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Activity of Anti-CD19 Chimeric Antigen Receptor T Cells Against B Cell Lymphoma Is Enhanced by Antibody-Targeted Interferon-Alpha

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An important emerging form of immunotherapy targeting B cell malignancies is chimeric antigen receptor (CAR) T cell therapy. Despite encouraging response rates of anti-CD19 CAR T cell therapy in B cell lymphomas, limited durability of response necessitates further study to potentiate CAR T cell efficacy. Antibody-targeted interferon (IFN) therapy is a novel approach in immunotherapy. Given the ability of IFNs to promote T cell activation and survival, target cell recognition, and cytotoxicity, we asked whether antibody-targeted IFN could enhance the antitumor effects of anti-CD19 CAR T cells. We produced an anti-CD20-IFN fusion protein containing the potent type 1 IFN isoform alpha14 (α14), and demonstrated its ability to suppress proliferation and induce apoptosis of human B cell lymphomas. Indeed, with the combination of anti-CD20-hIFNα14 and CAR T cells, we found enhanced cell killing among B cell lymphoma lines. Importantly, for all cell lines pretreated with anti-CD20-hIFNα14, the subsequent cytokine production by CAR T cells was markedly increased regardless of the degree of cell killing. Thus, several activities of CD19 CAR T cells were enhanced in the presence of anti-CD20-hIFNα14. These data suggest that antibody-targeted IFN may be an important novel approach to improving the efficacy of CAR T cell therapy.

Keywords: interferon, fusion protein, CAR T cells, immunotherapy, lymphoma, cytokine release

Introduction

Adoptive cell therapy (ACT), which includes chimeric antigen receptors (CARs), is a revolutionary form of immunotherapy in anti-cancer treatment. CARs are recombinant proteins with antigen recognition moieties and T cell activation domains that can be expressed by host T cells via viral transduction. The antigen recognition moiety of the resulting CAR T cell can redirect the specificity of the T cell to a cell surface differentiation or tumor-associated antigen. Specifically, the treatment of patients with CD19 CAR T cells has elicited objective tumor responses with tolerable toxicities, resulting in approval of CD19 CAR T cells for use in acute lymphoblastic leukemia (ALL) and diffuse large B cell lymphoma (DLBCL) (Brentjens and others 2013; Kochenderfer and others 2015; Turtle and others 2016; Abramson and others 2017; Neelapu and others 2017; Schuster and others 2017).

Despite the initial success with response rates in non-Hodgkin lymphoma (NHL) ranging between 59% and 84% (Abramson and others 2017; Neelapu and others 2017; Schuster and others 2017), longer follow-up has shown that the majority of patients relapse by 3–6 months (Abramson and others 2017; Neelapu and others 2017). Thus, given the limited durability of CAR T cell therapy, further studies are warranted to induce a sustained treatment response.

Interferons (IFNs) have both antiviral and immunostimulatory properties, acting as essential mediators of anti-cancer immunity (Parker and others 2016). IFN enhances CD8-positive T cell cytotoxicity, dendritic cell maturation (Papewalis and others 2008), protects T cells from NK cell attack (Crouse and others 2014; Xu and others 2014), and suppresses regulatory T cells (Bacher and others 2013; Anz and others 2015). Furthermore, type 1 IFNs increase T cell infiltration into tumors, promote memory T cell survival, and enhance recognition of cancer cells (Zitvogel and others 2015). Antibody-targeted IFN therapy harnesses the specificity of monoclonal antibodies to direct IFN directly to tumor sites, thereby minimizing systemic toxicity while maximizing the properties of IFN as an anticancer agent (Young and others

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2014). Anti-CD20-IFN fusion proteins were developed in our laboratory and previously have been shown to have superior antitumor effects *in vitro* and *in vivo* in both syngeneic and xenograft models (Xuan and others 2010; Trinh and others 2013). Importantly, the antitumor effects were achieved without systemic toxicity. A first-in-human phase I study of anti-CD20-hIFNα2 (IGN002) is now ongoing (NCT02519270) (Young and others 2016). Although IFNα2 has been most broadly studied clinically (Borden and others 2000), a recent study showed that among the 12 human IFNα subtypes, α14 has the strongest antiproliferative activity against cancer cells (Lavoie and others 2011). Therefore, for these studies we focused our attention on the fusion protein, anti-CD20-hIFNα14.

With the multitude of immunotherapeutic properties of IFN, we hypothesized that pretreatment of lymphoma tumor cells with anti-CD20-hIFNα14 would result in enhanced cell killing and increased production of cytokines during subsequent CAR T cell therapy. The goal of this study was to examine the effect of anti-CD20-hIFNα14 treatment on CD19-specific killing by CAR T cells in lymphoma cell lines of various histologies. To further characterize the functions of effector CAR T cells in combination with anti-CD20-hIFNα14, we examined their cytokine production during coculture with human B cell lymphomas. Indeed, we found increased killing of lymphoma cell lines when treated with the combination of anti-CD20-hIFNα14 and CAR T cells, and a concurrent marked increase in the production of proinflammatory cytokines. These data suggest that anti-CD20-hIFNα fusion proteins may be useful in improving the efficacy of CAR T cell therapy.

Materials and Methods

Cell lines

Raji, Daudi, DEL, Granta-519, Jeko-1, OCI-Ly2, OCI-Ly19, and RS-27 cell lines were obtained and cultured as previously described (Andorsky and others 2011). OVCAR-3 was a gift from Dr. Gottfried Konecny [University of California, Los Angeles (UCLA)]. Unless otherwise specified, tumor cells were cultured in RPMI 1640 medium (ThermoFisher Scientific, Waltham, MA) plus 10% heat-inactivated fetal calf serum (FCS; Omega Scientific, Tarzana, CA), 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, and 50 μM β-mercaptoethanol (''RPMI complete medium''; all supplements from ThermoFisher Scientific), at 37°C in 5% CO_2. OVCAR-3 was grown in RPMI supplemented with 20% fetal bovine serum (FBS) (Atlanta Biologics, Lawrenceville, GA) + 0.01 mg/mL bovine insulin (Sigma-Aldrich, St. Louis, MO).

Construction of expression vectors

The DNA sequence for human interferon α14 (GenBank accession No. NP002163.2) optimized for expression in Chinese hamster ovary cells was synthesized by DNA 2.0 with a *Bam*HI restriction site (GGATCC) added 5′ of the mature protein sequence, and an *Xba*I site (TCTAGA) added 3′ of the termination sequence. The hIFNα14 gene was cloned into the anti-huCD20 or anti-huCD138 expression vectors (Xuan and others 2010; Yoo and others 2015) as a *Bam*HI/*Xba*I cassette.

Protein production and purification

Protein production and purification were done using protein A Sepharose as previously described except the bound protein that was eluted with 0.1 M arginine pH 2.5 (Trinh and others 2013). Recombinant hIFNα14 reference standard was purchased from PBL InterferonSource (Piscataway, NJ). Rituximab was obtained from Genentech (South San Francisco, CA).

Determination of binding to CD20

Daudi cells were harvested by washing once in PBS + 2% bovine serum albumin (FACS buffer), and incubated on ice as follows (1) unstained, (2) 15 μg/mL mouse anti-human IgG1 Fd (Hamilton and Morrison 1993)+4 μL anti-mouse kappa phycoerythrin (PE) (Invitrogen/ThermoFisher Scientific), (3) 10 μg/mL rituximab +15 μg/mL mouse anti-human IgG1 Fd +4 μL anti-mouse kappa PE, or (4) 10 μg/mL anti-huCD20-hIFNα14 + 15 μg/mL mouse anti-human IgG1 Fd +4 μL anti-mouse kappa PE. Each sample was incubated for 1 h and washed twice with FACS buffer before the next incubation. All samples stained with anti-mouse kappa PE were incubated for 30 min. Samples were run on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

Flow cytometry for cell surface markers

Lymphoma cells were added to 6-well plates with medium or graded concentrations of rituximab (10 or 1 nM), anti-huCD20-hIFNα14 (10 or 1 nM), or rhIFNα14 (20 or 2 nM) and incubated for 24 or 48 h. Equimolar amounts of antibody and an equivalent rhIFNα14 dose (assuming 2 mol of IFN for every mol of fusion protein) were used. Cells were then stained with anti-human PD-L1/B7-H1 PE (clone MIH1) or anti-human CD19 PE (clone HIB19) (eBioscience/ThermoFisher Scientific) or appropriate isotype controls from BD Biosciences and analyzed using a BD FACSVerse flow cytometer (BD Biosciences) with FCS Express software (De Novo Software, Los Angeles, CA).

Metabolic activity assay

Daudi cells in RPMI complete medium were plated in triplicate in 96-well flat bottom plates. Antibodies were added in RPMI complete medium to wells in triplicate starting at 0.5 nM and serially diluted 1:5. Cells alone plus RPMI complete medium served as the no treatment control. Cells were incubated at 37°C for 72 h before performing the MTS assay (Promega, Madison, WI) according to the manufacturer's protocol. Absorbance was measured at 490 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT). Data were analyzed by nonlinear regression using Prism (GraphPad Software, Inc., La Jolla, CA) with the log [inhibitor] versus the response with a variable slope with the IC_{50} calculated. Data are expressed as the percentage of maximum metabolic activity of untreated cells. OVCAR-3 cells were assayed similarly, using RPMI 1640 medium +20% FBS +0.01 mg/mL bovine insulin (Sigma-Aldrich), and 1:10 antibody dilutions were made starting at 50 nM. The bioactivity of the antibodies was plotted adjusting for the fact that there are 2 IFNs per antibody molecule.

Apoptosis assay

Tumor cells were seeded in a 24-well plate in RPMI complete medium. Medium or graded concentrations of rituximab (10, 1, or 0.1 nM), anti-CD20-hIFNα14 (10, 1, or 0.1 nM) or rhIFNα14 (2 or 0.2 nM) were added at a final volume of 1 mL/well and incubated for 72 h. Apoptosis was assessed by annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining per manufacturer's protocol (Roche Applied Science, Indianapolis, IN) and stained cells were analyzed using a BD FACSCalibur flow cytometer (BD Biosciences) and FCS Express version 5 software (De Novo Software). Data are displayed as mean ± SD for triplicate values of % annexin V-positive cells.

Proliferation assay

Human lymphoma cell lines were incubated in 96-well U-bottom plates (Nunc, Rochester, NY) with medium, rituximab, anti-CD20-hIFNα14, or rhIFNα14 (starting at 10 or 1 nM and serially diluted 10-fold) for 72 h. DEL, a CD19-negative cell line, was used as a negative control for the tumor mixture assay. Equimolar amounts of antibody and an equivalent rhIFNα14 dose (assuming 2 mol of IFN for every mol of fusion protein) was used to compare to anti-CD20-hIFNα14 and plotted accordingly for direct comparison. Cells were pulsed with 1 μCi/well ^{3}[H]-thymidine (PerkinElmer, Waltham, MA) and harvested 8 h later. Incorporated radioactivity (counts per minute) was measured using a β-liquid scintillation analyzer (PerkinElmer) and percent proliferation was calculated as [cpm(expt)/cpm(untreated)] × 100 and shown as mean ± SD of quadruplicate values. Dose response curves were generated using nonlinear regression analysis using Prism (GraphPad software).

Preparation of CAR T cells

Primary human T cells. Peripheral blood mononuclear cells were isolated from blood from anonymous donors through Ficoll-Paque Plus (GE Healthcare Life Sciences, Marlborough, MA) density gradient separation. Dynabeads® Human T-Activator CD3/CD28 beads (ThermoFisher Scientific) were then used to activate T lymphocytes for 72 h. After 72 h, T cells were harvested for bead removal through a magnetic column system, and immediately incubated with lentiviral vectors for transduction (De Oliveira and others 2013). A portion of the activated T cells harvested were set aside and designated Mock (nontransduced) T cells to be used as an experimental control. All cells were cultured in RPMI 1640 plus 10% FBS (R10) with 10 ng/mL of rhIL-2 (R&D Systems, Minneapolis, MN) 24 h after lentiviral transduction, for up to 21 days.

Lentiviral vector construct and production. The lentiviral vector used has been described previously (Kowolik and others 2006; De Oliveira and others 2013; Larson and others 2017). In brief, the third-generation self-inactivating lentiviral vector utilized the pCCL-c backbone (Dull and others 1998) and contained the MND LTR U3 (MNDU3) (Challita and others 1995) promoter to deliver a single-chain variable fragment (scFv) specific for CD19 connected to CD28 costimulatory moiety and the intracellular domain of the human CD3ζ T cell intracellular domain (Cooper and others 2003). Lentiviral supernatant was created through triple transfection of 293T cells with gag/pol plasmid, VSV-G envelope plasmid, and the anti-CD19 CAR plasmid. High-titer vectors were produced by tangential flow filtration (Cooper and others 2011). Vector titer determination to define vector transduction units per volume (TU/mL) was performed through transduction of HT-29 cells with 3 independent dilutions of 10^{-1} vector, harvested after 72 h for ddPCR of the extracted DNA (Cooper and others 2011). For all determinations of vector copy number, the HIV-1 ψ region of the vector provirus was detected.

T cell lentiviral transduction and culture. After removal of immunomagnetic beads, $0.4–0.5 \times 10^{6}$ T cells/well of a nontissue culture-treated plate were incubated for 24 h in R10 medium with $1–1.5 \times 10^{8}$ TU/mL of lentiviral vector, in wells coated with RetroNectin (Clontech T100B). Cells were then removed and cultured in R10 with 10 ng/mL of rhIL-2 (R&D Systems) (De Oliveira and others 2013; Larson and others 2017). For the cytotoxicity assays, cells were used 14–21 days after transduction.

Cytotoxicity assays

Tumor mixture assay. CD19-negative and CD19-positive human lymphoma cells were harvested, washed in 1× PBS, and stained with 0.25 or 5 μM carboxyfluorescein succinimidyl ester (CFSE, ThermoFisher Scientific), respectively, for 10 min in a 37°C water bath. After incubation, 5 mL of FCS was added and cells centrifuged at 400 *g* for 7 min. Supernatant was removed and cells were washed 2 times with RPMI complete medium. Stained CD19-negative and -positive cell lines (targets) were mixed at an approximate 1:1 ratio, and plated in 96-well U-bottom plates at 10,000 cells/well. Day 14 posttransduction effectors (CD19 CAR or Mock T cells) were harvested, washed, and added at 125:1, 25:1, 5:1, or 1:1 effector:target (E:T) ratios. Plates were incubated for 2 h at 37°C in a 5% CO_2 humidified incubator. Cells were then stained with PI and analyzed immediately using a FACSVerse flow cytometer (BD Biosciences) and FCS Express (De Novo Software). Percent specific lysis = $100 \times [1 - (\text{controlCFSE}_{low}/\text{controlCFSE}_{high})/(\text{exptCFSE}_{low}/\text{exptCFSE}_{high})]$.

Fusion protein plus CAR T cell-killing assay. Human lymphoma cells were pretreated with either medium or graded concentrations of rituximab (10, 1, or 0.1 nM) or anti-CD20-hIFNα14 (10, 1, or 0.1 nM) for 18–24 h and incubated at 37°C in a 5% CO_2 humidified incubator. After incubation, cells were harvested and washed twice in cold 1 × PBS and kept on ice. Cell pellets were stained with 5 μM CFSE for 10 min in a 37°C water bath. After staining, 5 mL of FCS was added and cells centrifuged for 7 min at 400 *g*. Supernatants were removed and cells were washed twice in killing assay complete medium (RPMI complete medium plus 1 mM sodium pyruvate, 10 mM HEPES, and 1× MEM NEAA; ThermoFisher Scientific). Ten thousand target cells/well were plated onto 96-well U-bottom plates. Medium or graded concentrations of rituximab or anti-CD20-hIFNα14 were added back at the pretreatment concentrations to the appropriate wells for 30 min before CD19 CAR or Mock T cells (effectors) were harvested and added at the designated E:T ratios. For the experiments in which soluble fusion protein was not added back, CD19 CAR or Mock T cells were harvested and added at the designated E:T ratios with no additional treatment. Plates were spun at 200 *g* for 3 min and cocultured at 37°C for 24 h. After incubation, plates were spun at 400 *g* for 5 min and supernatant collected for multiplex cytokine ELISAs and/or cells were transferred to

V-bottom plates and spun at 400 *g* for 5 min and cell pellets washed twice in 200 μL/well of 1× PBS. Cells were stained with LIVE/DEAD far red fixable dead cell stain (ThermoFisher Scientific) per manufacturer's protocol and fixed using 1%–2% paraformaldehyde and transferred to cluster tubes (Corning, ThermoFisher Scientific). CountBright beads (ThermoFisher Scientific) were added (25 μL/tube) and 9,000–12,000 beads were acquired using a FACSVerse flow cytometer (BD Biosciences) in triplicate. Data were analyzed using FlowJo software (Tree Star, Inc.) and percent total killing calculated as [% dead target cells with treatment] − [% dead target cells without treatment].

Cytokine multiplex immunoassay

Supernatants from the cell-killing assays as described above were collected and analyzed for IFNγ, IL-2, IL-4, IL-6, IL-10, and TNFα by Ciraplex™ cytokine immunoassay kit (Aushon BioSystems, Billerica, MA) following the manufacturer's protocol at the indicated E:T ratios. Recombinant proteins were used to generate a standard curve and pg/mL concentrations graphed as mean ± SD of duplicate samples.

Statistical analysis

Apoptosis data were analyzed using the unpaired, 2-tailed Student's *t* test. Cell killing and cytokine secretion assays were analyzed using an unpaired *t* test with the Holm–Sidak correction method with Prism (GraphPad software). A *P* value less than 0.05 was considered statistically significant.

Results

Production and characterization of anti-CD20-hIFNα14 fusion protein

Anti-CD20-hIFNα14 consists of IFNα14 genetically fused by a SGGGGS linker to the end of the C_H3 domain of human IgG1 containing the V regions of the anti-CD20 antibody, rituximab (Xuan and others 2010) (Fig. 1A). As a non-CD20 targeting control, IFNα14 was similarly fused to an anti-CD138 antibody (Yoo and others 2015).

Anti-CD20-hIFNα14 and rituximab showed similar binding to CD20-expressing Daudi cells (Fig. 1B). Treatment of lymphoma cells with anti-CD20-hIFNα14 had minimal effect on the expression of CD19, with only slight downregulation among several cell lines tested, including Daudi, Raji, Granta-519, Jeko-1, and OCI-Ly2 (data not shown). There was no upregulation in PD-L1 expression under these same conditions (data not shown). The CD20-negative cell line, OVCAR-3 was used to evaluate the antiproliferative activity of the untargeted IFNα14 in the fusion protein. Anti-CD20-hIFNα14 retained IFNα14 activity with an IC50 of 115.2 pM, but is attenuated when compared to rhIFNα14 with an IC50 of 3.7 pM (Fig. 1C, left panel). However, with Daudi, an IFNα-sensitive CD20-positive cell line, anti-CD20-hIFNα14 showed superior growth inhibition compared to rhIFNα14 (IC50 of 0.47 pM compared to IC50 of 1.17 pM) (Fig. 1C, right panel). A nontargeted control fusion protein anti-CD138-hIFNα14 showed less activity (IC50 of 2.95 pM) compared to targeted anti-CD20-hIFNα14. Anti-CD20 (rituximab) did not inhibit the proliferation of Daudi under these conditions. Thus, anti-CD20-hIFNα14 has potent antiproliferative activity which is enhanced by targeting to the target cell surface.

Anti-CD20-hIFNα14 induces increased apoptosis among B cell lymphoma cell lines compared to rituximab

To evaluate whether anti-CD20-hIFNα14 was more effective in promoting apoptosis compared to equimolar concentrations of rituximab or equivalent concentrations of rhIFNα14, a panel of cell lines, including OCI-Ly19, Daudi, Jeko-1, and OCI-Ly2 was incubated with graded concentrations of anti-CD20-hIFNα14, rituximab, or rhIFNα14. At all concentrations, anti-CD20-hIFNα14 was more effective in causing apoptosis compared to rituximab with $P < 0.005$ for all cell lines (Fig. 1D). Even with its attenuated IFNα bioactivity, anti-CD20-hIFNα14 had comparable or improved effectiveness in causing apoptosis compared to rhIFNα14, except for OCI-Ly2 where rhIFNα14 was superior.

Anti-CD20-hIFNα14 inhibits proliferation of CD20-positive lymphoma cell lines

We then examined the growth inhibitory properties of anti-CD20-hIFNα14 against CD20-positive human B cell lymphoma lines representing different histologies. This panel included Burkitt lymphomas (Daudi, Raji), germinal center B cell (GCB) DLBCLs (OCI-Ly2, OCI-Ly19), mantle cell lymphomas (MCL) (Jeko-1, Granta-519), and an early passage DLBCL established in our laboratory (RS-27) (Fig. 2). Anti-CD20-hIFNα14 was more effective than rituximab at inhibiting the proliferation of all cell lines and similar to rhIFNα14. The CD20-negative anaplastic large cell lymphoma cell line (DEL), used as a negative control in the tumor mixture assay, showed no difference in percent proliferation when incubated with anti-CD20-hIFNα14 or rituximab. Given the activities of anti-CD20-hIFNα14, we hypothesized that CD19 CAR T cells may have enhanced killing when given in combination with anti-CD20-hIFNα14.

Anti-CD19 CAR T cells specifically lyse CD19-positive lymphoma cells

CD19 CAR T cells were produced as described (see Materials and Methods section). CD19 transduction of CAR T cells was verified by flow cytometry and ranged from 45% to 77%. Vector copy number ranged from 1.30 to 1.78 copies/cell. Initial cell killing experiments of CD19 CAR and Mock T cells utilized a tumor cell mixture of CD19-negative (DEL) and CD19-positive (Raji) cell lines. DEL and Raji cells were mixed at equivalent ratios and incubated with effector:target (E:T) ratios of 125:1, 25:1, 5:1, and 1:1 with either CD19 CAR or Mock T cells. Specific lysis was seen with CD19 CAR T cells but not Mock T cells for all E:T ratios. Thus, CD19 CAR T cells demonstrated antigen-specific killing of the CD19-positive cell line in an E:T dose-dependent manner. Mock T cells did not kill either cell line (Fig. 3). Several other paired CD19-negative and CD19-positive cell lines (SUP-M2/RS-27, DEL/Granta-519, H929/RS-27) showed similar specific lysis (data not shown), thereby confirming the CD19 CAR T cell specificity and dose-dependence.

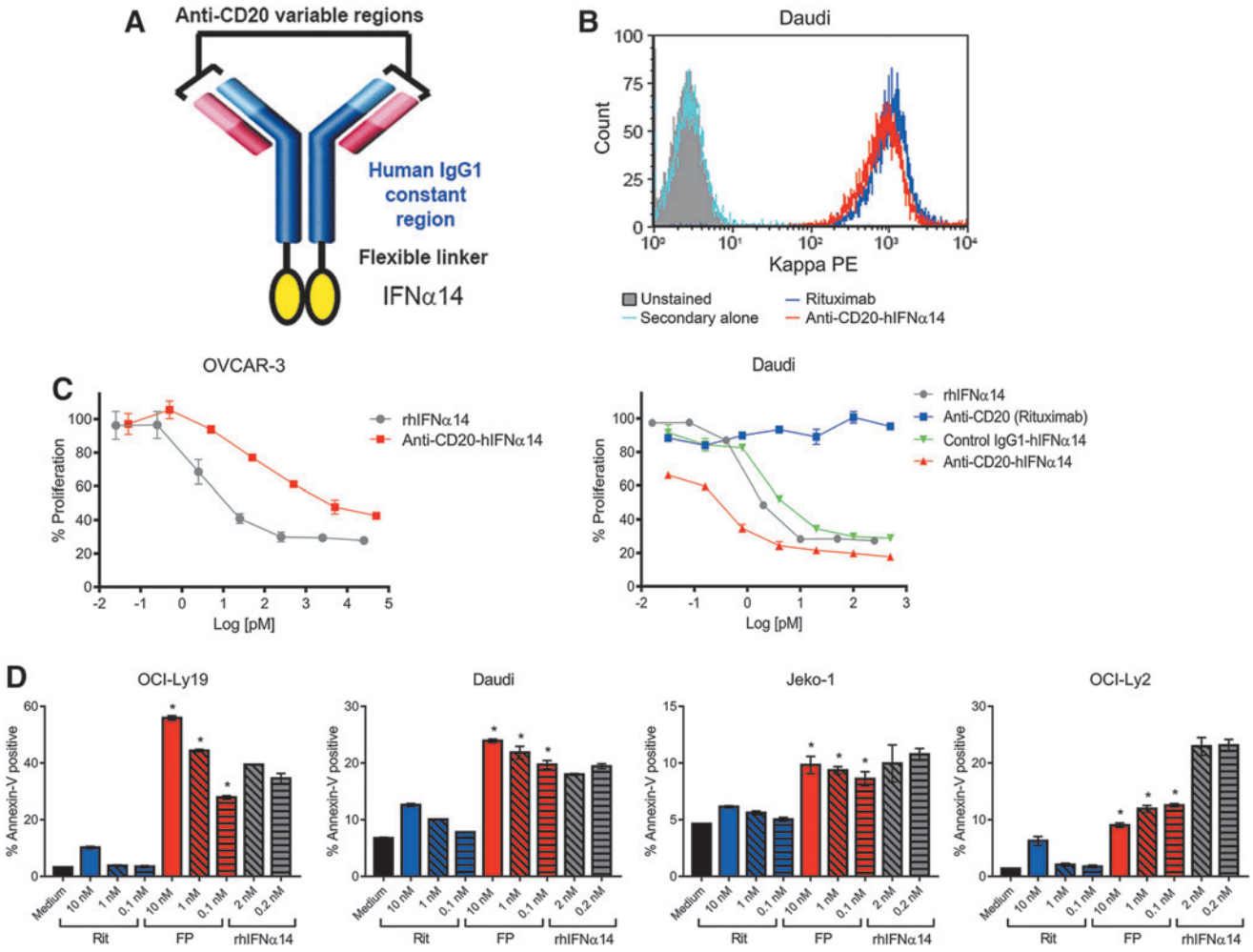

FIG. 1. Anti-CD20-hIFNα14 fusion protein characterization. **(A)** Diagram of the fusion protein, in which hIFNα14 was joined to the C-terminus of human γ1 heavy chains specific for CD20. The heavy chains were expressed with the appropriate light chain to generate the antigen-specific fusion protein. **(B)** Anti-CD20-hIFNα14 retains the ability to bind to CD20 similar to rituximab. Daudi tumor cells were treated with medium, rituximab, or anti-CD20-hIFNα14 fusion protein and analyzed by flow cytometry using FlowJo software. **(C)** Anti-CD20-hIFNα14 retains IFN activity and shows superior growth inhibition with antigen-specific targeting. OVCAR-3 (CD20-negative) or Daudi (CD20-positive) cells were treated with graded concentrations of recombinant IFNα14 (rhIFNα14), anti-CD20 (rituximab), nontargeted IgG1-hIFNα14 (anti-CD138-hIFNα14), or anti-CD20-hIFNα14 and incubated for 72 h before an MTS assay was performed to measure percent proliferation. Data are shown as percent proliferation of the mean ± SD of triplicates. **(D)** Anti-CD20-hIFNα14 induces more apoptosis than rituximab. Tumor cells were treated with medium or graded concentrations of rituximab, anti-CD20-hIFNα14, or rhIFNα14 as indicated and incubated for 72 h. Apoptosis was assessed by annexin V-FITC/PI staining and analyzed by flow cytometry. Data are displayed as mean ± SD for triplicate values of % annexin V-positive cells. Rit = rituximab and FP = anti-CD20-hIFNα14. $*P < 0.005$ comparing Rit and FP. PI, propidium iodide.

Anti-CD20-hIFNα14 plus CAR T cells demonstrate enhanced cell killing in cytotoxicity assays

We next evaluated whether anti-CD20-hIFNα14 could enhance the cytotoxic effects of CD19 CAR T cells against the lymphoma cell line panel described above. Tumor cells were pretreated with medium alone (no treatment), anti-CD20-hIFNα14, or rituximab at equimolar concentrations for 18–24 h and labeled with CFSE as described above. The CFSE-labeled tumor cells were then plated with medium, anti-CD20-hIFNα14, or rituximab at the equivalent pretreatment concentrations and CD19 CAR or Mock T cells added in varying E:T ratios and cocultured for 24 h. Combination of anti-CD20-hIFNα14 plus CD19 CAR T cells resulted in enhanced cell killing in the majority of the cell lines tested compared to rituximab or untreated cells (Fig. 4). Anti-CD20-hIFNα14 treated Daudi cells plus CD19 CAR T cells showed marked enhancement (nearly double) of cell killing at both 1 and 0.1 nM ($P < 0.05$). The fusion protein-treated combination group also showed significant total cell killing compared to rituximab or untreated cells in the OCI-Ly19 cell line, particularly at 10 nM treatment ($P < 0.05$). At all E:T ratios, fusion protein pretreatment resulted in statistically significant increased total cell killing of the Granta-519 cell line at 1 nM and OCI-Ly2 at 10 nM ($P < 0.05$). Pretreatment of the RS-27 cell line with anti-CD20-hIFNα14 at lower E:T ratios showed modest, yet statistically significant increased cell killing with anti-CD20-hIFNα14 plus CAR T cells when compared to

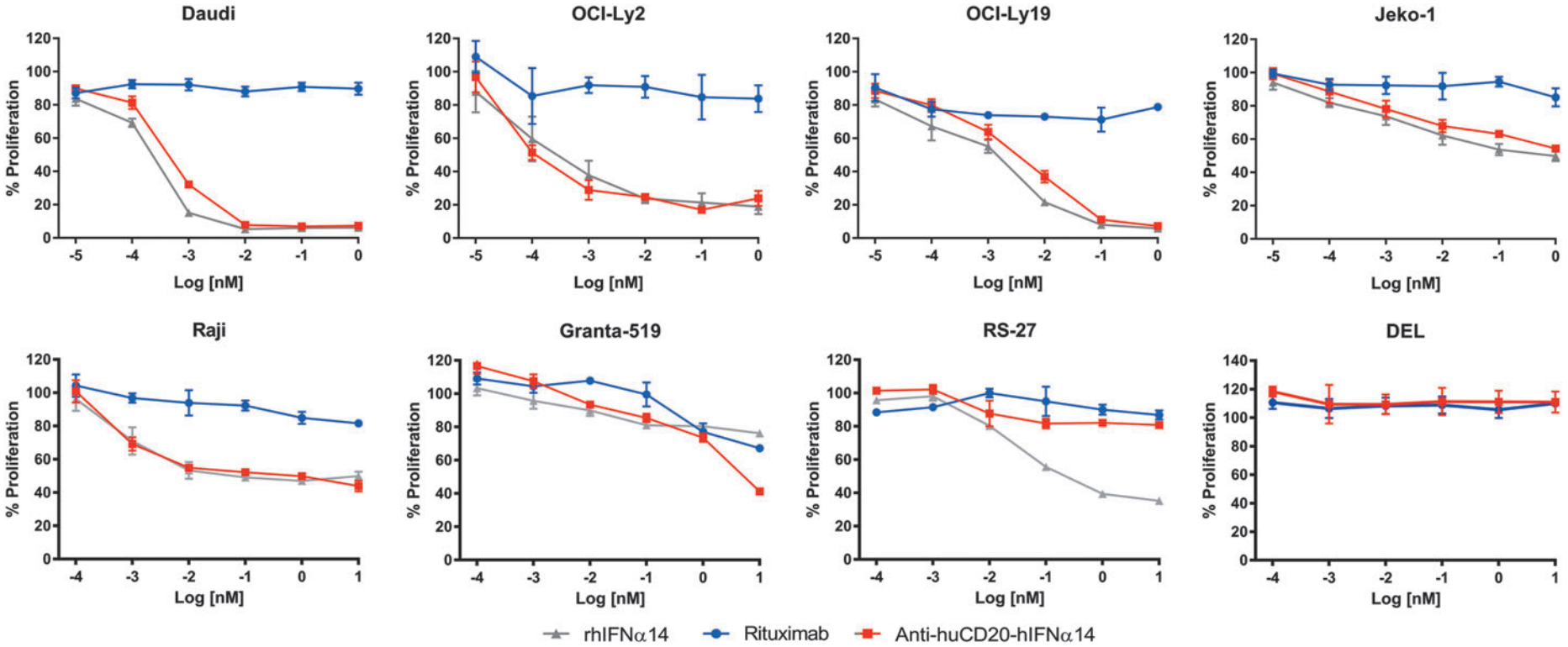

FIG. 2. Anti-CD20-hIFNα14 potently inhibits the proliferation of human B cell lymphomas compared to rituximab. Tumor cells were treated with medium, rituximab, anti-CD20-hIFNα14, or rhIFNα14 as shown and incubated for 72 or 24 h (DEL). Cells were pulsed with ^{3}[H]-thymidine and incorporated radioactivity (counts per minute) was measured using a β-liquid scintillation analyzer. Percent proliferation was calculated and shown as mean ± SD of quadruplicate values.

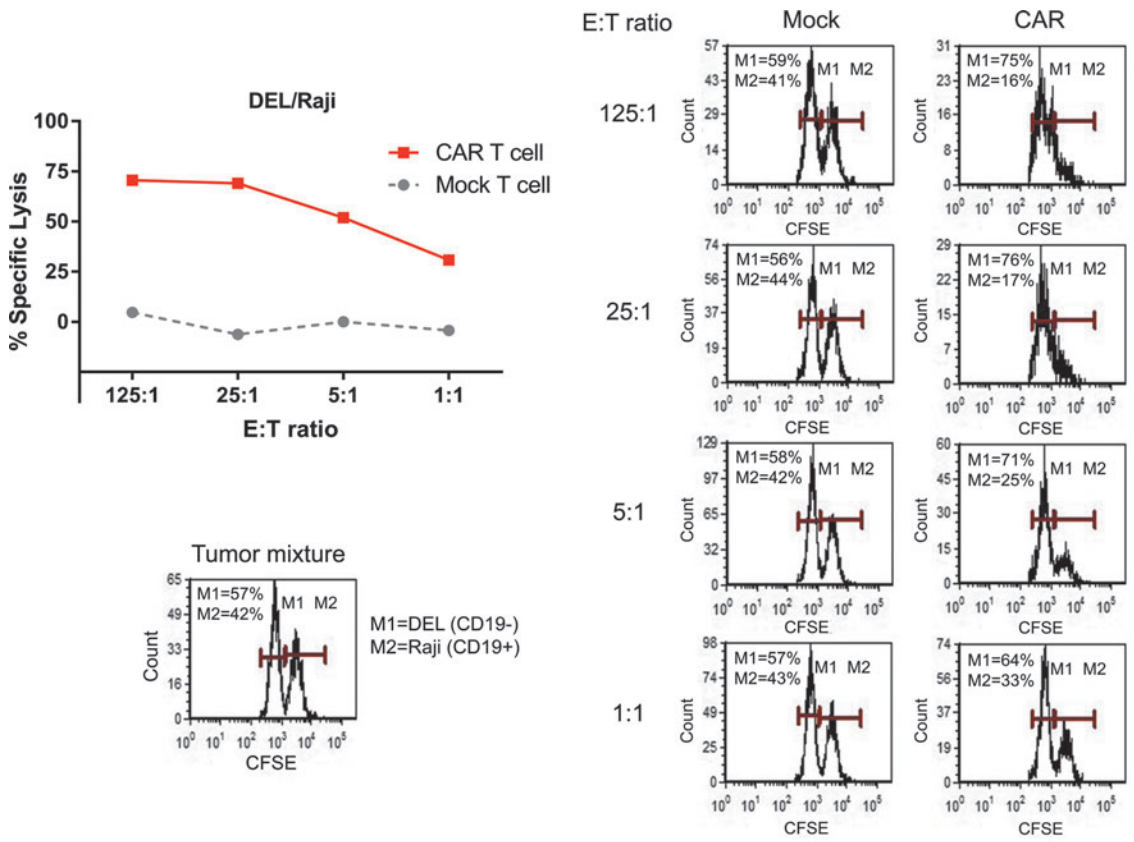

FIG. 3. Cytolytic activity of CD19 CAR T cells against lymphoma cells. Mixtures of DEL (CD19-negative, CFSE low) and Raji (CD19-positive, CFSE high) tumor cells (targets) were plated with day 14 posttransduction CD19 CAR or Mock T cells (effectors) at the designated effector:target (E:T) ratios. Plates were incubated for 2 h at 37°C and then cells were stained with PI and analyzed immediately by flow cytometry and shown on the *right* in histograms as % gated of M1 or M2 and on *left* as % specific lysis. Tumor mixture cells alone with no added effector cells are shown below the % specific lysis graph.

rituximab plus CAR T cells. The fusion protein pretreatment did not show significantly enhanced cell killing at any of the concentrations tested in Jeko-1. Higher concentrations of fusion protein did not necessarily lead to higher levels of cytotoxicity (for example Granta-519 at 10 nM). When tumor cells were treated with Mock T cells, only background levels of cell killing were noted. Overall, the addition of anti-CD20-hIFNα14 increased the tumor cell killing by effector CAR T cells even in these short-term overnight cocultures.

Anti-CD20-hIFNα14 treatment of lymphoma cells causes increased cytokine secretion by CD19 CAR T cells

To further explore the effects of anti-CD20-hIFNα14 on the response of CD19 CAR T cells to lymphoma cells, we evaluated the supernatants from the CAR and Mock tumor cell-killing assays for cytokines, including IFNγ, IL-2, TNFα, IL-10, IL-4, and IL-6. With the OCI-Ly2 cell line, despite only modest enhancement of cell killing by CAR T cells at 10 nM of anti-CD20-hIFNα14 and no enhancement at 1 and 0.1 nM (data not shown), there was a substantial increase in the release of cytokines compared to rituximab and no treatment, $P < 0.05$ (Fig. 5). Overall, cytokine production did not appear to be dependent on the dose of anti-CD20-hIFNα14 and in some cases the lowest dose resulted in the largest enhancement of cytokine secretion. CD19 CAR T cells in the absence of tumor cells, but with added rituximab or anti-CD20-hIFNα14, showed only background levels of cytokines. Mock T cells combined with anti-CD20-hIFNα14 showed only minimal increases in cytokine secretion (data not shown). Target lymphoma cells without the addition of T cells, but treated with rituximab or anti-CD20-hIFNα14 fusion protein secreted negligible amounts of cytokines (data not shown).

Surprisingly, even among the cell lines that did not show enhanced killing, there was a significant increase in cytokine production by the CAR T cells when comparing anti-CD20-hIFNα14 treatment to rituximab or tumor alone. For all of the cell lines tested, anti-CD20-hIFNα14 plus CD19 CAR T cell combination therapy showed a significant increase in IFNγ, IL-2, and IL-10 production compared to rituximab or untreated cells. Among the fusion protein plus CAR T cell-treated cell lines, 66% showed a significant increase in TNFα, and 50% showed a significant increase in IL-4 and IL-6 (Fig. 6). Thus, anti-CD20-hIFNα14 uniformly increased cytokine release by CD19 CAR T cells, regardless of whether there was enhanced cell killing.

Limited anti-CD20-hIFNα14 exposure shows enhanced CAR T cell killing and increased cytokine production similar to prolonged coculture

To replicate *in vivo* conditions, where anti-CD20-hIFNα14 targets to tumor cells and unbound fusion protein clears, we performed *in vitro* assays with anti-CD20-hIFNα14 pretreatment without adding anti-CD20-hIFNα14 back to the

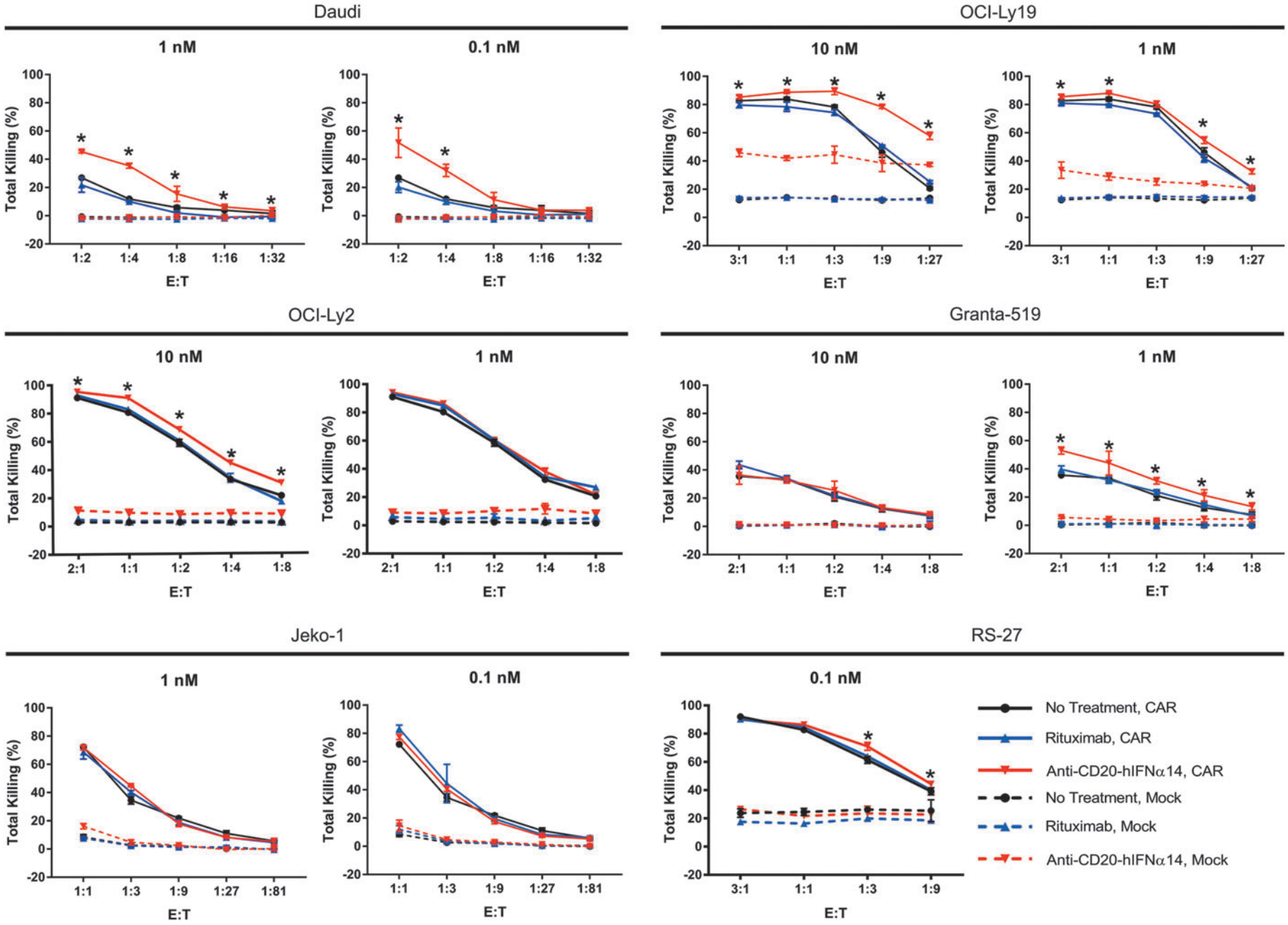

FIG. 4. Anti-CD20-hIFNα14 in combination with CD19 CAR T cells enhances cell killing compared to rituximab. Human lymphoma cell lines were pretreated with medium or with the indicated concentrations of rituximab or anti-CD20-hIFNα14 fusion protein for 18–24 h. Cells were CFSE stained and plated in triplicate. Medium, rituximab, or anti-CD20-hIFNα14 at the same pretreatment concentrations were added back to the appropriate wells and incubated for 30 min before CD19 CAR or Mock T cells were added at the designated E:T ratios and cocultured for 24 h. Cells were analyzed by flow cytometry and % total killing calculated. $*P < 0.05$ when comparing rituximab and anti-CD20-hIFNα14-treated cell lines in combination with CD19 CAR T cells.

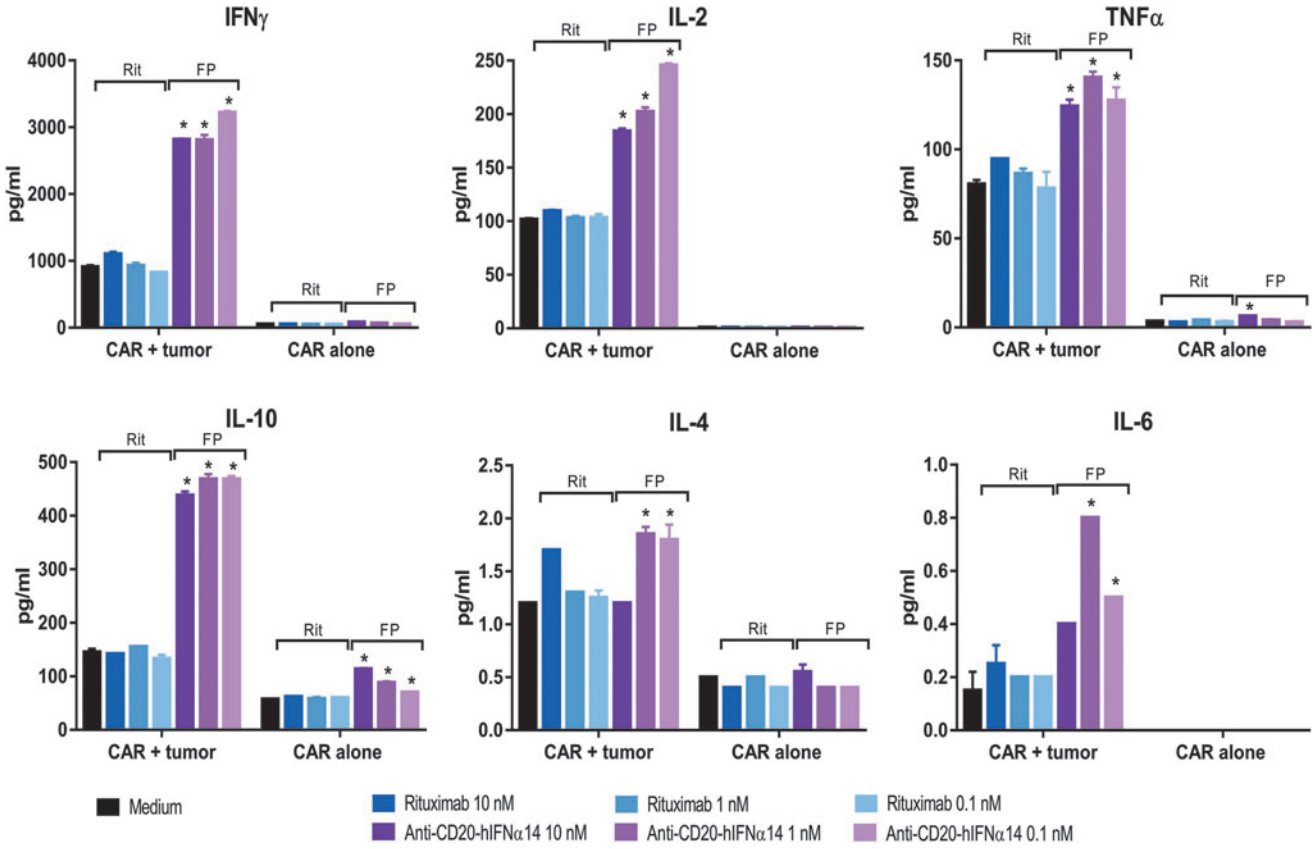

FIG. 5. The combination of anti-CD20-hIFNα14 plus CD19 CAR T cells results in substantially increased cytokine production by effector T cells targeting OCI-Ly2 lymphoma cells. Supernatants from the 24 h coculture of the cell-killing assay, as previously described, were collected and analyzed for IFNγ, IL-2, TNFα, IL-10, IL-4, and IL-6 by Ciraplex™ cytokine immunoassay kit. The tumor cells were treated at the indicated concentrations of rituximab or anti-CD20-hIFNα14 and CD19 CAR T cells added at an E:T ratio of 1:3. Recombinant cytokines were used to generate a standard curve and pg/mL concentrations graphed as mean ± SD of duplicate samples. $*P < 0.05$ when comparing rituximab and anti-CD20-hIFNα14 combination treatment. Rit = rituximab and FP = anti-CD20-hIFNα14.

coculture. Daudi, Jeko-1, and OCI-Ly2 tumor cells were treated with medium, anti-CD20-hIFNα14, or rituximab at the indicated concentrations for 18–24 h and then cocultured with CAR or Mock T cells as above without adding soluble fusion protein. Under these conditions effector CAR T cells would only be influenced by cell-bound fusion protein or antibody. Similar to the experiments in which soluble fusion protein was added to the coculture, enhanced cell killing was seen with the anti-CD20-hIFNα14 plus CAR T cell combination (Fig. 7). Markedly increased cytokine production was also observed independent of the degree of cytotoxicity (Fig. 7).

Discussion

There is great unmet need for improving the ability of adoptively transferred T cells to infiltrate into cancers and then achieve optimal tumor-killing potency. Many patients' cancers resist infiltration by T cells, and in others the cells become inactivated or weakened upon reaching the tumor microenvironment. While CAR T cell therapy has been shown to be effective in several types of lymphomas and leukemias, most patients do not achieve durable remissions or cures with CAR T cell therapy alone (Abramson and others 2017; Neelapu and others 2017). Potential explanations for the unsustained responses may include loss of CD19 expression, CAR T cell exhaustion/target cell expression of PD-L1, lack of proliferation or survival, and poor trafficking of CAR T cells to the tumor site. Among the successive generations of CAR T cells, the first generation CAR T cells failed to induce adequate cytokine production and T cell expansion, resulting in suboptimal antitumor effects. The second generation CAR T cells, with the addition of a costimulatory molecule to the CD3ζ signaling domain, resulted in increased cytokine production and improved tumor regression (Kershaw and others 2013; Slaney and others 2014). Cytokine production appears to be necessary for optimal antitumor effects of CAR T cells. Thus, by combining antibody-IFN fusion protein therapy with CAR T cells, therapeutic efficacy may be improved.

There are several strategies that have been proposed for boosting ACT in cancer, but none has the unique properties of antibody-IFN fusion proteins. Specifically, anti-CD20-hIFN fusion proteins and CD19 CAR T cells utilize a 2-pronged attack against lymphoma cells with both CD19 and CD20 lymphoma-associated antigens being targeted. Antibody-IFN fusion proteins can localize to all sites of tumor in the body, thus permitting potentiation of ACT. By treating the patient with antibody-IFN fusion proteins, the IFN reaching the tumor sites can result in immunologic reactions that could be

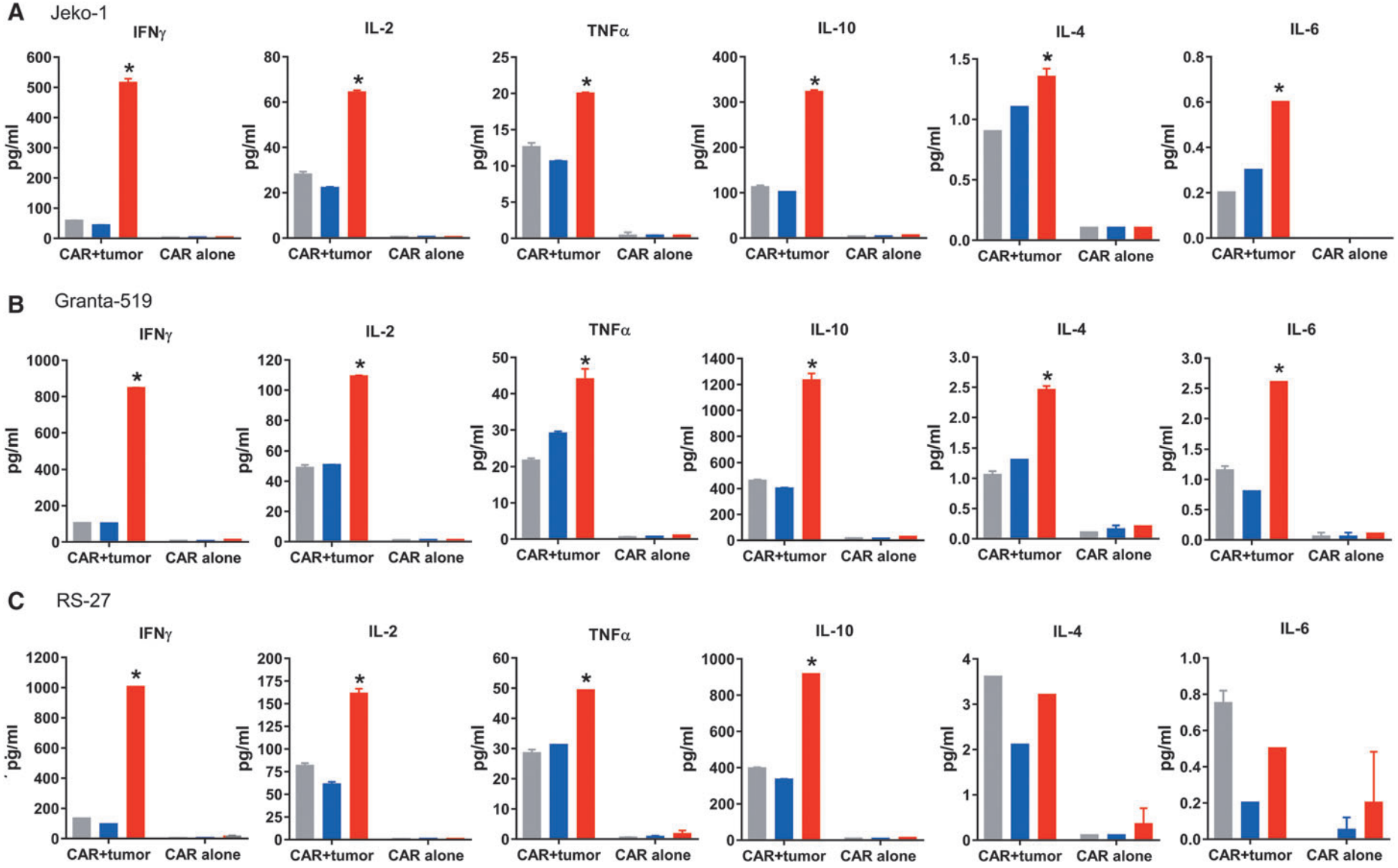

FIG. 6. Anti-CD20-hIFNα14-treated tumor cells in combination with CD19 CAR T cells elicit increased cytokine production compared to rituximab when targeting multiple B cell lymphomas. Assays were carried out as described in Fig. 5. Tumor cells were treated with medium or the indicated concentrations of rituximab or anti-CD20-hIFNα14: **(A)** Jeko-1 with 0.1 nM and E:T 1:1, **(B)** Granta-519 with 10 nM and E:T 2:1, **(C)** RS-27 with 0.1 nM and E:T 1:1, **(D)** Daudi with 0.1 nM and E:T 1:2, and **(E)** OCI-Ly19 with 1 nM and E:T 1:9. $*P < 0.05$ when comparing rituximab and anti-CD20-hIFNα14 combination treatment.

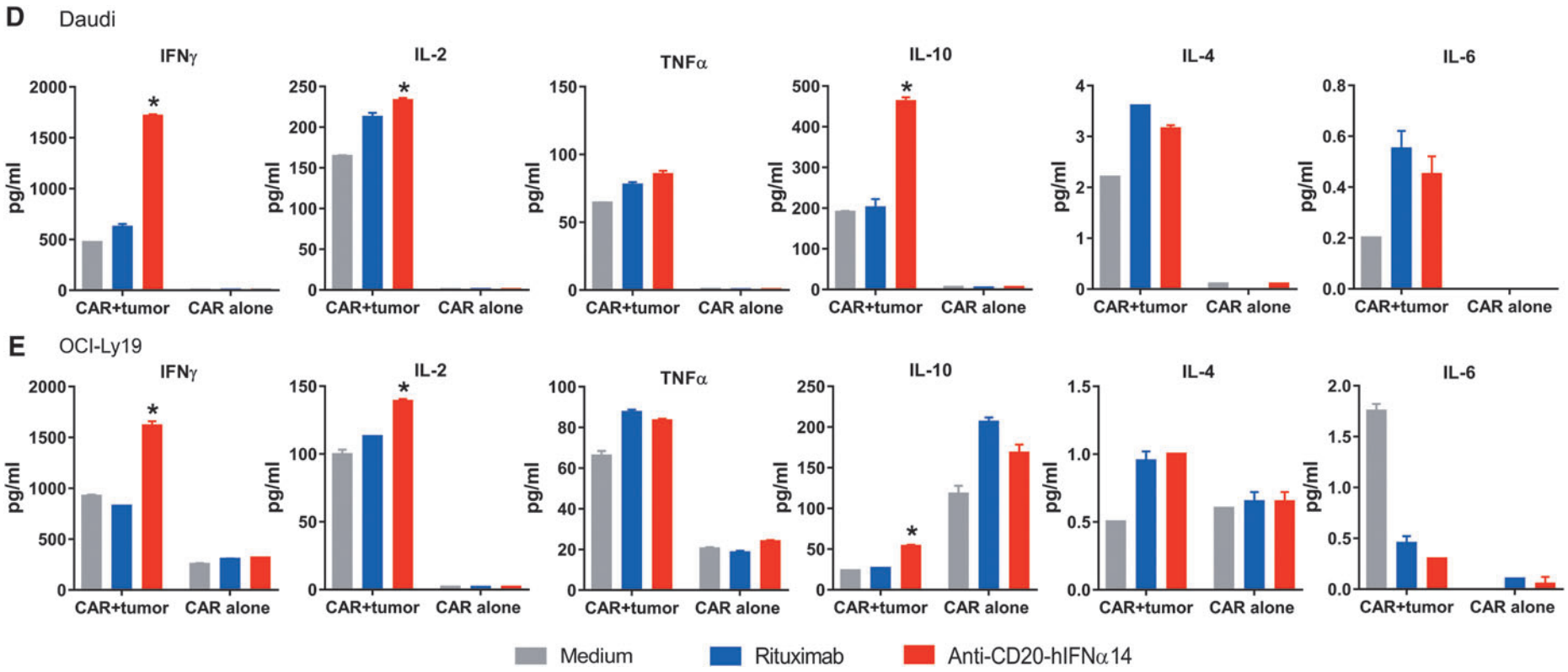

FIG. 6. (Continued)

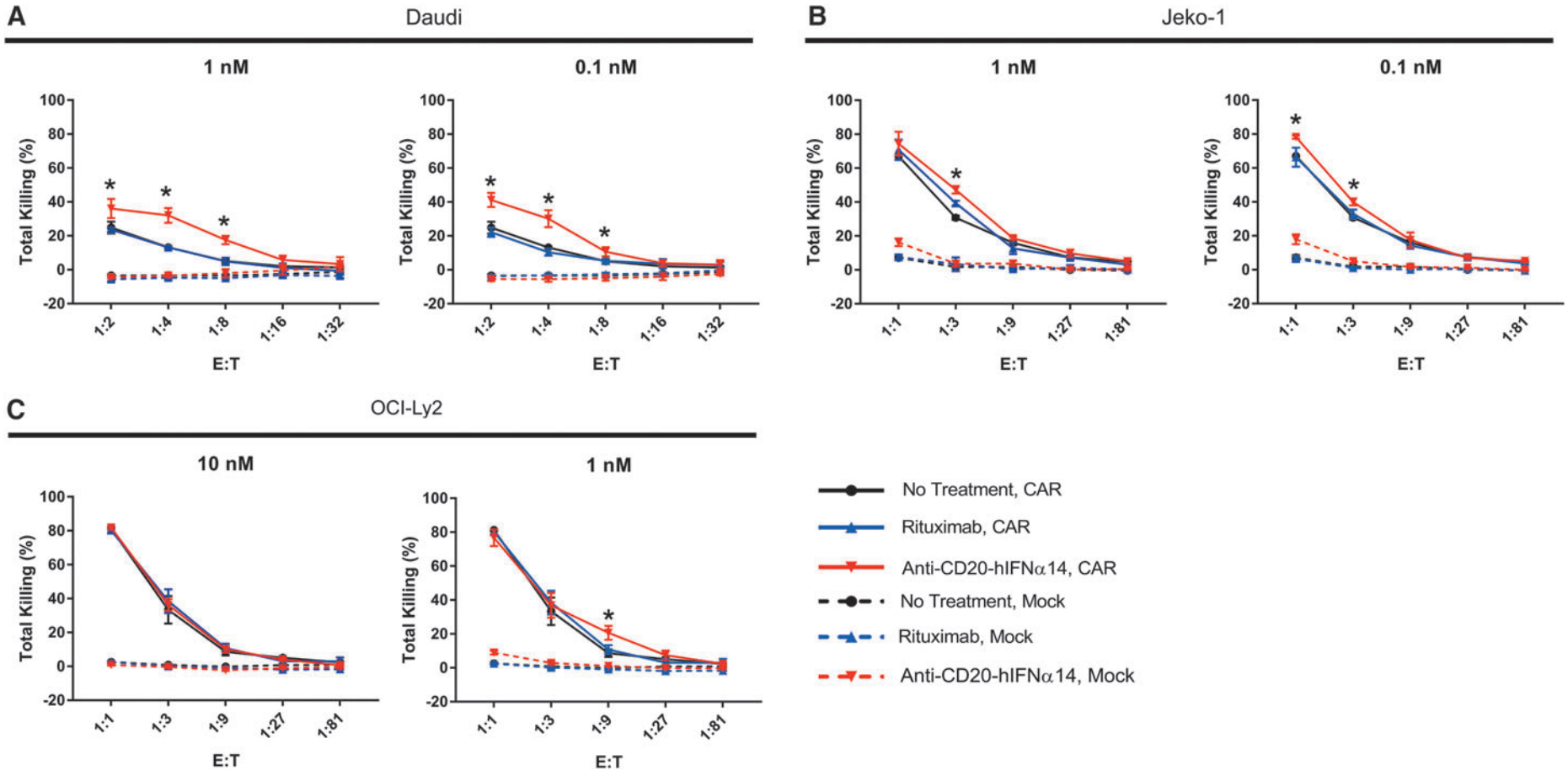

FIG. 7. Anti-CD20-hIFNα14 pretreatment only of lymphoma cells in combination with CD19 CAR T cells shows enhanced cell killing and increased cytokine production when compared to rituximab. **(A–C)** Assays were carried out as described in Fig. 4 with the exception that medium was added back to the wells instead of pretreatment concentrations of rituximab or anti-CD20-hIFNα14. Assays for cytokine production were carried out as described in Fig. 5 for **(D)** Jeko-1 with 0.1 nM and E:T 1:1, **(E)** OCI-Ly2 with 10 nM and E:T 1:1, and **(F)** Daudi with 0.1 nM and E:T 1:2. $*P < 0.05$ when comparing rituximab and anti-CD20-hIFNα14 combination treatment.

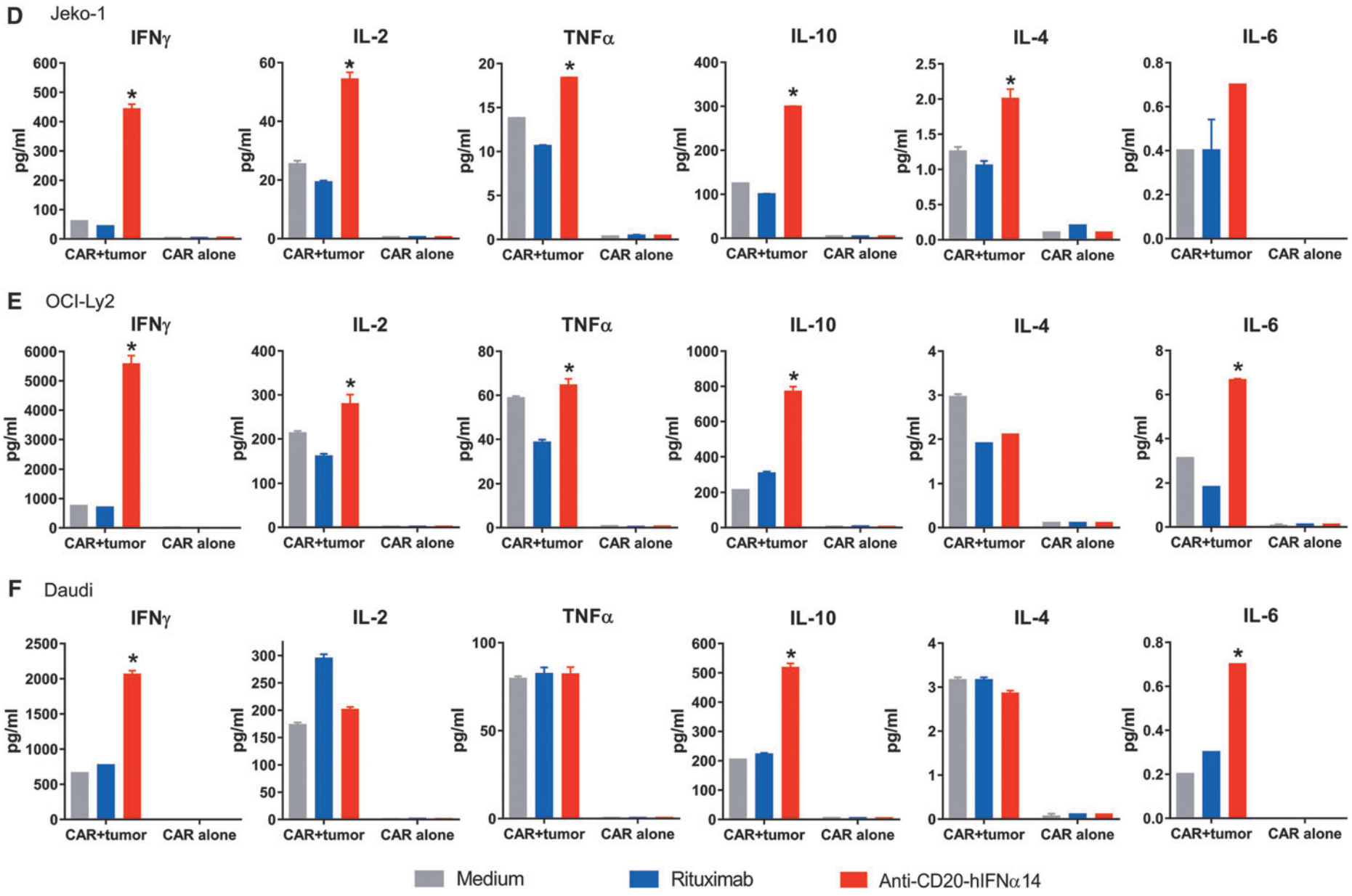

FIG. 7. (Continued)

expected to weaken the tumor cells by inhibiting their growth, altering their expression of cell surface molecules, thereby making them more recognizable to T cells (eg, adhesion, costimulation and human leukocyte antigen molecules), inducing local production of other cytokines and chemokines that promote T cell infiltration into tumors, and activating T cells that localize to the tumor site to attain more potent cytolytic functions.

We showed that pretreatment of lymphoma cells with anti-CD20-hIFNα14 can lead to sensitization for CAR T cell lysis and enhanced cytokine production. Interestingly and more biologically relevant, similar results were obtained when anti-CD20-hIFNα14 pretreated lymphoma cells (performed for Daudi, Jeko-1, and OCI-Ly2) were cocultured with CAR T cells only without further addition of anti-CD20-hIFNα14. This indicates that tumor cell-bound anti-CD20-hIFNα14 is responsible for enhancing CAR T cell effector functions. The observed enhanced tumor cell killing and increased cytokine production by CAR T cells, coupled with the known ability of IFNs to promote T cell infiltration and activation within tumors, suggest that this combined approach may contribute to a significant improvement in CAR T cell efficacy (Zitvogel and others 2015).

With the combination of anti-CD20-hIFNα14 and CAR T cells, we have found significant direct killing, but in a greater proportion of cell lines with differing histologies, including Burkitt, GCB DLBCL, and MCL lines, we have found a substantial enhancement in cytokine release with the combination therapy. Cytokine release syndrome (CRS) is a well-documented side effect of CAR T cell therapy, characterized by secretion of large quantities of cytokines (including IL-6, TNFα, and IFNγ) and is associated with T cell activation (Davila and others 2014). The condition seems to correlate with tumor type and burden, genetic polymorphisms, and perhaps certain vector constructs (Lee and others 2014; Xu and Tang 2014). With CRS, there is infiltration and recruitment of T cells to the tumor bed. Whether this increase in cytokines correlates with efficacy remains unanswered. However, extrapolating from the ALL experience, it appears that higher CRS grades are associated with greater CAR T cell expansion and persistence in responders (Porter and others 2015; Mueller and others 2017). In patients where CRS is symptomatic, the condition can be addressed by supportive care, corticosteroids, and anti-IL-6 therapy, tocilizumab. We have shown a significant increase in cytokine release when tumor cells are pretreated with anti-CD20-hIFNα14 compared to when tumor cells are treated with CAR T cells alone, which may correlate with enhanced T cell activation and efficacy *in vivo*.

We observed improvement in CAR T cell activity in direct killing and cytokine production in the presence of anti-CD20-hIFNα14. However, the limitations of these *in vitro* experiments include a brief coculture of fusion protein, CAR T cells, and target cells, and thus may underestimate the effects of combination therapy. Even though this *in vitro* system does not represent the intact tumor microenvironment, we were still able to show enhanced direct killing of target cells and a clear escalation in cytokine release by CAR T cells with combination therapy. Even in the absence of enhanced cell killing, there was increased cytokine production, thereby suggesting the potential for even greater cytotoxicity and activation of T cells. Future *in vivo* studies with antibody-targeted IFN therapy plus CD19 CAR T cells in syngeneic lymphoma/leukemia models are thus indicated to further explore the potential of this combination therapeutic approach. CAR T cells are at the forefront of cancer immunotherapy, but obtaining a high frequency of durable remissions and cures remains a challenge. These experiments highlight how antibody-targeted IFN can sensitize tumor cells for lysis and augment CAR T cell activation and cytokine production. Thus, combining antibody-targeted IFN with CAR T cells or other forms of ACT may be a promising new approach for treating patients with B cell lymphomas and other cancers.

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Author Disclosure Statement

No competing financial interests exist.

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