001). A statistical trend toward reduction in tumor burden was observed at day 28 between the MAb 4D5-treated animals and those treated with CD8+ NKT cells redirected with F(ab')2 HER2xCD3, where tumor signals were less intense (P = 0.06).

Long-term survival of tumor xenografts correlated inversely to the light intensity measured. Animals treated with CD8+ NKT cells redirected with F(ab')2 HER2xCD3 were found to have both the highest survival rate at week 21 (Fig. 6A) and the lowest light intensity at day 28 (Fig. 5A; P = 0.001). Control animals treated with saline or F(ab')2 HER2xCD3 alone died within 12 weeks and 16 weeks, respectively, and showed highest light intensity at day 28 (Fig. 5B; Fig. 6B).

A statistical trend was found in the overall survival at week 21 between the animals treated with CD8+ NKT cells in combination with F(ab')2 HER2xCD3 (70% survival) and those treated with MAb 4D5 alone (30% survival; P = 0.11).

**DISCUSSION**

Antibody-based therapies against growth factor receptors on cancer cells have been very successful, and HER2 represents an important candidate target for studying antitumor immunity because it is preferentially expressed on malignant cells. HER2-directed therapy with MAbs has resulted in enhanced survival; however, the majority of patients eventually relapse (5). Therefore, more effective therapeutic strategies are clearly needed. To study the biological activity of HER2-directed cellular therapies we performed sensitive and quantitative *in vitro* assays and developed an *in vivo* assay using the light imaging properties of the luciferase gene transfected into HER2-expressing ovarian carcinoma cells. With these assays we demonstrated rapid regression of tumor growth with *in vitro* generated CD8+ NKT cells redirected with F(ab')2 HER2xCD3.

Because immunotherapy with BsAb can augment the cytotoxic potentials of many effector cells, investigators have redirected cells of the innate immune system to FCγ-receptors (e.g., NK cells, monocytes, macrophages, and granulocytes) and T-lymphocytes. Such attempts to redirect FCγ-receptor-expressing immune cells against HER2 are currently being evaluated in clinical trials using systemically administered BsAb targeting CD16 (BsAb 2B1) or CD64 (BsAb MDX-H210) on effector cells (12, 13, 34–36). TCR/CD3-directed BsAb have the ability to directly trigger cytotoxic activity of effector cells (37). However, resting T cells need induction signals to transform to the effector phase of tumor cell killing (38, 39). Because the CD3 antigen is a key molecule for activating human or murine T cells from a variety of tissue sources that the majority of redirected cytotoxicity is mediated by the CD3+ T cells had almost none. Furthermore, the biological activity of CD8+ NKT cells...
was found to be dependent on cytotoxic molecules such as perforin as demonstrated by accelerated degradation of their lytic granules after treatment with CMA or by inhibition of Ca\(^{2+}\)-dependent granule exocytosis after treatment with EGTA.

To better understand the molecular signaling pathway by which CD8+ NKT cells mediate redirected cytotoxicity, specific immune inhibition experiments were performed. As shown previously, the immunosuppressants CsA or FK506 can modestly suppress the direct cytotoxicity of CD8+ NKT cells against the lymphoma cell line OCI-Ly8 (43). Moreover, in similar experiments we found a strong inhibition after treatment of CD8+ NKT cells with db-cAMP or by blocking cellular adhesion molecules (LFA-1/ICAM-1). In contrast, when CD8+ NKT cells were redirected with F(ab\(^\prime\))\(_2\)HER2xCD3, neither the immunosuppressants, db-cAMP, nor the MAbs against LFA-1 or ICAM-1 adhesion molecules were able to significantly inhibit their immune effector function. The scientific basis of this observation is yet unclear, but one might propose that different signal transduction pathways can be used by non-MHC-restricted lymphocytes. We suggest that a classical pathway is sensitive to recognition of adhesion molecules (LFA-1/ICAM-1) and is dependent on calcineurin function, whereas alternative pathways can be recruited through artificial nonspecific stimulation of the TCR/CD3 antigen.

The latter we believe to be important for non-MHC-restricted cellular immunotherapy with CD8+ NKT cells because this pathway was able to overcome tumor cell resistance as demonstrated by experiments in which effector cells were cross-linked with F(ab\(^\prime\))\(_2\)HER2xCD3 to tumor cells. This is a novel observation, and it can be hypothesized that non-MHC-restricted cellular immunotherapy in combination with BsAb is more effective than tumor-specific CTL against HER2-overexpressing tumors with respect to tumor escape mechanisms, i.e., induction of T-cell anergy.

For a long time animal studies have been hampered by the difficulty of monitoring tumor growth or immunotherapeutic interventions in living animals. To better visualize the kinetics of tumor-cell clearance in response to adoptive transferred CD8+ NKT cells in xenografts we used bioluminescent real-time imaging. The constitutively expressed reporter gene encoding the firefly photoprotein luciferase was introduced into the chromosomes of HER2-expressing tumor cells. The photons of light were transmitted through tissue and detected with a photon counting camera system. Using this technology we performed comparative analysis of antibody-based HER2 directed therapies in living animals. A dramatic response could be observed as early as 48 h after therapy with CD8+ NKT cells redirected with BsAb F(ab\(^\prime\))\(_2\)HER2xCD3. Ninety-six h after initiation of adoptive immunotherapy, 50% of the animals receiving two therapy cycles with CD8+ NKT cells redirected with F(ab\(^\prime\))\(_2\)HER2xCD3 showed a complete resolution of tumor signal (80% at day 28). This rapid response to immunotherapy has, of yet, never been demonstrated and indicates the power of this treatment strategy. HER2-directed therapy with MAb 4D5 led to a gradual but slower decline of tumor signal over several logs with 50% of animals showing complete resolution at day 28. The application of F(ab\(^\prime\))\(_2\)HER2xCD3 alone showed no response, which might be the combined result of lack of functional T lymphocytes in scid mice, low antibody concentration at the tumor site, and a weak tumor penetration ability of the BsAb itself. Survival could be predicted by noninvasive imaging of tumor-bearing animals at several time points. Animals treated with redirected CD8+ NKT cells showed prolonged survival compared with other treated groups of animals including those treated with saline or effector cells without F(ab\(^\prime\))\(_2\)HER2xCD3. Additional studies are in progress to evaluate effector cell survival and trafficking to tumor sites by introducing the luciferase gene into the CD8+ NKT cells, and visualizing animals in the presence and absence of unmarked tumor cells with and without BsAb.

In summary, our proposed immunotherapeutic approach using CD8+ NKT cells redirected with F(ab\(^\prime\))\(_2\)HER2xCD3 resulted in rapid, and in most instances sustained, eradication of HER2-expressing tumor cells in a scid mouse model. This combination compared favorably to HER2-directed treatment with MAb 4D5 with respect to the rapidity of tumor response and overall survival. Our protocol for ex vivo expansion of CD8+ NKT cells results in the generation of large numbers of cytotoxic effector cells, which appear not to mediate graft-versus-host disease, and show in vivo activity without the additional administration of IL-2. This holds promise for cellular immunotherapies in combination with BsAbs against a variety of neoplastic diseases. Rapid tumor targeting with highly efficient tumor clearance is particularly important for the treatment of small numbers of neoplastic cells present in minimal disease states. Therefore, an immunotherapeutic approach such as that proposed here might prove beneficial in the context of a peritransplant setting or in combination with conventional chemotherapies. Finally, the in vivo detection of bioluminescent reporter genes, as shown in this study, visualizes the dynamics of immunotherapeutic interventions to an extent not possible previously.

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