T Lymphocytes Expressing a CD16 Signaling Receptor Exert Antibody-Dependent Cancer Killing

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Abstract
To expand applications for T-cell–based immunotherapy in cancer, we designed a receptor that binds the Fc portion of human immunoglobulins and delivers activation signals. The construct included the high-affinity CD16 (FCGR3A) V158 variant, CD8ε hinge, and transmembrane domains, along with signaling domains from CD3ζ and 4-IBB (TNFRSF9), forming a chimeric receptor termed CD16V-BB-ζ. After retrovirus-mediated expression in human T cells, CD16V-BB-ζ bound humanized antibodies with higher affinity than a control receptor containing the more common F158 variant. Engagement of CD16V-BB-ζ provoked T-cell activation, exocytosis of lytic granules, and sustained proliferation, with a mean cell recovery after 4-week coculture with Daudi lymphoma cells and rituximab of nearly 70-fold relative to input cells. In contrast, unbound antibody alone produced no effect. CD16V-BB-ζ T cells specifically killed lymphoma cells and primary chronic lymphocytic leukemia cells in combination with rituximab at a low effector:target ratio, even when assayed on mesenchymal cells. Trastuzumab triggered CD16V-BB-ζ–mediated killing of HER2 (ERBB2)+ breast and gastric cancer cells; similar results were obtained with an anti-GD2 antibody in neuroblastoma and osteosarcoma cells. Furthermore, coadministration of CD16V-BB-ζ T cells with immunotherapeutic antibodies exerted considerable antitumor effects in vivo. Signaling mediated by 4-IBB-CD3ζ induced higher T-cell activation, proliferation, and cytotoxicity than CD3ζ or FcRγII, and the receptor was expressed effectively after mRNA electroporation without viral vectors, facilitating clinical translation. Our results offer preclinical proof of concept for CD16V-BB-ζ as a universal, next-generation chimeric receptor with the potential to augment the efficacy of antibody therapies for cancer.

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Introduction
Immunotherapy is a promising option for cancer treatment because of its potential to evade genetic and cellular mechanisms of drug resistance, and to target tumor cells while sparing normal tissues. T lymphocytes can exert major antitumor effects, as demonstrated by results of allogeneic hematopoietic stem cell transplantation for hematologic malignancies, where T-cell–mediated graft-versus-host disease is inversely associated with disease recurrence, and immunosuppression withdrawal or infusion of donor lymphocytes can contain relapse (1–5). The expression of chimeric signaling receptors with antibody recognition properties can skew the reactivity of T lymphocytes towards cancer cells: ligation of the cognate target results in T-cell activation and triggers cytotoxicity (6–10). Recent results of clinical trials with infusions of chimeric receptor-expressing autologous T lymphocytes provided compelling evidence of their clinical potential (11–16).

Another approach to cancer immunotherapy is the administration of monoclonal antibodies, which can exert cytotoxicity through a variety of mechanisms, including induction of proapoptotic signals, complement fixation, and antibody-dependent cell cytotoxicity (ADCC; refs. 17–22). A major role is played by the latter mechanism, which results from the engagement of Fc receptors (FcγR) expressed on the surface of natural killer (NK) cells and other cells, such as neutrophils and macrophages, by the Fc portion of the antibody (23). Polymorphisms of FcγR genes can have marked functional consequences, which, in turn, influence response to antibody treatment. To this end, allotypes of the gene coding FcγRIIIa (FCRG3A or CD16), expressed by NK cells, can result in receptors having either a phenylalanine (F) or a valine (V) residue at position 158; CD16 V158, which occurs in a minority of individuals, has higher Fc binding and is associated with increased tumor cell killing and superior responses in patients (18, 24–32).

We sought to combine the anticancer potential of T-cell- and antibody therapy. T lymphocytes expressing ωζ T-cell receptors (the vast majority of T cells) lack activating FcγR and do...
not mediate ADCC (23). We reasoned that expression of a chimeric receptor composed of FcR and T-cell-signaling molecules should confer ADCC capability to these cells and, if so, they should significantly augment the antitumor potential of monoclonal antibodies, regardless of the targeted tumor antigen. We here describe the antibody-guided antitumor activity of T lymphocytes expressing such receptor.

Materials and Methods

Cells
The human cell lines Daudi and Ramos (B-cell lymphoma), Jurkat (T-cell acute lymphoblastic leukemia), and CHLA-255, NB1691 and SK-N-SH (neuroblastoma) were available at St. Jude Children's Research Hospital (Memphis, TN). MCF-7 and SK-BR-3 (breast carcinoma), and U2-OS (osteosarcoma) were obtained from the American Type Culture Collection; MKN7 (gastric carcinoma) was from the National Institute of Biomedical Innovation (Osaka, Japan). Daudi, CHLA-255, NB1691, SK-N-SH, SK-BR-3, MCF-7, U2-OS, and MKN7 were also transduced with a murine stem cell virus (MSCV)-internal ribosome entry site (IRE)-green fluorescent protein (GFP) retroviral vector containing the firefly luciferase gene (33) and selected for GFP expression with a FACSaria (BD Biosciences) or a MoFlo cell sorter (Beckman Coulter). Cell lines were characterized by the providers for molecular and/or gene expression features; the cell marker profile of leukemia and lymphoma cell lines was periodically tested by flow cytometry to ensure that no changes had occurred. Cell lines were expanded after receipt, cryopreserved and cells for experiments were obtained from recently thawed vials. Peripheral blood or bone marrow samples from newly diagnosed and untreated patients with B-chronic lymphocytic leukemia (CLL) were obtained following informed consent and approval from the Domain Specific Ethics Board governing Singapore's National University Hospital.

Peripheral blood samples were obtained from deidentified by-products of platelet donations from adult donors. Mononuclear cells were enriched by centrifugation on Accu-Prep Human Lymphocytes Cell Separation Media (Accurate Chemical & Scientific Corp.), and cultured with anti-CD3/CD28 beads (Invitrogen) in RPMI-1640 with 10% FBS, antibiotics, 100 IU/mL interleukin (IL)-2 (Roche) for 3 days. On day 4, T cells were purified by negative selection with a mixture of CD14, CD16, CD19, CD36, CD56, CD123, and CD235a antibodies and magnetic beads (Pan T Cell Isolation Kit II; Miltenyi Biotec; purity, >98%). Purified T cells were maintained in the above medium, with the addition of 100 IU/mL IL-2 every other day.

Plasmids, virus production, and gene transduction
The pMSCV-ires-GFP, pEQ-PAM3(-E), and pRDF were obtained from the St. Jude Children’s Research Hospital Vector Development and Production Shared Resource (10). The FCGR3A (CD16) and FCER1G cDNA were obtained from Origene; the FCGR3A V158 variant was generated using site-directed mutagenesis by PCR. The CD8ζ hinge and transmembrane domain, and the intracellular domains of 4-1BB and CD3ζ were subcloned from an anti-CD19/4BB-CD3ζ cDNA previously made in our laboratory (10). These molecules were assembled in various combinations (Supplementary Fig. S1), using splicing by overlapping extension by PCR (SOE-PCR). The constructs and expression cassette were subcloned into EcoRI and MluI sites of the MSCV-IRES-GFP vector.

Preparation of retroviral supernatant and transduction were performed as previously described (10, 34). Briefly, T cells were incubated with RD114-pseudotyped retroviral supernatant in the presence of RetroNectin (Takara) at 37°C for 24 hours and then maintained in RPMI-1640 with FBS, antibiotics, and 100 IU/mL IL-2 until the time of the experiments, 7 to 21 days after transduction.

Surface expression of CD16 was analyzed by flow cytometry using R-phycocerythrin (PE)-conjugated anti-human CD16 (clones B73.1 or 3G8, BD Biosciences). Western blotting was performed as previously described (34), using a mouse anti-human CD3ζ (clone SD3; BD Biosciences) and then with a goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). Antibody binding was revealed by using the Amersham ECL Prime detection reagent (GE Healthcare).

mRNA electroporation
The pVAX1 vector (Invitrogen) was used as a template for in vitro mRNA transcription. The CD16V-BB-ζ cDNA was subcloned into EcoRI and XbaI sites of pVAX1. The corresponding mRNA was transcribed in vitro with T7 mScript mRNA production system (CellScript; ref. 35). For electroporation, we used the Amaxa Nucleofector (Lonza); 1 × 10⁶ of purified T cells activated with 200 IU/mL IL-2 overnight were mixed with 200 μg/mL mRNA in Cell Line Nucleofector Kit V, transferred into the processing chamber, and transected using the program X-001. Immediately after electroporation, cells were transferred from the processing chamber into a 24-well plate and then cultured in RPMI-1640 with 10% FBS, antibiotics, and 100 IU/mL IL-2.

Antibody binding, cell activation, conjugation, and cell proliferation assays
To measure the chimeric receptors’ antibody-binding capacity, T lymphocytes (5 × 10⁵) transduced with chimeric receptors or a vector containing GFP only were incubated with rituximab (Rituxan, Roche; 0.1–1 μg/mL), trastuzumab (Herceptin; Roche; 0.1–1 μg/mL) or purified human IgG (R&D Systems; 0.1–1 μg/mL) for 30 minutes at 4°C. After washing twice with PBS, cells were incubated with goat anti-human IgG conjugated to PE (Southern Biotechnology Associates, Birmingham, AL) for 10 minutes at room temperature and cell staining was measured using an Accuri C6 flow cytometer (BD Biosciences).

To prepare immobilized antibody, we added 100 μL of antibody (1 μg/mL) to the wells of a 96-well plate (Costar) that was then kept at 4°C for 16 hours; the plate was washed once with PBS before use. Expression of IL-2 receptors was measured by staining with anti-CD25 PE (BD Biosciences). To determine whether antibody binding to the receptor promoted cell aggregation, CD20 (MS4A1)-positive Daudi cells were labeled with CellTrace calcein red-orange AM (Invitrogen) and then incubated with rituximab (0.1 μg/mL) for 30 minutes at
4°C. After washing twice in PBS, Daudi cells were mixed with Jurkat cells transduced with the chimeric receptor or mock-transduced at 1:1 E:T ratio in 96 round bottom plates (Costar) for 60 minutes at 37°C. The proportion of cells forming heterologous cell aggregates (calcein AM-GFP double positive) was determined by flow cytometry. To measure cell proliferation, 1 × 10^6 of T cells transduced with the chimeric receptor or mock-transduced were placed in the wells of a 6-well plate (Costar) in RPMI-1640 with FBS, antibiotics and 50 IU/mL IL-2. Daudi, SK-BR-3, or NB1691 cells were treated with Streck cell preservative (Streck Laboratories, Memphis, TN; ref. 36), respectively (all at 0.1 or hu14.18K322A (gift of Dr. James Allay, Children’s GMP, were treated with Streck cell preservative (Streck Laboratories) to stop proliferation and labeled with rituximab, trastuzumab, or hu14.18K322A (gift of Dr. James Allay, Children’s GMP, Memphis, TN; ref. 36), respectively (all at 0.1 μg/mL), for 30 minutes at 4°C. They were added to the wells, at 1:1 ratio with T cells, on days 0, 7, 14, and 21. Then, the number of viable T cells after culture was measured by flow cytometry.

**CD107a degranulation and cytotoxicity assays**

To determine whether CD16 cross-linking caused exocytosis of lytic granules, we placed chimeric receptor- and mock-transduced T cells (1 × 10^5) into each well of a rituximab-coated 96-well flat bottom plate and cultured for 4 hours at 37°C. In some experiments, T cells were cocultured with Daudi cells preincubated with rituximab. Anti-human CD107a PE antibody (BD Biosciences) was added at the beginning of the cultures; one hour later GolgiStop (0.15 g/mL), and plated into 96-well round bottom plates (Costar). T cells were added at various E:T ratios as described (10); numbers of viable CLL cells after culture were determined by flow cytometry.

**Xenograft experiments**

Daudi cells expressing luciferase were injected intraperitoneally (i.p.: 0.3 × 10^6 cells/mouse) in NOD.Cg-Prkdcsdef IL2rgtm1Wjl/Sj (NOD/scid IL2RGnull) mice (Jackson Laboratories). Some mice received rituximab (150 μg) i.p. Four days after Daudi inoculation, with or without intraperitoneal injection of T cells on days 5 and 6. T cells had been activated with anti-CD3/CD28 beads for 3 days, transduced with the CD16-V-BB-ζ receptor, resuspended in RPMI-1640 plus 10% FBS and then injected at 1 × 10^6 cells per mouse. Rituximab injection was repeated weekly for 4 weeks, with no further T lymphocyte injection. A group of mice received tissue culture medium instead of rituximab or T cells. All mice received intraperitoneal injections of 1,000–2,000 IU of IL-2 twice a week for 4 weeks. Similar experiments were also performed with mice engrafted with NB1691 cells (i.p.: 0.3 × 10^6 cells/mouse) and treated with hu14.18K322A (25 μg).

Tumor engraftment and growth was measured using a Xenogen IVIS-200 system (Caliper Life Sciences). Imaging commenced 5 minutes after intraperitoneal injection of an aqueous solution of D-luciferin potassium salt (3 mg/mouse) and photons emitted from luciferase-expressing cells were quantified using the Living Image 3.0 software.

**Results**

**Expression of the CD16V-BB-ζ receptor**

We generated a V158 variant of the FCGR3A (CD16) gene and combined it with the hinge and transmembrane domain of CD8ζ, the T-cell stimulatory molecule CD3ζ, and the costimulatory molecule 4-1BB (Fig. 1A), which we previously found to significantly enhance chimeric receptor-mediated T-cell activation (10). We used an MCSV retroviral vector containing the CD16V-BB-ζ construct and GFP to transduce peripheral blood T lymphocytes from 12 donors: median GFP expression in CD3+ cells was 89.9% (range, 75.3–97.1%); in the same cells, median chimeric receptor surface expression as assessed by anti-CD16 staining was 83.0% (67.5–91.8%; Fig. 1B). T lymphocytes from the same donors transduced with a vector containing only GFP had a median GFP expression of 90.3% (67.8–98.7%) but only 1.0% (0.1–2.7%) expressed CD16 (Fig. 1B). Expression of the receptor did not differ significantly between CD4+ and CD8+ T cells: 69.8 ± 10.8% CD4+ cells were CD16+ after transduction with CD16V-BB-ζ, as compared with 77.6% ± 9.2% CD8+ cells (Supplementary Fig. S2).

CD16V-BB-ζ—transduced T lymphocytes expressed CD3ζ at levels much higher than those expressed by mock-transduced cells: mean (±SD) of the mean fluorescence intensity was 45,985 ± 16,365 in the former versus 12,547 ± 4,296 in the latter (P = 0.027 by t test; n = 3; Fig. 1B). We also determined the presence of the chimeric protein by Western blotting probed with an anti-CD3ζ antibody. CD16V-BB-ζ—transduced T lymphocytes expressed a chimeric protein of approximately 25 kDa under reducing conditions, in addition to the endogenous CD3ζ of 16 kDa; under nonreducing conditions, the CD16V-BB-ζ
protein was shown to be expressed as either a monomer or a dimer of 50 kDa (Fig. 1C).

**Antibody-binding capacity of V158 versus F158 CD16 receptors**

To test the capacity of the CD16V-BB-ζ chimeric receptor to bind immunoglobulin (Ig), we transduced peripheral blood T lymphocytes from 3 donors: CD16V-BB-ζ-expressing T lymphocytes were coated with the antibody after incubation with rituximab (Fig. 2A). Similar results were obtained with trastuzumab and human IgG (not shown).

We then compared the Ig-binding capacity of the CD16V-BB-ζ receptor, which contained the high-affinity V158 polymorphism of FCRG3A (CD16), to that of an identical receptor containing the F158 variant instead ("CD16F-BB-ζ"). After transducing Jurkat cells with either receptor, we incubated them with rituximab and an anti-human Ig PE antibody (binding rituximab) and related the PE fluorescence intensity to that of GFP. As shown in Fig. 2B, at any given level of GFP, cells transduced with the CD16V-BB-ζ receptor had a higher PE fluorescence intensity than those of cells transduced with the CD16F-BB-ζ receptor, indicating that the former had a significantly higher antibody-binding affinity. Trastuzumab and human IgG were also bound by CD16V-BB-ζ receptors with a higher affinity (Supplementary Fig. S3).

To determine whether antibody binding to the CD16V-BB-ζ receptor could promote aggregation of effector and target cells, we mixed Jurkat cells expressing CD16V-BB-ζ (and GFP) at a 1:1 ratio with the CD20⁺ Daudi cell line (labeled with Calcein AM red-orange) for 60 minutes, and measured the formation of GFP-Calcein doublets with or without addition of rituximab. In 3 experiments, 39.0% ± 1.9% of events in the coculture were doublets if Jurkat cells expressed CD16V-BB-ζ receptors and rituximab was present (Fig. 2C and D). In contrast, there were less than 5% doublets with human IgG instead of rituximab, or with mock-transduced Jurkat cells regardless of whether rituximab was present.

**Binding of Ig to CD16V-BB-ζ induces T-cell activation, degranulation, and cell proliferation**

We assessed whether CD16V-BB-ζ receptor cross-linking by an immobilized antibody could induce activation signals in T lymphocytes. Indeed, T lymphocytes transduced with CD16V-BB-ζ markedly increased IL-2 receptor expression (CD25) when cultured on plates coated with rituximab, whereas no changes were detected in the absence of antibody, or in mock-transduced cells regardless of whether the antibody was present (Fig. 3A and B).

In addition to expression of IL-2 receptors, CD16V-BB-ζ receptor cross-linking triggered exocytosis of lytic granules in T lymphocytes, as detected by CD107a staining. Thus, in 6 experiments in which T lymphocytes from 4 donors were either seeded onto microtiter plates coated with rituximab (n = 3) or cocultured with Daudi cells in the presence of rituximab (n = 3), T lymphocytes expressing CD16V-BB-ζ became CD107a⁺ (Fig. 3C).

T lymphocytes expressing CD16V-BB-ζ expanded in the presence of rituximab and Daudi cells (at a 1:1 ratio with T lymphocytes): in 3 experiments, mean T-cell recovery after 7 days of culture was 632% (±97%) of input cells; after 4 weeks of culture, it was 6,877% (±1,399%; Fig. 3D). Of note, unbound rituximab, even at a very high concentration (1–10 mg/mL), had no significant effect on cell proliferation in the absence of target cells, and no cell growth occurred without rituximab, or in mock-transduced T cells regardless of the presence of the
antibody and/or target cells (Fig. 3D). Thus, CD16V-BB-ζ receptor cross-linking induces signals that result in sustained proliferation.

**T lymphocytes expressing CD16V-BB-ζ mediate ADCC in vitro and in vivo**

The observation that CD16V-BB-ζ cross-linking provoked exocytosis of lytic granules implied that CD16V-BB-ζ T lymphocytes should be capable of killing target cells in the presence of specific antibodies. Indeed, in 4-hour in vitro cytotoxicity assays, CD16V-BB-ζ T lymphocytes were highly cytotoxic against the B-cell lymphoma cell lines Daudi and Ramos in the presence of rituximab: more than 50% target cells were typically lysed after 4 hours of coculture at a 2:1 E:T ratio (Fig. 4A and Supplementary Fig. S4). In contrast, target cell killing was low in the absence of the antibody or with mock-transduced T cells (Fig. 4A and Supplementary Fig. S4). Notably, the effector cells used in these experiments were highly enriched with CD3+ CD56+ NK cells. Rituximab-mediated cytotoxicity of CD16V-BB-ζ T lymphocytes was also clear with CD20+ primary CLL cells (n = 5); cytotoxicity typically exceeded 70% after 4 hours of coculture at a 2:1 E:T ratio (Fig. 4B). Bone marrow mesenchymal stromal cells have been shown to exert immunosuppressive effects (38, 39). To test whether this would affect the cytotoxic capacity of CD16V-BB-ζ T lymphocytes, we cocultured them with CLL cells in the presence of bone marrow-derived mesenchymal stromal cells for 24 hours at a 1:2 E:T. As shown in Fig. 4C, mesenchymal cells did not diminish the killing capacity of the ADCC-mediating lymphocytes.

Next, we determined whether different immunotherapeutic antibodies could trigger similar cytotoxicities against tumor cells expressing the corresponding antigen. Thus, we tested the cytotoxicity of CD16V-BB-ζ T lymphocytes against solid tumor cells expressing HER2 (the breast cancer cell lines MCF-7 and SK-BR-3 and the gastric cancer cell line MKN7) or GD2 (the neuroblastoma cell lines CHLA-255, NB1691 and SK-N-SH, and the osteosarcoma cell line U2-OS). We used the antibodies trastuzumab to target HER2 and hu14.18K322A to target GD2. CD16V-BB-ζ T lymphocytes were highly cytotoxic against these cells in the presence of the corresponding antibody (Fig. 4A and Supplementary Fig. S4). In experiments with NB1691, we also tested whether cytotoxicity could be achieved at even lower E:T ratios by prolonging the culture to 24 hours. Cytotoxicity exceeded 50% at 1:8 ratio in the presence of hu14.18K322A (Supplementary Fig. S5A). To further test the specificity of the CD16V-BB-ζ–mediated cell killing, we cultured the CD20+ Daudi cells with CD16V-BB-ζ T lymphocytes and antibodies of different specificity: only rituximab mediated cytotoxicity, while there was no increase in cytotoxicity in the presence of trastuzumab or hu14.18K322A (Supplementary Fig. S5B). Importantly, CD16V-BB-ζ-mediated cell killing

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**Figure 2. Antibody-binding capacity of CD16V-BB-ζ receptors.** A, T lymphocytes transduced with a vector containing GFP (Mock) or GFP and CD16V-BB-ζ were incubated with rituximab for 30 minutes; the amount of antibody bound was visualized with a goat-anti human IgG antibody conjugated to phycoerythrin (GAH IgG) and flow cytometry. B, Jurkat cells transduced with CD16V-BB-ζ (V158) or CD16F-BB-ζ (F158) were incubated with rituximab for 30 minutes. The plot compares the relation between mean fluorescence intensity (MFI) of GFP and MFI of GAH IgG obtained with cells expressing the two receptors. C, mock- or CD16V-BB-ζ–transduced Jurkat cells were cocultured with Daudi cells labeled with calcein AM orange-red in the presence of rituximab. Cell aggregates are quantified in the top right quadrants of each dot plot. D, summary of the aggregation assays illustrated in C (mean ± SD of 3 experiments). Aggregation measured with Jurkat cells transduced with CD16V-BB-ζ in the presence of rituximab (Ab) was significantly higher than that measured in the three other culture conditions (P < 0.001 by t test).
with immunotherapeutic antibodies was not inhibited by the presence of unbound monomeric IgG, even if this was present at a concentration 1,000 times higher than that of the cell-bound immunotherapeutic antibody (Supplementary Fig. S5C).

To gauge the antitumor capacity of CD16V-BB-ζ T lymphocytes in vivo, we performed experiments with NOD/scid IL2RGnull mice engrafted with luciferase-labeled Daudi cells. We measured tumor growth by live imaging in mice receiving CD16V-BB-ζ T lymphocytes plus rituximab, and compared their outcome to mice receiving either rituximab or T lymphocytes alone, or no treatment. Tumor cells expanded in all mice except those that received rituximab followed by CD16V-BB-ζ T lymphocytes (Fig. 5). All 5 mice treated with this combination were still in remission more than 120 days after tumor injection, in contrast to 0 of 12 mice that were untreated or received antibody or cells alone. A strong antitumor activity was also observed in mice engrafted with the neuroblastoma cell line NB1691 and treated with hu14.18K322A and CD16V-BB-ζ T lymphocytes (Supplementary Fig. S6).

Comparison of CD16V-BB-ζ with other receptors

In line with their higher affinity for Ig, CD16V-BB-ζ receptors induced significantly higher T cell proliferation and ADCC than that triggered by the lower affinity CD16F-BB-ζ receptors (Supplementary Fig. S7).

Next, we compared the function of T cells bearing CD16V-BB-ζ with that of T cells expressing other receptors with different signaling properties. These included a receptor with no signaling capacity (CD16V-truncated), one with CD3ζ but no 4-1BB (CD16V-ζ), and a previously described receptor that combined CD16V with the transmembrane and cytoplasmic domains of FcεRIg (CD16V-FcεRIg; ref. 40; Supplementary Fig. S1). After retroviral transduction in activated T cells, all receptors were highly expressed (Supplementary Fig. S8). CD16V-BB-ζ induced significantly higher activation, proliferation, and specific cytotoxicity than all other constructs (Fig. 6).

Finally, we determined how ADCC exerted by T cells expressing CD16V-BB-ζ compared with the cytotoxicity of T cells expressing a chimeric antigen receptor. For this purpose, we transduced T cells with an anti-CD19 chimeric receptor ("anti-CD19-BB-ζ") composed by an anti-CD19 ScFv and transmembrane and signaling domains identical to those of CD16V-BB-ζ (10). T cells expressing this receptor were tested against Daudi cells (which express high levels of CD19 in addition to CD20) in parallel to T cells expressing CD16V-BB-ζ in the presence of rituximab (Ab) than in the other conditions (P < 0.0001). D, mock- or CD16V-BB-ζ-transduced T lymphocytes were cultured alone, or with rituximab with or without Daudi cells for up to 4 weeks. Symbols indicate percentage of cell recovery as compared with the number of input cells (mean ± SD of experiments with T cells from three donors).
Expression of CD16V-BB-ζ receptors by mRNA electroporation

In all the above experiments, CD16V-BB-ζ expression was enforced by retroviral transduction. We tested whether an alternative method, electroporation of mRNA, could also confer ADCC capacity to T lymphocytes. We electroporated activated T lymphocytes from 2 donors and obtained high expression efficiencies: 55% and 82% of T lymphocytes became CD16+ 24 hours after electroporation (Fig. 7A). In the second donor, receptor expression was also tested on day 3, when it was 43%, a result similar to those of previous experiments with another receptor where expression persisted for 72 to 96 hours (35). ADCC was activated in T lymphocytes electroporated with CD16V-BB-ζ mRNA: in the presence of rituximab, 80% Ramos cells were killed after 4 hours at a 2:1 E:T ratio, whereas cells electroporated without mRNAs were ineffective (Fig. 7B).

Discussion

We developed a chimeric receptor that endows T lymphocytes with the capacity to exert ADCC. When the CD16V-BB-ζ receptor is engaged by an antibody bound to tumor cells, it triggers T-cell activation, sustained proliferation, and specific cytotoxicity against cancer cells targeted by antibody. CD16V-BB-ζ T lymphocytes were highly cytotoxic against a wide range of tumor cell types, including B-cell lymphoma, breast and gastric cancer, neuroblastoma and osteosarcoma, as well as primary CLL cells. Cytotoxicity was entirely dependent on the presence of a specific antibody bound to target cells; unbound antibodies did not provoke nonspecific cytotoxicity nor affected cytotoxicity with cell-bound antibodies. CD16V-BB-ζ T cells also killed CLL cells when these were cultured on mesenchymal cell layers, regardless of the known immunosuppressive effects of this microenvironment (38, 39). Moreover, CD16V-BB-ζ T lymphocytes infused after rituximab eradicated B-cell lymphoma cells engrafted in immunodeficient mice, and had considerable antitumor activity in mice engrafted with neuroblastoma cells in the presence of an anti-GD2 antibody. In sum, T cells expressing CD16V-BB-ζ effected strong ADCC in vitro and in vivo.

The affinity of CD16 for the Fc portion of Ig is a critical determinant of ADCC and, thus, influences clinical responses.
to antibody immunotherapy. Hence, considerable efforts are being made to further enhance the affinity of Fc fragments for FcγR, for example, by glycoengineering (23, 41). To construct our receptor, we selected the FCRG3A (CD16) gene with the 158V polymorphism. This variant encodes a receptor with higher binding affinity for Ig and has been shown to mediate superior ADCC (18, 24–32). Indeed, in side-to-side comparisons with a chimeric receptor containing the more common F158 variant, the CD16V-BB-ζ had a significantly higher capacity to bind human Ig Fc, and induced more vigorous proliferation and cytotoxicity, evoking results of recent studies addressing the role of affinity in chimeric antigen receptor function (42, 43). Clemenceau and colleagues described a receptor containing the CD16 158V combined with the transmembrane and cytoplasmic domains of FcεRIγ, and found that selected T-cell clones expressing this receptor could lyse Epstein–Barr virus derived B-lymphoblastoid cells in the presence of rituximab (40). Current “second generation” chimeric receptors combine a stimulatory molecule with a costimulatory one to augment signaling and prevent activation-induced apoptosis. Therefore, we combined CD16 V158 with a stimulatory molecular tandem constituted by CD3ζ and 4-1BB (CD137). We previously found the addition of the costimulatory molecule 4-1BB to CD3ζ significantly improved the expansion and cytotoxicity of T lymphocytes when incorporated into a chimeric receptor directed against CD19 (10). This was also the case with the CD16V-BB-ζ receptor, which induced a markedly superior T-cell activation, proliferation, and cytotoxicity than did receptors acting through CD3ζ alone, or of FcεRIγ. Of note, in side-by-side cytotoxicity assays targeting cells expressing both CD19 and CD20, CD16V-BB-ζ plus rituximab was more effective than a receptor with identical signaling properties but binding directly to CD19.

The clinical potential of genetically modified T cells expressing receptors that recognize antigens of the surface of tumor cells and can transduce stimulatory signals is being increasingly demonstrated by results of clinical trials (11–16). Most notably, significant tumor reductions and/or complete remissions have been reported in patients with B-cell malignancies who received autologous T lymphocytes expressing chimeric antigen receptors against CD19 or CD20, CD16V-BB-ζ plus rituximab was more effective than a receptor with identical signaling properties but binding directly to CD19.

Figure 5. T lymphocytes expressing CD16V-BB-ζ receptors exert antitumor activity in vivo. NOD-SCID-IL2Rγnull mice were injected intraperitoneally with 3 × 10^5 Daudi cells labeled with luciferase. Rituximab (150 μg) was injected intraperitoneally once weekly for 4 weeks starting on day 4. In four mice, no other treatment was given, whereas in five other mice, the first rituximab injection was followed by T lymphocytes expressing CD16V-BB-ζ receptors (1 × 10^7 i.p.; n = 5) on days 5 and 6; the other two groups of four mice each received CD16V-BB-ζ T lymphocytes preceded by intraperitoneal injection of RPMI-1640 instead of rituximab or intraperitoneal injection of RPMI-1640 medium only (Control). A, results of in vivo imaging of tumor growth. Each symbol corresponds to one bioluminescence measurement; lines connect all measurements in one mouse. B, representative mice (2 per group) for each experimental condition. Ventral images on day 3 were processed with enhanced sensitivity to demonstrate the presence of tumors in mice of the CD16V-BB-ζ + rituximab group. Mice were euthanized when bioluminescence reached 5 × 10^11 photons/second. C, overall survival comparisons of mice in the different treatment groups.

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advantageous given immunoevasive mechanisms exploited by tumors, as illustrated by the recent report of a leukemia relapse driven by a subclone lacking the marker targeted by a chimeric receptor with single specificity (44). Antibody-directed cytotoxicity could be stopped whenever required by simple withdrawal of antibody administration. Because the T cells expressing CD16V-BB-ζ are only activated by antibody bound to target cells, soluble immunoglobulin should not exert any

Figure 6. CD16V-BB-ζ induces higher T-cell activation, proliferation, and cytotoxicity than CD16V receptors with different signaling properties. A, CD25 mean fluorescence intensity (MFI) by flow cytometry plotted against green fluorescent protein (GFP) MFI in T lymphocytes expressing different chimeric receptors after 48-hour coculture with Daudi cells and rituximab (0.1 μg/mL). CD25 expression with CD16V-BB-ζ was significantly higher than that triggered by CD16V-ζ, CD16V-FcRy, or CD16V with no signaling capacity (CD16V-trunc.; P < 0.0001). B, T lymphocytes transduced with various CD16V receptors were cultured with Daudi, SK-BR-3, or NB1691 cells in the presence of rituximab, trastuzumab, and hu14.18K322A, respectively (all at 0.1 μg/mL). Symbols indicate percentage of cell recovery as compared with number of input cells (mean ± SD of 3 experiments); cell counts for weeks 1–3 of culture were significantly higher with CD16V-BB-ζ receptors than with all other receptors by paired t test for all 3 cultures (P < 0.0001). C, 4-hour ADCC of T lymphocytes expressing various CD16V receptors or mock-transduced T cells against Daudi, SK-BR-3, and NB1691 in the presence of rituximab, trastuzumab, and hu14.18K322A, respectively. Symbols are mean ± SD of triplicate cultures at the E:T shown. Cytotoxicities with CD16V-BB-ζ receptors were significantly higher than those with all other receptors (P < 0.0001 by t test in all comparisons), whereas cytotoxicities of lymphocytes mock-transduced or transduced with the CD16V-truncated receptor were not significantly different (P > 0.05) from each other; cytotoxicity with CD16V-FcRy was significantly higher than that with CD16-ζ against Daudi (P = 0.006) and SK-BR-3 (P = 0.019); lymphocytes expressing either receptor had higher cytotoxicities than those mock-transduced or transduced with CD16V-truncated (P < 0.01 for all comparisons).

Figure 7. Expression of CD16V-BB-ζ receptors by mRNA electroporation. A, activated T lymphocytes were electroporated with CD16V-BB-ζ mRNA or without mRNA (mock); expression of CD16 was tested 24 hours later by flow cytometry. B, cytotoxicity of mock or CD16V-BB-ζ electroporated T cells was tested against the Ramos cell line in the presence of rituximab. Symbols show mean ± SD percentage cytotoxicity (n = 3; P < 0.001).
stimulation on the infused T cells. Nevertheless, we suggest that it would be important to test the clinical safety of this strategy by using mRNA electroporation to express the CD16V-BB-ζ receptor transiently, thus limiting any potential autoimmune reactivity. In our study, we found that mRNA electroporation can express the receptor very effectively.

Antibody therapy has become standard-of-care for many cancer subtypes; its clinical efficacy is mostly determined by its capacity to trigger ADCC through the engagement of Fc receptors (18, 45). The main effectors of ADCC are NK cells but their function can be impaired in patients with cancer. For example, it was reported that trastuzumab-mediated ADCC of gastric cancer cells overexpressing HER2 was significantly lower with peripheral blood mononuclear cells from patients with gastric cancer and advanced disease as compared with that obtained with samples from patients with early disease or healthy donors (46). Moreover, responses are likely to be influenced by other factors, including the genotype of NK cell inhibitory receptors and their ligands (47). The results of our study suggest that the infusion of autologous T cells genetically engineered with the CD16V-BB-ζ receptor should significantly boost ADCC. Because the combined CD3ζ4-1BB signaling also causes T-cell proliferation, there should be an accumulation of activated T cells at the tumor site, which may further potentiate their activity. CD16V-BB-ζ receptors can be expressed by mRNA electroporation not only in activated T lymphocytes but also in resting peripheral blood mononuclear cells, a procedure that would take only a few hours from blood collection to infusion of CD16V-BB-ζ-expressing cells and is therefore well suited for clinical application.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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