Gene-Modified Human α/β-T Cells Expressing a Chimeric CD16-CD3ζ Receptor as Adoptively Transferable Effector Cells for Anticancer Monoclonal Antibody Therapy

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Abstract
The central tumoricidal activity of anticancer monoclonal antibodies (mAb) is exerted by FcγRIIIa (CD16)-expressing effector cells in vivo via antibody-dependent cell-mediated cytotoxicity (ADCC), as observed for natural killer (NK) cells. In practice, chemotherapy-induced leukopenia and exhaustion of NK cells resulting from ADCC often hamper the clinical efficacy of cancer treatment. To circumvent this drawback, we examined in vivo the feasibility of T cells, gene-modified to express a newly generated affinity-matured (188V/V) chimeric CD16-CD3ζ receptor (cCD16ζ-T cells), as a transferable alternative effector for cancer mAb therapy. cCD16ζ-T cells were readily expandable in ex vivo culture using anti-CD2/CD3/CD28 beads and recombinant human interleukin-2 (rhIL-2), and they successfully displayed ADCC-mediated tumoricidal activity in vitro. During ADCC, ligation of opsonized cancer cells to the introduced cCD16ζ-T cells stimulated the effector cells to produce proinflammatory cytokines and release toxic granules through the activation of the Nuclear factor of activated T cells (NFAT) pathway after phosphorylation of the CD3ζ chain. In parallel, these stimulated cCD16ζ-T cells transiently proliferated and differentiated into effector memory T cells. In contrast, NK cells activated by rhIL-2 displayed similar ADCC activity, but failed to proliferate. Human cCD16ζ-T cells infused concomitantly with anti-CD20 mAb synergistically inhibited the growth of disseminated Raji cells, a CD20+ lymphoid malignancy, and displayed ADCC-mediated tumoricidal activity in vivo. Treatment with cCD16ζ-T cells transiently expanded NK cells (6,7) and γδ-T cells (8) have been tested in adoptive cell transfer cancer therapy alone or in combination with tumor antigen-specific mAbs. However, no reliable clinical procedure has yet been established for ex vivo expansion of autologous effector cells to the numbers that are therapeutically sufficient (9). Instead, culture-induced killer (CIK) cells (10) and lymphokine-activated killer (LAK) cells (NCT 01329354; ClinicalTrials.gov), both mostly comprise NK cells, are being investigated for adoptive cell therapy in combination with rituximab in patients with refractory CD20+ immune cells including natural killer (NK) cells, γδ-T cells, macrophages, and neutrophils against the opsonized cancer cells. NK cells comprise the crucial population of these effector cells (4).

Introduction
Monoclonal antibodies (mAb) specific for tumor antigens are efficacious in the treatment of cancers (1); these include anti-CD20 mAb (rituximab) for CD20+ lymphoid malignancies (2) and anti-Her2/Neu mAb (trastuzumab) for Her2/Neu+ solid tumors (3). A major mechanism for the tumoricidal activity is antibody-dependent cell-mediated cytotoxicity (ADCC), which is executed by the Fc receptor–bearing innate immune cells including natural killer (NK) cells, γδ-T cells, macrophages, and neutrophils against the opsonized cancer cells. NK cells comprise the crucial population of these effector cells (4).

In practice, as anticancer treatment proceeds, a decline in the clinical efficacy of the mAb therapy is often observed. This decline in efficacy could be attributed to the decrease in the number of active effector cells during the ADCC process from a combination of chemotherapy-induced leukopenia and NK cell exhaustion (5). As CD16-expressing effector cells play a critical role in ADCC, we hypothesized that the combined therapeutic regimen of replenishing these cells together with the specific anticancer mAb may improve cancer treatment. Ex vivo–expanded NK cells (6,7) and γδ-T cells (8) have been tested in adoptive cell transfer cancer therapy alone or in combination with tumor antigen-specific mAbs. However, no reliable clinical procedure has yet been established for ex vivo expansion of autologous effector cells to the numbers that are therapeutically sufficient (9). Instead, culture-induced killer (CIK) cells (10) and lymphokine-activated killer (LAK) cells (NCT 01329354; ClinicalTrials.gov), both mostly comprise NK cells, are being investigated for adoptive cell therapy in combination with rituximab in patients with refractory CD20+
Lymphomas, although no conclusive results have yet been reported.

Recent laboratory studies have demonstrated that both circulating CD16\(^+\) \(\alpha/\beta\)-T cells and CD16\(^+\) \(\gamma/\delta\)-T cells are involved in ADCC-mediated antiviral activity \textit{in vivo}, independently of human leukocyte antigen (HLA) restriction (11–13). The binding affinity of the Fc\(\gamma\)RIIIa-158.Val/Val) homodimer (158V/V) to immunoglobulin G (IgG)1 and IgG3 is much higher than that of the Fc\(\gamma\)RIIIa-158.Phe/Phe) homodimer (158F/F; ref. 14), and that the Fc\(\gamma\)RIIIa-158FV biallelic polymorphism influences the efficacy of NK cell activation (15, 16). Unlike that of NK and \(\gamma/\delta\)-T cells, the \textit{ex vivo} expansion and gene-modification of CD3\(^+\) T cells are clinically established for the application to adoptive cell therapy (17). Therefore, we hypothesized that therapeutically infused CD3\(^+\) T cells, gene-modified to express CD16 with the 158V/V amino acid substitution, might be able to mediate ADCC against opsonized cancer cells and virus-infected cells \textit{in vivo}. In addition, these effector cells could be used in combination with almost all currently available ADCC-mediated anticancer mAbs. A similar concept was proposed by Clémenceau and colleagues (18), who validated \textit{in vitro} the applicability of CD3\(^+\) T cells gene-modified to express a Fc\(\gamma\)RIIIa-158(V/V)-Fc\(\varepsilon\)RIg chimera receptor for ADCC against CD20\(^+\) lymphoma cells in combination with rituximab, although the \textit{in vivo} validation has not been described yet. Regarding the risk of leukemogenesis of infused gene-modified T cells, results from clinical trials using retroviral or lentiviral vector gene-modified T cells for adoptive cell therapy have proved the long-term safety of this approach (19, 20). Therefore, we believe that adoptive transfer of gene-modified T cells expressing the CD16 complex, designed to mediate ADCC, might be clinically acceptable and possibly enhance the clinical efficacy of mAb therapy against cancers.

In the present study, we used lentivirus vectors to establish gene-modified CD3\(^+\) T cells expressing the Fc\(\gamma\)RIIIa-158(V/V)-CD3\(\zeta\) chimera receptor (cCD16\(\zeta\)-T cells) as the adoptive transfer effector cells for ADCC, and examined their function in detail both \textit{in vitro} and \textit{in vivo}. The cCD16\(\zeta\)-T cells were

Figure 1. Functional validation of the chimeric CD16(158V/V)-CD3\(\zeta\) receptor. A, lentiviral vector construct of chimeric CD16(158V/V)-CD3\(\zeta\). Asc1 and Sal1 are restriction enzymes. SP, ECD, TM, and ICP denote the signal peptide, extracellular domain, transmembrane portion, and intracellular portion, respectively. aa158-V indicates amino acid substitution at position 158 to Valine. Number of amino acids at each site is indicated in parentheses. B, parent Jurkat/MA/CD8a/luc cells (left), and cCD16\(\zeta\)-gene-transduced Jurkat/MA/CD8a/luc cells (right) are shown. C, assessment of target-responsive phosphorylation status of the CD3\(\zeta\) chain (CD247) in cCD16\(\zeta\)-Jurkat/MA/CD8a/luc cells by flow cytometry. Solid line denotes the response to Raji cells in the presence of 1 \(\mu\)g/mL of rituximab. Dotted line denotes the response to Raji cells alone. Dashed line indicates the response to K562 cells similarly treated with rituximab as a negative control. Shaded area indicates the response to 1 \(\mu\)g/mL of rituximab alone. D, target-responsive luciferase production mediated by cCD16\(\zeta\)-Jurkat/MA/CD8a/luc cells. The introduced cCD16\(\zeta\) receptor successfully discriminated rituximab-opsonized Raji cells and successfully activated the NFAT pathway to produce luciferase, which was obviously inhibited by the anti-CD16 F(ab\(^2\))2 mAb, 3G8, in a dose-dependent manner. Clear bar denotes luciferase production in the response to each target alone. Black bar indicates the response to rituximab alone, rituximab-opsonized Raji cells, or similarly treated K562 cells. Gray bar denotes the response to rituximab-opsonized Raji cells in the presence of various concentrations of 3G8.
Figure 2. Validation of cCD16z-T cells. A, surface CD16 expression of cCD16z-T cells assessed by flow cytometry (right). NGM-T cells, non-gene-modified T cells similarly treated using anti-CD2/CD3/CD28 beads and rIL-2 (left). B, expandability of cCD16z-T cells in ex vivo culture with 50 U/mL of rIL-2. Proliferation of expanded cCD16z-T cells from 13 different donors relative to the number on day 2 is shown. Mean number is shown as a solid circle (●). Error bars indicate SEs. Proliferation on day X was calculated as number of cCD16z-T cells on day X/that on day 2. C, time course of the CD8+/CD4+ T-cell ratio among cultured cCD16z-T cells (n = 3). Error bars indicate SDs. D, levels of CD20 expression on Raji and Daudi cells (top). Solid line indicates Raji, dotted line indicates Daudi, and the shaded area indicates K562 cells as a negative control. The 51Cr-release assay (bottom) indicated that cCD16z-T cells successfully and equally exerted ADCC activity against rituximab-opsonized Raji (black circles) and Daudi (gray circles) cells, but not against K562 cells (clear circles) in accordance with the dose of rituximab. Effector cells established from 3 different donors were used at an E:T ratio of 5:1. Experiments were conducted in triplicate. Error bars represent SDs. E, levels of Her2/neu expression on SKOV3 and MCF-7 cells. Solid line indicates SKOV3, dotted line indicates MCF-7 and shaded area indicates K562 cells (top). Similarly to D, cCD16z-T cells exerted ADCC activity against trastuzumab-opsonized SKOV3 (black circles) and MCF-7 cells (gray circles), but not against K562 cells (clear circles) in accordance with the dose of trastuzumab. In addition, the level of Her2/neu expression on tumor cells also limited the ADCC activity. Error bars represent SDs. F, levels of CCR4 expression on MT-4 and ATN-1 cells. Solid line indicates MT-4, dotted line indicates ATN-1, and shaded area indicates K562 cells (top). Similarly to D and E, cCD16z-T cells exerted ADCC activity against mogamulizumab-opsonized MT-4 (black circles) and ATN-1 (gray circles), but not against K562 cells (clear circles) in accordance with the dose of mogamulizumab. The level of CCR4 expression on tumor cells again limited the ADCC activity. Error bars represent SDs. G, when the rituximab dose was fixed at 0.1 mg/mL, which was lower than the pharmacologic range, the number of effector cCD16z-T cells also regulated the ADCC activity against rituximab-opsonized Raji cells. n = 3, E:T ratio = 5:1. Error bars represent SDs. H, similarly to G (n = 3, E:T ratio = 5:1), at a fixed dose of 0.1 μg/mL of trastuzumab, also lower than the pharmacologic range, the number of effector cells again regulated the ADCC activity against trastuzumab-opsonized SKOV3 cells. Error bars represent SDs. I, similarly to G and H (n = 3, E:T ratio = 5:1), at a fixed dose of 0.1 μg/mL of mogamulizumab, also lower than the pharmacologic range, the number of effector cells again regulated the ADCC activity against mogamulizumab-opsonized ATN-1 cells. Error bars represent SDs. J, as was shown in G and H (n = 3, E:T ratio = 5:1), ADCC activity mediated by cCD16z-T cells against rituximab-opsonized Raji cells was significantly inhibited by 3G8, an anti-CD16 F(ab’2) mAb (P < 0.01). K562 was used as a negative control. Error bars represent SDs.
readily expandable in ex vivo short-term culture using recombinant human interleukin-2 (rhIL-2), and successfully displayed tumoricidal activity via ADCC both in vitro and in vivo. Our experimental observations indicate that this approach could be a potential option for enhancing the clinical efficacy of currently available mAb therapy against cancers.

Materials and Methods

Cells

Approval for this study was obtained from the Institutional Review Board of Ehime University Hospital (Ehime, Japan). Written informed consent was provided by all the healthy volunteers and patients with B-cell lymphomas in accordance with the Declaration of Helsinki. The HEK 293T cell line (RIKEN BioResource Center) was maintained in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% FBS, 2 mmol/L L-glutamine (Sigma-Aldrich), and was used for the production of infectious lentivirus particles. The CD20⁺ lymphoma cell lines Raji (21) and >Daudi (21), the human ovarian carcinoma cell lines SKOV3 (22) and K562 [American Type Culture Collection (ATCC)], the human T-lymphotropic virus type-I (HTLV-1) (22) and K562 [American Type Culture Collection (ATCC)], the human T-cell leukemia (ATL) cell line cnull (NOG) female mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan; ref. 27) and maintained in the institutional animal facility at Ehime University (Ehime, Japan).

Flow cytometry

Transfectants obtained using cCD16⁺ gene transfer, non-gene-modified T cells, NK cells isolated from PBMCs using MACS beads (Miltenyi Biotec), and cell lines were labeled with anti-CD3, anti-CD4, anti-CD8, anti-CD20, anti-CD45, anti-CD56, anti-CD62L, anti-CD194 (also known as HER-2; BioLegend) mAbs. Flow cytometry was conducted using MACS beads (Miltenyi Biotec) were cultured in GT-T503 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, and penicillin/streptomycin (Biowest). The human breast cancer cell line MCF-7 (ATCC) was maintained in DMEM with 10% FBS, 2 mmol/L L-glutamine, and penicillin/streptomycin (Biowest). The Jurkat/MA cell line is a Jurkat subclone previously established by Calogero and colleagues that lacks the endogenous T-cell receptor (TCR) expression and function of the Jurkat receptor 4 (CCR4) mAb exploiting ADCC activity against CCR4⁺ ATl tumor cells (26), was purchased from Kyowa-Hakko-Kirin, Ltd.

Establishment of cCD16⁺ gene-transduced T cells

CD3⁺ T cells isolated from PBMCs of healthy volunteers and patients with B-cell lymphomas using MACS beads (Miltenyi Biotec) were cultured in GT-T503 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, and penicillin/streptomycin (Biowest). The Jurkat/MA cell line is a Jurkat subclone previously established by Calogero and colleagues that lacks the endogenous T-cell receptor (TCR) expression and function of the Jurkat receptor 4 (CCR4) mAb exploiting ADCC activity against CCR4⁺ ATl tumor cells (26), was purchased from Kyowa-Hakko-Kirin, Ltd.

Construction of a plasmid carrying the chimeric FcyRIII-CR3 chain

A lentiviral vector expressing a novel chimeric CD16 with a 158V/V-CD3⁺ (cCD16⁺) construct was synthesized (Fig. 1A). cDNA encoding the signal peptide and extracellular domain of CD16 (accession no.: NM_000569) with a gene alteration at F158V was directly connected to the CD3⁺ gene (accession no.: NM_198053) at the second amino acid (P) of the extracellular domain. After codon-optimization (GeneArt), the cCD16⁺ construct was inserted into the lentiviral vector, pRRLSIN.cPPT. MSCV/GFP.WPRE (25).

Chemicals

Rituximab (Rituxan) and trastuzumab (Herceptin) were purchased from Chugai Pharmaceutical Co., Ltd. Mogamulizumab (Poteligeo), a new defucosylated anti-C-C chemokine receptor 4 (CCR4) mAb exploiting ADCC activity against CCR4⁺ ATl tumor cells (26), was purchased from Kyowa-Hakko-Kirin, Ltd.

Mice

All in vivo mouse experiments were approved by the Ehime University Animal Care Committee. Six-week-old NOD/scid/γc−/− (NOD) female mice were purchased from the Central Institute for Experimental Animals (Kanagawa, Japan; ref. 27) and maintained in the institutional animal facility at Ehime University (Ehime, Japan).

Figure 3. Cellular outputs mediated by cCD16⁺ T cells during ADCC. A, cCD16⁺ T cells from 3 different donors produced proinflammatory cytokines in exclusive response to rituximab-opsonized Raji cells, as determined by ELISA assay. Clear bar depicts IL-2 and filled bar depicts IFN-γ. Error bars represent SDs. A less than detectable level. NGM-T cells, non-gene-modified T cells. B, to demonstrate the reproducibility, an experiment similar to that in A was conducted against trastuzumab-opsonized SKOV3 cells. cCD16⁺ T cells (n = 3) exclusively produced IL-2 (clear bar) and IFN-γ (filled bar) in response to trastuzumab-opsonized SKOV3 cells. Error bars represent SDs. NGM-T cells, non-gene-modified T cells. C, cCD16⁺ T cells released cytotoxic granules exclusively in response to rituximab-opsonized SKOV3 cells. Error bars represent SDs. NGM-T cells, non-gene-modified T cells. C, cCD16⁺ T cells released cytotoxic granules exclusively in response to trastuzumab-opsonized SKOV3 cells, but not K562 cells. D, cCD16⁺ T cells released cytotoxic granules again exclusively in response to trastuzumab-opsonized SKOV3 cells, but not K562 cells. E, CFSE dilution assay demonstrated the proliferative response mediated by cCD16⁺T cells in response to trastuzumab-opsonized Raji cells (middle), but not to similarly treated K562 cells as a negative control (bottom). OKT3 as a positive control led to obvious proliferation of cCD16⁺T cells, but rituximab alone did not (top). A representative result from three experiments using different donor-derived effector cells is shown. F, after ligation with rituximab-opsonized Raji cells, the indicated dose of rituximab and E/T ratio, cCD16⁺ T cells transiently proliferated for 7 days, then declined thereafter. On the other hand, similarly treated K562 cells (□) did not. G, in this series of experiments, during the proliferative response to rituximab-opsonized Raji cells in similar experiments, cCD16⁺ T cells tended to differentiate toward CD45RA+ /CD62L− effector memory T cells.
Luciferase production by Jurkat/MA cells engineered to express cCD16ζ receptor upon ligation with opsonized cancer cells

To demonstrate the functionality of the introduced cCD16ζ receptor in gene-modified T cells, CD16ζ Jurkat/MA/CD8ζ/luc cells were isolated, expanded, and subjected to luciferase assay as described previously (28). One million CD16ζ Jurkat/MA/CD8ζ/luc cells were coincubated with 1 × 10^6 Raji cells or negative control K562 cells, with or without rituximab (1 µg/L), for 12 hours in advance at an effector:target ratio of 1:1. For blocking experiments, the F(ab')2 fragment of the anti-human CD16-specific mAb 3G8 (Ancell) was added at several concentrations (10, 25, and 50 µg/mL).

CFSE dilution assay

To assess the proliferative response to opsonized target cells mediated by effector cells during the ADCC process, cCD16ζ-T cells or NK cells labeled with CFSE (Molecular Probes Inc.) were cocultured with Raji, primary lymphoma cells, or K562 cells with or without 1 µg/mL of rituximab. After 4 days, CFSE dilution among cCD16ζ-T cells or NK cells was assessed by flow cytometry as described previously (28).

In vitro ADCC assay

To determine the ADCC activity mediated by the cCD16ζ-T cells or NK cells, a standard 51Cr-release assay was performed (29). Briefly, 10^5 target cells were labeled with 5 × 10^3 51Cr (Na2CrO4: MP Bio Japan) and incubated at various concentrations of each mAb or effector/target (E:T) ratios with effector cells in 200 µL of culture medium in 96-well round-bottomed plates. After 4 hours of incubation with effector cells at 37°C, 100 µL of the supernatant was collected from each well. The percentage of specific lysis was calculated as: (experimental release cpm – spontaneous release cpm)/(maximal release cpm – spontaneous release cpm) × 100 (%). In some experiments, we conducted ADCC assays with a fixed E:T ratio of 5:1 or a 0.1 µg/mL concentration of mAb. For blocking experiments, 10 µg/mL 3G8 mAb was used.

In vitro complement-dependent cytotoxicity assay

To exclude the complement-dependent cytotoxicity (CDC) activity of rituximab in a xenografted mouse model, we examined in vitro the rituximab-mediated CDC activity against 1 × 10^7/well Raji cells in 10% mouse serum using the 51Cr-release assay during 1.5 hours of culture with various concentrations of rituximab. Experiments were conducted in triplicate.

IFN-γ and IL-2 secretion assay

Fifty thousand cCD16ζ-T cells were coincubated with Raji, Daudi, SKOV3, MCF-7, or K562 cells for 24 hours with or without 2 µg/mL of rituximab or trastuzumab. cCD16ζ-T cells treated with 1 µg/mL of OKT-3 (BioLegend) and 1 µmol/L of ionomycin (Cell Signaling Technology) were used as a positive control. IFN-γ and IL-2 in culture supernatants were measured using an ELISA kit (Thermo Scientific).

CD107a assay

CD107a expression mediated by cCD16ζ-T cells in response to opsonized cancer cells was examined as described previously (30). Briefly, 1 × 10^5 target cells were preincubated with or without 4 µg/mL of rituximab for 20 minutes were incubated with 2 × 10^5 cCD16ζ-T cells for 3 hours in a 96-well round-bottomed plate. After being labeled with fluorescein isothiocyanate (FITC)–conjugated CD107a mAb (BioLegend), anti-CD3, anti-CD8 (BD Biosciences), and anti-cCD16 (BioLegend) mAbs, the cells were analyzed by flow cytometry.

In vivo antitumor activity mediated by intravenously infused cCD16ζ-T cells in a xenografted mouse model

To examine the in vivo antitumor effect of cCD16ζ-T cells, we prepared luciferase gene-transduced Raji cells (Raji/luc) whose CD20 expression was equal to that of the parental Raji cells. All NOG mice of ages 6 weeks (n = 11) were exposed to 1 Gy of irradiation, and then intravenously inoculated with 5 × 10^5 Raji/luc cells via tail vein on day 0. These mice were divided into three cohorts: (i) nontreated mice (n = 3), (ii) mice subjected to intravenous administration of 5 × 10^6 cCD16ζ-T cells alone (n = 3), and (iii) mice subjected to intravenous administration of both 5 × 10^6 cCD16ζ-T cells and 40 µg/body of rituximab (n = 5). Intravenous administration of cCD16ζ-T cells with or without rituximab was done twice on days 4 and 24. In this series of experiments, no exogenous rhIL-2 was administered. Serial acquisition of the luciferase photon counts of inoculated Raji/luc cells was carried out as described previously (31). The photon count relative to that on day 4 before cell therapy, which indicated the residual tumor mass burden, was calculated for each mouse. In some experiments, mice were similarly treated with activated NK cells from...
healthy donors (n = 3) using 3-day culture with 50 U/mL of rhIL-2. The same experiments were carried out twice independently.

Table 1. Patient demographics

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age, y</th>
<th>Sex</th>
<th>Pathologic diagnosis</th>
<th>CD20+ tumor cells (%)</th>
<th>Previous Tx. (number)</th>
<th>WBC (/μL)</th>
<th>CD3+ T cell</th>
<th>NK cell</th>
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<td>NHL (DLBCL); CD20(+)CD79a(+)CD3(-)CD56(-)CD10(-)Bcl-2(+)</td>
<td>96.9%</td>
<td>None</td>
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<td>1.43%</td>
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<td>R-CHOP (5)</td>
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Abbreviations: DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; M, male; NHL, non-Hodgkin lymphoma; R-CHOP, rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine, prednisolone; Tx, treatment.

a Determined by flow cytometer.

Statistical analysis

Data were analyzed using the statistical computing package SPSS 15.0J for Windows. Data were expressed as mean ± SE or
SD. Differences were assessed using paired t test or one-way ANOVA followed by Tukey post hoc analysis. Overall survival rate was estimated by the Kaplan–Meier method and analyzed using the log-rank test. Differences at P < 0.05 were considered significant.

Results

Functional validation of chimeric CD16 with the 158V/V-CD3ζ receptor

The newly devised cCD16ζ gene construct (Fig. 1A) was lentivirally introduced into Jurkat/MA/CD8α/luc cells (cCD16ζ-Jurkat/MA/CD8α/luc; Fig. 1B). CD3ζ phosphorylated at 1:42Y tended, without statistical significance, to increase in cCD16ζ-Jurkat/MA/CD8α/luc cells ligated with opsonized CD20+ Raji cells in the presence of 1 μg/mL of rituximab (MFI = 8.34 ± 3.4, mean ± SE), but not in cCD16ζ-Jurkat/MA/CD8α/luc cells ligated with Raji cells alone (MFI = 3.23 ± 1.4, mean ± SE), 1 μg/mL of rituximab alone (MFI = 3.15 ± 1.6, mean ± SE), or similarly treated K562 cells as a control with 1 μg/mL of rituximab (MFI = 3.58 ± 1.4, mean ± SE; Fig. 1C). Thus, the introduced cCD16ζ construct successfully recognized the rituximab-opsonized Raji cells, evoking phosphorylation of the CD3ζ chain. Subsequently, activation of the NFAT pathway in the cCD16ζ-Jurkat/MA/CD8α/luc was triggered in response to the rituximab-opsonized Raji cells, as demonstrated by the maximal production of luciferase, which was inhibited by the anti-CD16 (Fab’/2) mAb (3G8) in a dose-dependent manner (Fig. 1D).

In summary, the introduced cCD16ζ receptor in T cells successfully recognized the opsonized target cells and transmitted the stimulatory signal to the intracellular NFAT pathway to activate T cells via phosphorylation of the CD3ζ chain. Therefore, we used this cCD16ζ construct in all subsequent experiments.

ADCC activity of cCD16ζ-T cells against opsonized cancer cells

Lentivirally gene-modified cCD16ζ-T cells successfully expressed CD16 on their surface (96.2% of cCD16ζ-T cells vs. 0.6% of NGM-T cells; Fig. 2A). cCD16ζ-T cells were readily expandable in ex vivo culture, showing a 3.4 ± 0.8 x 10^5-fold increase on day 14, and a 1.01 ± 0.25 x 10^3-fold increase on day 28 (mean ± SE; Fig. 2B). The absolute cCD16ζ-T cell count was 7.82 ± 1.83 x 10^3 on day 2 of culture (mean ± SE), eventually increasing to 11.9 ± 5.1 x 10^3 on day 28. The CD8α T-cell population became more predominant in ex vivo-expanded cCD16ζ-T cells as culture progressed, 32.3% ± 3.2% on day 2, 69.7% ± 3.1% on day 14, and 87.2% ± 2.0% on day 28 (mean ± SE; Fig. 2C). cCD16ζ-T cells derived from 3 healthy individuals displayed ADCC activity in vitro against opsonized CD20+ lymphoma cell lines (Raji and Daudi) with rituximab, but not similarly treated K562 cells (Fig. 2D). cCD16ζ-T cells similarly displayed ADCC activity against the opsonized Her2/neu breast cancer cell line MCF-7 and the ovarian cancer cell line SKOV3 with trastuzumab (Fig. 2E), and against the opsonized CCR4+ HTLV-1 infection-immortalized CD4+ T cell line MT-4 and the ATL cell line ATN-1 with mogamulizumab (Fig. 2F), but not against similarly treated K562 cells, respectively. The ADCC activities observed in the presence of rituximab, trastuzumab, and mogamulizumab were uniformly dependent on the dose of the antibody. In addition, the cell-surface expression level of target antigen also limited the ADCC activity mediated by cCD16ζ-T cells. The ADCC-mediated cytocidal activity displayed by cCD16ζ-T cells became almost maximal in the presence of 0.1 μg/mL therapeutic antibodies. Therefore, we examined the effect of effector cell number on this ADCC activity in the presence of 0.1 μg/mL of each mAb (n = 3). As shown in Fig. 2G–I, the cytocidal activity mediated by cCD16ζ-T cells against opsonized target cells in the presence of 0.1 μg/mL of each mAb was dependent on the number of effector cells. Again, in a blocking test using the Raji–rituximab combination as a representative, the ADCC activity mediated by cCD16ζ-T cells against similarly opsonized Raji cells was inhibited significantly by the 3G8 mAb at 10 μg/mL (Fig. 2J).

Responsive functionalities displayed by cCD16ζ-T cells following ligation with opsonized cancer cells

As a result of recognizing opsonized Raji and Daudi, but not K562 cells in the presence of rituximab, cCD16ζ-T cells, but not NGM-T cells, produced high amounts of both IFN-γ and IL-2 (Fig. 3A). This observation was reproducible using the combination of SKOV3, MCF-7, and trastuzumab (Fig. 3B). In addition, cCD16ζ-T cells exhibited cytotoxic degranulation, reflected as CD107a expression, exclusively in response to rituximab-opsonized Raji and trastuzumab-opsonized SKOV3, but not similarly opsonized K562, cells in both CD4+ and CD8α T-cell subsets of cCD16ζ-T cells (Fig. 3C and D). These cellular outputs mediated by cCD16ζ-T cells were directly involved in the cytocidal activity. In addition, the CFSE dilution assay demonstrated a proliferative response mediated by cCD16ζ-T cells in response to rituximab-opsonized Raji cells, but not rituximab alone or similarly treated K562 cells in the presence of rituximab (Fig. 3E). The proliferation mediated by cCD16ζ-T cells lasted for 7 days following single ligation with rituximab-opsonized Raji cells, but not with similarly treated K562 cells (Fig. 3F). This observation was replicated using the SKOV3–

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trastuzumab combination (data not shown). Furthermore, after single ligation of rituximab-opsonized Raji cells, the effecter memory (CD45RA-CD62L-) subset of cCD16<sup>+</sup>-T cells predominated (66.4% ± 5.0%), in comparison with those ligated with rituximab alone (25.7% ± 10.6%), or similarly treated K562 cells (39.6% ± 3.3%; mean ± SD; Fig. 3G). Thus, both the proliferative response and differentiation into an effecter memory T-cell subset might contribute to the persistence of therapeutically infused cCD16<sup>+</sup>-T cells following the recognition of opsonized cancer cells in vivo.

**cCD16<sup>+</sup>-T cells generated from PBMCs of patients with CD20<sup>+</sup> B-cell lymphomas successfully display ADCC activity against rituximab-opsonized autologous lymphoma cells**

PBMCs and autologous tumor cells were obtained from 3 patients with CD20<sup>+</sup> B-cell lymphomas. The demographics of these patients are shown in Table 1. As shown in Fig. 4A, cCD16<sup>+</sup>-T cells generated from all 3 patients successfully killed rituximab-opsonized, but not un-opsonized autologous lymphoma cells. Raji and K562 were used as a positive and negative control, respectively. Next, limited by the number of lymphoma cells available, cCD16<sup>+</sup>-T cells from 2 of 3 patients (nos. 1 and 2) were subjected to CFSE dilution assay. The cCD16<sup>+</sup>-T cells from these 2 patients similarly proliferated in response to rituximab-opsonized autologous lymphoma cells, as did the positive control, rituximab-opsonized Raji cells (Fig. 4B). Collectively, cCD16<sup>+</sup>-T cells generated from patients functioned similarly as those from healthy individuals.

**Activated NK cells exert ADCC activity comparable with that of cCD16<sup>+</sup>-T cells, but do not proliferate in response to opsonized tumor cells**

We found that CD3<sup>+</sup>CD56<sup>+</sup>CD16<sup>+</sup> NK cells accounted for 13.6% ± 2.0% (mean ± SE) of the peripheral blood lymphocytes (PBL) from healthy individuals (n = 10), but only 3.7% ± 1.6% (mean ± SE) from patients with lymphoma (n = 5). Thus, due to the difficulty in preparing a sufficient number of NK cells from patients with lymphoma, we only compared the functionality of NK cells with that of cCD16<sup>+</sup>-T cells from patients with CD20<sup>+</sup>-T cells generated from the same healthy donors (n = 3). After activation using a 3-day culture with 50 U/mL of rhIL-2, NK cells became positive for CD69 expression and capable of exerting the ADCC activity against rituximab-opsonized Raji cells to a degree comparable with that of cCD16<sup>+</sup>-T cells (Fig. 5A–C). We then examined the proliferative activity in response to rituximab-opsonized Raji cells mediated by these activated NK cells. After ligation with opsonized tumor cells and in the absence of additional rhIL-2, unlike the cCD16<sup>+</sup>-T cells (Fig. 3E and F), these activated NK cells did not proliferate (Fig. 5D and E).

**Successful inhibition of the growth of disseminated CD20<sup>+</sup> lymphoma cells by adoptively transferred cCD16<sup>+</sup>-T cells in combination with rituximab in a mouse xenograft tumor model**

NOG mice intravenously inoculated with Raji/luc cells were used to assess the in vivo therapeutic effect of cCD16<sup>+</sup>-T cells. First, we confirmed that rituximab did not exert CDC activity against Raji cells in the presence of serum from un-inoculated NOG mouse (Fig. 6A), as reported previously (32). As shown in Fig. 6B, in the Raji/luc xenograft tumor model, NOG mice were treated with cCD16<sup>+</sup>-T cells and rituximab intravenously twice (on days 4 and 24 after tumor cell inoculation; n = 9), tumor growth was significantly suppressed during the 5-week observation period (P < 0.01) in comparison with that of non-treated mice (n = 3), mice treated with rituximab alone (n = 3), mice treated with NGM-T cells (n = 3), mice treated with cCD16<sup>+</sup>-T cells alone (n = 3), or rhIL-2–treated NK cells (n = 3). Serial examination of mice in all cohorts using in vivo bioluminescence assay up to day 14 also demonstrated the suppression of tumor growth mediated by the combined regimen of cCD16<sup>+</sup>-T cells and rituximab (Fig. 6C). This result was reflected in the survival period of the mice; all mice in the control cohort died within 14 days and all mice treated with activated NK cells and rituximab died within 18 days, whereas mice treated with cCD16<sup>+</sup>-T cells and rituximab survived for more than 35 days (data not shown). In some experiments, mice were euthanized on day 14 when almost all mice in the control cohorts had died, and residual Raji/luc cells in autopsied organs were assessed using luciferase assay (Fig. 6D and E). In mice treated with cCD16<sup>+</sup>-T cells alone (solid bar), massive tumor burdens were detected in the liver, spleen, lung/heart, and uterus/ovary/fallopian tube. In contrast, in mice treated with cCD16<sup>+</sup>-T cells and rituximab (clear bar), tumor growth was suppressed in all the organs.

We examined the persistence of human NK cells and Raji cells in mouse spleen and bone marrow using flow cytometry (Supplementary Fig. S1). On day 14, a number of CD20<sup>+</sup> Raji cells in the bone marrow were observed in a mouse treated with NK cells (Supplementary Fig. S1C, left), but not in a mouse...
treated with cCD16ζ-T cells (Supplementary Fig. S1D). Furthermore, as shown in Supplementary Fig. S1C (right), in another mouse treated with NK cells on day 18, Raji cells increased in number in both the spleen and the bone marrow, but the number of NK cells remained constant.

These observations suggested that cCD16ζ-T cells infused along with rituximab successfully inhibited the growth of CD20⁺ Raji cells in vivo, and that their tumor-suppressive activity was superior to that of NK cells.

Discussion

Cancer antigen-specific mAbs have demonstrated efficacy in the treatment of cancer (1). The central tumoricidal machinery of this strategy in vivo is ADCC executed by FcyRII (CD16)–expressing effector cells, such as NK cells (4). In practice, however, a decline in the clinical efficacy of this treatment is often observed, and this involves both chemotherapy-induced leukopenia and NK cell exhaustion due to ADCC evoked by the applied mAb (5). Given that effector cells play important roles in ADCC, instead of escalating the dose of therapeutic mAb, replenishment of effector cells might circumvent this drawback. In the present study, we focused on three factors: (i) the expression level of target antigen on the surface of cancer cells, (ii) the dose of mAb used, and (iii) the number of effector cells restricting the ADCC activity (Fig. 2D–I). When the number of effector cells was fixed, the maximal ADCC mediated by cCD16ζ-T cells was achieved (Fig. 2D and E) at a rituximab, trastuzumab, or mogamulizumab concentration of 0.1 to 1.0 μg/mL, which is lower than the clinically effective concentrations in serum (26, 33, 34). When each mAb concentration was fixed at 0.1 μg/mL, ADCC activities against opsonized tumor cells were dependent on the number of cCD16ζ-T cells (Fig. 2G–I). At the clinically relevant concentrations of mAbs, the number of effector cells seems to be a crucial determinant of the efficacy of this form of therapy.

However, the anticancer effect mediated by ex vivo–expanded NK cells in clinical trials seems controversial (9), and the clinical efficacy of such cells administered along with anticancer mAbs, that is, as infused effector cells for ADCC, has not yet been evaluated clinically. There is a need to establish a standard procedure to expand clinically acceptable NK cells (6, 7). The other potential candidate CD16⁺ γδ-T cells (8) still await clinical evaluation. A clinical trial of adoptive cell therapy using culture-induced killer cells (CIK) in conjunction with rituximab against refractory lymphomas is ongoing (NCT01329354: ClinicalTrials.gov). In this setting, the central effector cells among infused CIKs have been defined as NK cells (10), although no conclusive results have been reported yet.

With regard to clinical feasibility, T cells have a proven record in terms of ex vivo expansion and genetic modification for adoptive therapy (17). Therefore, in the present study, we generated CD3⁺ CD16⁺ T cells gene-modified to express the chimeric CD16 (158V/V)-CD3ζ receptor as an adoptive transfer effector cell mediating ADCC in the setting of anticancer mAb therapy. It is known that CD16 naturally associates with immunoreceptor tyrosine-based activating motif (ITAM)–containing homodimers of FcRγ in human NK cells (35). Although in this study we did not make a direct comparison with the chimeric CD16/FcRγ construct, in view of the lower proliferation mediated by activated NK cells against opsonized Raji cells (Fig. 5A–E), we expected this chimeric CD16 (158V/V)-CD3ζ to show some degree of enhancement of proliferative activity after ligation with opsonized tumor cells. The chimeric CD16 (158V/V)-CD3ζ construct introduced into Jurkat/MA cells successfully recognized opsonized Raji cells in the presence of rituximab. This opsonized cell ligation led to the phosphorylation of the CD3ζ chain (Fig. 1C), followed by activation of the NFAT pathway, as reflected by luciferase production, which was blocked by the anti-CD16 (Fab’)2 mAb (3G8; Fig. 1D). As expected, through this mechanism, during ADCC, the cCD16ζ-T cells produced IFN-γ and IL-2 (Fig. 3A and B), released cytotoxic granules (Fig. 3C and D), proliferated (Fig. 3E and F), and differentiated into an effector memory T-cell phenotype (Fig. 3G). The former two cellular outputs directly accounted for tumoricidal activity, and the latter two cellular activities were able to contribute to the durable antitumor effect observed in vivo. To our knowledge, no previous reports describing a similar concept have included in vivo experimental observations (18, 34). Therefore, after confirming that rituximab-induced CDC was not present in NOG mouse serum (Fig. 6A), we used an in vivo bioluminescence assay to demonstrate that cCD16ζ-T cells concomitantly infused with rituximab significantly inhibited the growth of disseminated Raji/luc cells in NOG mice, compared with the other cohorts (P < 0.01; Fig. 6B and C). The luciferase assay of autopsied organs from mice treated simultaneously with cCD16ζ-T cells and rituximab demonstrated inhibition of tumor growth in the liver, spleen, lung/heart, and uterus/ovary/fallopian tube (Fig. 6D and E). Although cCD16ζ-T cells and activated NK cells displayed comparable ADCC activity in vitro (Fig. 5B and C), cCD16ζ-T cells demonstrated a superior tumor-suppressive effect in vivo (Fig. 6B and C and Supplementary Fig. S1). In Fig. 6B, slower tumor progression was observed in mice treated with cCD16ζ-T cells plus rituximab. The progression of the Raji/luc tumor in NOG mice was more obvious in head lesions, but remained localized and suppressed at truncal sites (Supplementary Fig. S2A). Furthermore, in a cCD16ζ-T–treated mouse on day 32, we observed durably persistent cCD16ζ-T cells, defined as hCD45⁺/CD3ζ⁺/CD16⁺, accounting for 9.0% of total spleen cells and 0.14% of total bone marrow cells, but no CD20⁺ Raji cells (Supplementary Fig. S2B), and these cCD16ζ-T cells retained their responsiveness against rituximab-opsonized Raji cells (Supplementary Fig. S2C). In addition, Raji cells in mice showing tumor progression were still positive for CD20 (data not shown). Taken together, these data indicate that NOG mice inoculated intravenously with Raji cells may be more likely to develop head metastasis, and that head lesions seem to be less accessible to cCD16ζ-T cells (and/or rituximab) than those in the truncal sites. The localization and persistence of effector cells or the loss of CD20 by Raji cells may be specific to this particular model.

In this series of in vivo experiments, exogenous rhIL-2, which had been defined as an important determinant in this type of murine experiment (36, 37), was not administered. Our
findings strongly suggested that the proliferation and differentiation mediated by the infused cCD16ζ-T cells in response to rituximab-opsonized Raji cells (Fig. 3E–G) contributed to the persistent efficacy observed in vivo. Overall, the data suggest that our newly generated cCD16ζ-T cells would be promising for clinical application.

At present, chimeric antigen receptor (CAR)—transduced T cells, whose target recognition is mediated by an introduced single-chain variable fragment (scFv) of cancer-associated antigen-specific mAb, especially in CD19-CAR T-cell therapy for CD19+ leukemia, have shown clinical efficacy and safety (38, 39). However, the range of applications for the CAR strategy remains narrow, and the preparation of a huge series of CAR-T cells covering various kinds of cancer does not seem realistic. From this viewpoint, cCD16ζ-T cells might be advantageous, because almost all anticancer mAbs currently available for ADCC would be theoretically applicable.

Because the first-generation CAR constructs containing only CD3ζ were unable to exert fully the anticipated antitumor effect (40), the incorporation of a costimulatory molecule, such as 4-1BB, into the cytoplasmic portion of our chimeric CD16ζ-CD3ζ construct, may improve the persistence and antitumor trafficking in vivo, and possibly providing a better clinical outcome (41). Therefore, we plan to develop a “second-generation” chimeric CD16ζ-CD3ζ construct. In the present study, we have successfully demonstrated that our therapeutic approach using novel cCD16ζ-T cells as adoptive transfer anticancer effector cells is feasible, and can mediate ADCC both in vitro and in vivo. Although further studies are warranted, our experimental observations strongly suggest that cCD16ζ-T cells might provide another option to improve the currently available anticancer immunotherapy.

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Gene-Modified Human α/β-T Cells Expressing a Chimeric CD16-CD3 ζ Receptor as Adoptively Transferable Effector Cells for Anticancer Monoclonal Antibody Therapy

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