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14. ABSTRACT

Major advances have been made in two main areas. Firstly, Receptor tyrosine kinase-like orphan receptor-1 (ROR1) was identified as a tumor antigen expressed on ovarian cancer (OvCa), but not expressed on normal tissue(s). Second generation chimeric antigen receptors (CARs) were designed with CD3z and either CD28 or CD137 endodomains fused to the antigen-binding region of a ROR1-specific monoclonal antibody (clone 4A5). CARs were stably expressed in T cells following *Sleeping Beauty* transposition and propagation on $ROR1^+$ artificial antigen presenting cells (aAPC). Re-directed cytolysis of $ROR1^+$ OvCa cell lines by CAR^+ T cells was demonstrated. Secondly, the anti-tumor activity of γδ T cells was harnessed to kill OvCa. aAPC were used to expand a polyclonal population of γδ T cells for immunotherapy. OvCa xenografts were eliminated by polyclonal γδ T cells.

15. SUBJECT TERMS

ROR1, γδ T cells, adoptive T cell therapy, ovarian cancer, chimeric antigen receptor (CAR)

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INTRODUCTION

Ovarian cancer, hypoxia, and tumor antigens. Relapsed ovarian cancer (OvCa) is refractory to current therapies in many cases and remains a lethal disease for women worldwide.[1, 2] Advanced ovarian tumors have significant areas that are hypoxic (low O_2 concentration), which can lead to therapeutic escape and relapse in women who initially respond to therapy.[3-5] Therapies targeting tumor-associated antigens (TAAs) expressed in both hypoxic and normoxic regions of ovarian tumors could dramatically increase survivorship in women with advanced disease. c-Met, hepatocyte growth factor receptor (HGFR), has been identified as a candidate TAA because of its well-described expression on OvCa that increases in hypoxia.[6-8] However, c-Met is also expressed on normal tissues, such as liver and lung,[9] thereby complicating its utility as a TAA for cell-based therapy. In contrast to c-Met, the TAA receptor tyrosine kinase-like orphan receptor-1 (ROR1) is not expressed on normal adult tissues, but is expressed on a variety of cancers, including OvCa.[10-12] ROR1 expression was maintained in hypoxia in OvCa cell lines (**Figure 1**), suggesting that targeting ROR1 may be a safe and effective strategy for OvCa immunotherapy.

Chimeric Antigen Receptors. Chimeric antigen receptors (CARs) are employed to re-direct T-cell specificity to TAAs. They are fashioned by fusing T-cell signaling endodomains (CD3ζ, CD28, and CD137) to a single chain variable fragment (scFv) derived from a monoclonal antibody (mAb) with specificity to a cell surface TAA. Upon docking with TAA the introduced CAR activates genetically modified T cells outside of the constraints of major histocompatibility complex (MHC) interaction with the endogenous T-cell receptor (TCR). The CAR contains both signal 1 (CD3ζ) and signal 2 (CD28 or CD137) to achieve full T-cell activation. Clinical trials are investigating the therapeutic efficacy of CAR-based therapies.[13] Patients with refractory B-cell malignancies who were recently treated with CD19-specific CAR^+ T cells employing a CD137 endodomain achieved complete responses.[14, 15] Thus, ROR1 was targeted in this study with CAR^+ T cells expressing either CD28 (ROR1RCD28) or CD137 (ROR1RCD137) endodomains (**Appendix 1**).

Adoptive T-cell therapy, graft-versus-host disease, and γδ T cells. Allogeneic adoptive T-cell therapy (allo-ACT) is a cancer treatment modality used in clinical trials. Allo-ACT is particularly useful when autologous T

cells cannot be generated, *e.g.*, after/during chemotherapy.[16, 17] However, there is the risk of graft-versus-host disease (GvHD) in allo-ACT, where donor TCRαβ inappropriately recognizes the host MHC leading the infused T cells to attack normal tissues. It is difficult to predict GvHD incidence because specificity of the donor-derived TCRαβ chains are uniquely educated within individuals.[18] T cells can be dichotomized into two main subsets based on their TCRs. The conventional αβ T cells express TCRαβ and constitute ~95% of the circulating T-cell pool. In contrast, γδ T cells are less frequent (1-10% of peripheral-blood-derived T cells) and are characterized by TCRγδ. While αβ T cells recognize peptides in the context of MHC, γδ T cells do not necessarily recognize MHC and most of the antigens they recognize are outside of MHC presentation altogether. Therefore, γδ T cells have a reduced risk for GvHD and appear to be a safer option for allogeneic allo-ACT compared with T cells expressing αβTCR.[19]

γδ **T-cell numeric expansion and utility in cancer therapy.** Bisphosphonates have been used to propagate mono- and oligo-clonal subsets of human peripheral blood-derived γδ T cells. The utility of clinical-grade bisphosphonates in γδ T-cell expansion was serendipitously discovered when patients with bone diseases, e.g. osteoporosis, sarcoma, etc., were treated with bisphosphonates to regenerate their bone and sizeable expansions of γδ T cells were subsequently observed *in vivo*.[20] The bisphosphonate-derived Vδ2 T cells are reactive to metabolites in the cholesterol synthesis pathway and display natural anti-tumor reactivity, because tumors are commonly dependent upon cholesterol-rich lipid rafts for growth factor signaling. Moreover, γδ T cells are regarded in general as a critical part of the natural anti-tumor response.[21, 22] Clinical trials have investigated the efficacy of bisphosphonates in tumor therapy with some promising results in both hematological and solid cancers, including OvCa, but were not curative.[23] In contrast, long term survivorship of patients following allogeneic TCRαβ-depleted hematopoietic stem-cell transplantation (HSCT) was predictive based on increased frequency of peripheral γδ T cells that were primarily of Vδ1 origin.[24-26] Polyclonal γδ T cell expansion has not been achieved, which limits the ability to use both anti-tumor subsets of γδ T cells in anti-cancer therapy. A major problem with attempting to ex *vivo* propagate γδ T cells to clinically-appealing numbers is that conventional strategies to expand αβ T cells, *e.g.*, with high-dose IL-2 and OKT3 (anti-CD3), do not support long-term growth of γδ T cells in tissue culture. Furthermore, there are very limited starting quantities of γδ T cells (1-10% of peripheral T cells). The expansion schema used in our lab centers around using K562-derived artificial antigen presenting cells (aAPC) that express cytokines, co-stimulatory molecules,

and TAA as feeder cells to support T-cell growth to clinically relevant numbers. K562 cells are natural targets for allogeneic γδ T cells, and preliminary data suggests that γδ T cells can be expanded with similar kinetics as αβ T cells in compliance with current good laboratory practice (cGLP) and current good manufacturing practices (cGMP) and thus can be rapidly extrapolated into clinical trials. The first clinical trials using *Sleeping Beauty* transposition and aAPC technology is currently open at MD Anderson for treating B cell leukemia with allogeneic αβ T cells re-directed to leukemia with a CD19-specific CAR.

***Sleeping Beauty* transposition and aAPC-based T-cell expansion.** As with the CD19-specific CAR, ROR1 CARs can be expressed in T cells with the *Sleeping Beauty* transposase/transposon gene transfer system. *Sleeping Beauty* transposition is a non-viral gene cut-and-paste mechanism where the transposase enzyme is transiently expressed in the cell, makes excisions at inverted repeats flanking the transposon containing the CAR, and integrates the excised transposon into TA repeats within the genome.[27] After a few days, the transposase enzyme is no longer expressed, and long-term expression of the transposon can be readily achieved. Nucleofection is used to electroporate peripheral blood mononuclear cells (PBMC) with CAR transposon and *Sleeping Beauty* transposase, and stimulations with γ-irradiated aAPC are applied weekly to the cells along with recombinant interleukin-2 (IL-2) and interleukin-21 (IL-21).[28] The aAPC culture imposes selective pressure for CAR^+ T-cell propagation through (i) corresponding antigen expression, (ii) co-stimulation, and (iii) cytokine support, and after four weeks the culture contains >90% CAR^+ T cells at clinically relevant numbers (**Appendix 2**). The electroporated/propagated CAR^+ T cells kill tumors and secrete pro- inflammatory cytokines in a CAR- and antigen-restricted manner, and can allow for conditional production of pro-inflammatory molecules in a canonically immunosuppressive tumor microenvironment.

IL-17 and ovarian cancer. IL-17-polarized T cells exhibit anti-tumor efficacy and are sought after cell lineage for adoptive T cell therapy. More specifically, IL-17 expression and presence of IL-17 producing cell types in tumors are positively associated with survivorship in ovarian cancer.[29, 30] IL-17 is well described to induce pro-inflammatory processes, which can be advantageous in cancer therapy.[31] $CD4^+$ T cells, $CD8^+$ T cells, and γδ T cells have been described as IL-17 producers, and are named Th17, Tc17, and Tγδ17, respectively.[32] Importantly, Th17 cells are inversely correlated with regulatory T cells (Tregs), and Tregs often promote tumor

growth.[33, 34] It was recently shown that inducible co-stimulator (ICOS) polarized $CD4^+$ T cells towards Th17 lineage, and that CD28 co-stimulation reversed the polarization and made the $CD4^+$ T cells express interferon- γ (IFNγ).[35] This is important for the current aAPC expansion strategy because the aAPC express CD86, a ligand for CD28, and most aAPC-expanded T cells are restricted to IFNγ production and not IL-17. γδ T cells are also known to polarize towards either IFNγ or IL-17 producing lineages, although the co-stimulatory requirements are unknown. CD27 expression predicts for IFNγ producing γδ T cells, whereas $CD27^{neg}$ γδ T cells are commonly IL-17 producing lineages.[36] Optimization of the co-stimulatory molecules on the aAPC could allow skewing towards ideal cytokine production and anti-tumor effect that can be induced in the hypoxic environment by T cells.

Summary. This proposal aims to accomplish (i) targeting of ovarian cancer with ROR1-specific T cells under conditions of normoxia and hypoxia and (ii) optimization of the co-stimulation requirements for maximum anti-tumor efficacy and pro-inflammatory cytokine production by the T cells, especially IL-17, in the ovarian tumors. *Sleeping Beauty* gene transfer and aAPC technology will be integral in evaluating the hypotheses, and data generated by this study will serve as the basis for initiating a clinical trial for the safe and effective treatment of disease-resistant ovarian cancer.

BODY

We have adapted our specific aims from the Statement of Work as described below.

Task 1: Development of bi-specific T cells to recognize EBV and ovarian CA cells

- Generate EBV-specific T cells LCL (Mos. 1-2)
- Refine c-Met-specific CAR and propagate c-Met-specific T cells on K562-derived artificial antigen presenting cells (Mos. 1-2)
- Generate EBV and c-Met-bi-specific T cells (Mos. 2-6)
- Functional analysis for redirected specificity targeting ovarian tumor (Mos. 4-8)
- Develop and obtain functional analyses of TK transgene (Mos. 6-8)
- Interim analysis (Mos. 9)

It was determined that ROR1 was a superior TAA compared to cMet, and we have switch the SOW to reflect targeting ROR1 instead of cMet in the Specific Aims beloe. ROR1 is not expressed on normal tissues whereas cMet is highly expressed in number of normal tissues, including liver and lung. Thus, there is no longer a need for TK transgene for conditional ablation in the event of deleterious targeting of cMet on normal cells because ROR1 expression is absent on normal tissues. We have also advanced our understanding of T-cell biology to generate T cells expressing $\gamma\delta$ T-cell receptor (TCR) instead of $\alpha\beta TCR^{+}$ T cells as a cellular template for expressing ROR1-specific CAR. This obviates the need to express CAR in EBV-specific T cells, because the TCR repertoire is no longer an issue for allogeneic transplant with T cells.

Task 2: Development of conditionally-activated T cells to recognize ovarian CA cells

- Develop c-Met-specific CAR fused to ODDD (Mos. 9-14)
- Functional analysis for redirected specificity targeting ovarian tumor cells under hypoxia (Mos. 12-16)
- Interim analysis (Mos. 16).

Targeting ROR1 instead of cMet obviates the need for conditional expression of the CAR with ODDD, because ROR1 is not expressed on normal tissues. Thus, growth in hypoxia to enforce CAR expression is no longer

needed. We did investigate the immunobiology of T cells under conditions of hypoxia, since this mimics the tumor microenvironment.

- **Task 3: To evaluate relative ability of ovarian CA-specific T cells using PET as a biomarker for molecular imaging to determine T-cell persistence and assess anti-tumor response *in vivo*.**
- Develop and evaluate imaging probes for PET and bioluminescence (Mos. 16-17)
- Adoptive transfer of T cells and assess persistence by PET and bioluminescence (Mos. 17-23)
- Adoptive transfer of T cells and assess anti-tumor effect targeting ovarian CA (Mos. 17-23)
- Conditional elimination of infused genetically modified T cells (Mos. 17-23)
- Interim analysis (Mos. 24).

We were able to generate T cells expressing firefly luciferase and undertook BLI, but we did not complete PET-based imaging.

Our data generating ROR1-specific CAR^+ T cells and $\gamma\delta TCR^+$ T cells completed our work to date in which we provide compelling pre-clinical data that CAR^+ T cells targeting ROR1 and $\gamma\delta TCR^+$ T cells can be generated using an approach amenable to their clinical translation.

We **hypothesize** that cell-based therapies for OvCa can be generated by (i) targeting ROR1 with CAR^+ T cells and (ii) expanding polyclonal γδ T cells for clinical use where optimization of their endogenous co-stimulation will maximize anti-tumor efficacy.

Specific Aim #1: To evaluate the ability of ROR1-specific CAR to re-direct the specificity of T cells to kill OvCa.

Experimental Plan: Recent clinical trials treating refractory B-cell malignancies with CD19-specific CAR^+ T cells achieved complete responses using a CD137 endodomain instead of chimeric CD28.[15] It is unknown whether the same would apply to targeting ROR1. Thus, two second generation CAR transposons were generated with CD28 (ROR1RCD28) and CD137 (ROR1RCD137) endodomains. Healthy donor-derived peripheral blood mononuclear cells (PBMC) were electroporated with *Sleeping Beauty* transposase and either ROR1RCD28 or ROR1RCD137 transposons then stimulated with $ROR1^+$ aAPC, IL-2, and IL-21. Sham

electroporations will also be sorted and stimulated in parallel with OKT3 (agonistic CD3 antibody)-loaded aAPC to propagate CAR^{neg} T cells for negative controls. Extended phenotypic analysis for CD3, CD4, CD8, CD56, CD27, CD28, CD62L, CCR7, CD38, CD95, TCRγδ, and TCRαβ were evaluated by flow cytometry. Interferon-γ (IFNγ) expression were evaluated by intracellular cytokine staining following TCR stimulation (PMA/Ionomycin) and CAR stimulation from co-culture with EL4-$ROR1^{neg}$ and EL4-$ROR1^{+}$ tumor targets. Ovarian cancer cell lines (A2780, EFO21, EFO27, IGROV1, OC314, and UPN251) were profiled for ROR1 expression in normoxia (20% O_2) and hypoxia (1% O_2). Four-hour CRA was used to evaluate cytotoxicity against the OvCa and EL4 tumor targets in normoxia and hypoxia. OC314 ($ROR1^{+}$) cell line was genetically modified to express mKate and *firefly Luciferase* (*ffLuc*) to measure tumor burden by fluorescence and bioluminescent imaging (BLI), respectively. *In vivo* tumor clearance of established OC314-mKate-*ffLuc* xenografts (intraperitoneal, i.p.) by CAR^{+} T cells (i.p.) in immunocompromised (NOD.*scid*.$\gamma c^{-/-}$; NSG) mice were monitored by non-invasive BLI following subcutaneous (s.c.) D-Luciferin administration. Immunohistochemistry and fluorescence will be used to corroborate BLI data post-mortem.

Progress Report Part Specific Aim#1. Before proceeding with ROR1 CAR studies, it was important to evaluate ROR1 surface expression on OvCa as a function of oxygen tension. The following ovarian cancer cell lines were kindly given to us by Dr. Bast (UTMDACC): A2780, EFO21, EFO27, IGROV1, OC314, and UPN251. Identities of all cell lines were confirmed by STR Fingerprinting (Characterized Cell Line Core, UTMDACC) and were found to be mycoplasma free. Flow cytometry was used to analyze ROR1 antigen levels on the available OvCa cell lines following three days growth in normoxia (20% O_2) or hypoxia (1% O_2). As anticipated, ROR1 was expressed on 5 of 6 cell lines tested and expression was maintained or increased in hypoxic conditions (**Figure 1**). This was the first report of ROR1 expression in hypoxia to our knowledge, and corroborated previous reports of ROR1 expression in OvCa.[12] After establishing that ROR1 is expressed in both high and low oxygen tension, OvCa can now be targeted with ROR1-specific CAR^{+} T cells.

Complete anti-tumor responses have been achieved with CD19-specific CAR^{+} T cells signaling through CD137 endodomain.[14, 15] Whether the same would be true for ROR1-specific CAR^{+} T cells in the treatment of OvCa is unknown. Thus, ROR1-specific CARs were constructed with CD137 endodomain (ROR1RCD137) and the canonical CD28 endodomain (ROR1RCD28) and cloned into *Sleeping Beauty* (SB) transposons (**Appendix 1**). Peripheral blood mononuclear cells (PBMC) were electroporated with plasmids encoding SB transposons and SB

transposase using Nucleofection and were stimulated in weekly recursive additions of γ-irradiated aAPC along with exogenous interleukin-2 and -21 (IL-2 and IL-21) supplementation (**Appendix 2**). aAPC were phenotyped for antigens (CD19 and ROR1), membrane bound IL15 (fused to IL-15Rα), co-stimulatory molecules (CD86 and CD137L), and Fc receptors (CD32 and CD64) before stimulations (**Appendix 3**). After 36 days of expansion on aAPC, $>10^9$ CAR^+ T cells were propagated from 10^6 CAR^+ T cells at the start of the culture (**Figure 2**). Stable CAR expression was observed with both ROR1RCD28 and ROR1RCD137 indicating that there was imposed selective pressure for CAR expression though cognate antigen expression on aAPC (**Figure 3**). Sham electroporations were taken forward in parallel to propagate CAR^{neg} T cells with OKT3(agonistic CD3 antibody)-loaded aAPC for negative controls. EL4 is a murine T cell lymphoma cell line used to test specificity of CAR^+ T cells with limited allo-reactivity from the endogenous T cell receptor (TCR). Thus, EL4 ($ROR1^{neg}$) cells were genetically modified to express recombinant ROR1 (EL4-$ROR1^+$) and these cells were used along with OvCa cell lines negative (A2780) or positive (EFO27) for ROR1 expression (**Figure 1**) in standard 4-hour chromium release assays to assess CAR-specific cytolysis. Both ROR1RCD28 and ROR1RCD137 displayed minimal killing of EL4-$ROR1^{neg}$ cells (**Figure 4A**) but significantly higher killing of EL4-$ROR1^+$ cells compared to CAR^{neg} T cells (**Figure 4B**). Similarly, A2780 ($ROR1^{neg}$) OvCa cells were lysed at the same levels by CAR^+ T cells as CAR^{neg} T cells (**Figure 4C**), whereas EFO27 ($ROR1^+$) OvCa cells were significantly killed by CAR^+ cells compared to CAR^{neg} T cells (**Figure 4D**). Thus, CAR^+ T cells were specifically re-directed to ROR1 expressed on OvCa. As ROR1 expression on OvCa has only recently been elucidated and has not yet been tested as a target for OvCa, ROR1-specific CAR T cells can thus be used for the first time in OvCa treatment.

Specific Aim #2: To assess the inherent cytolysis of γδ T cells against OvCa. *Experimental Plan:* Healthy donor-derived PBMC were paramagnetically sorted for γδ T cells with TCRγ/δ isolation kit then stimulated weekly with aAPC feeder ells and exogenous IL-2 and IL-21. The other fraction of the paramagnetic bead sorting, comprising mainly of αβ T cells, were stimulated with OKT3-loaded aAPC to propagate αβ T cells for negative staining controls. Extended phenotypic analysis of cell surface markers (as in Aim#1 with TCRδ1, TCRδ2, and TCRγ9) was performed at the end of the culture period by flow cytometry to assess TCR distribution and T cell, homing, and memory phenotype in the culture. TCRγδ allele expression was evaluated by our direct TCR expression array (DTEA) using digital probes to quantify mRNA expressing TCR chains and validated by

flow cytometry. Cytokine release following TCR stimulation (PMA/Ionomycin) and co-culture with OvCa cell lines was assessed by Luminex. Standard 4-hour CRA was used to evaluate cytotoxicity towards OvCa cell lines detailed in Aim#1 with healthy donor-derived B cells serving as negative controls. CAOV3 cell line was genetically modified to express mKate and *firefly Luciferase* (*ffLuc*) as done for OC314 in Aim#1. The ability of i.p. injected γδ T cells to eliminate i.p. established CAOV3-mKate-*ffLuc* xenografts in NSG mice was evaluated by BLI during the course of the experiment then immunohistochemistry and fluorescence were used to corroborate BLI data post-mortem.

Progress Report Specific Aim #2. We established that γδ T cells propagated on aAPC in parallel with CD19-specific CAR^+ γδ T cells and maintained a polyclonal distribution of γ and δ TCR chains.[37] This was a major advance as only one subset (Vγ9Vδ2) of γδ T cells had been previously expanded for human application, yet other γδ T-cell subsets exhibit anti-tumor immunity. Thus, studies were initiated to evaluate whether aAPC would drive γδ T-cell proliferation in the absence of CAR^+ T cells and if these γδ T cell would express polyclonal TCRγδ repertoire. PBMC were first depleted of NK cells with CD56 microbeads on paramagnetic columns and then unlabeled γδ T cells were purified from other cells labeled with TCRγ/δ+ isolation kit attached to magnetic columns. Recursive weekly stimulations on aAPC were performed with exogenous administration of IL-2 and IL-21. γδ T cells were in limited quantities in the starting PBMC (3.2% ± 1.2%; mean ± SD; n=4), but after sorting and expansion for 22 days on aAPC the cultures were highly pure for γδ T cells (97.9% ± 0.6%) as assessed by co-expression of $CD3^+$ and $TCR\gamma\delta^+$ (**Figure 5A**). Cultures yielded $>10^9$ γδ T cells from $<10^6$ total cells in three weeks of co- culture (**Figure 5B**), which represented $4.9x10^3$ ± $1.7x10^3$ fold change over the culture period (**Figure 5C**). Similar results were seen with γδ T cells sorted from umbilical cord blood (UCB) by fluorescence activated cell sorting (FACS) and stimulated as was done with PBMC (data not shown). Altogether this suggested the aAPC, not CAR, led to growth of γδ T cells in the culture system.

Only three TCRγδ chain-specific antibodies are commercially available limiting the detection of γδ T cell repertoire outside of Vδ1, Vδ2, and Vγ9. Thus, the direct T cell expression array "DTEA" was utilized to detect TCR mRNA in the γδ T-cell cultures and flow cytometry was used to corroborate mRNA data, when applicable. All three Vδ alleles, i.e. Vδ1, Vδ2, and Vδ3, were detected by both flow cytometry (**Figure 6A**) and DTEA (**Figure 6C**) with similar frequencies following the trend of Vδ1>Vδ3>Vδ2. The Vδ3 subset was shown to be present within the $V\delta1^{neg}V\delta2^{neg}$ population with DTEA analysis which allowed for inferential detection by

flow cytometry.[37] Most Vδ2 cells paired with Vγ9 (**Figure 6B**), as expected, and DTEA revealed that Vγ2, Vγ5, Vγ7,

Vγ8 (two alleles), Vγ10, and Vγ11 mRNA were expressed along with Vγ9 in the aAPC-expanded γδ T cell cultures (**Figure 6D**). All work was performed under GLP for direct clinical translation. Thus, we report the first clinically-relevant expansion of polyclonal γδ T cells for human use.

To determine whether γδ T cells would foster an inflammatory environment during therapy, a multiplex analysis (27-Plex Luminex) of cytokines and chemokines was performed on T cells following culture on aAPC. Phorbol myristate acetate (PMA) and Ionomycin mimic TCR activation by stimulating protein kinase C (PKC) and increasing intracellular Ca^{2+} to activate phospholipase C (PLC), respectively.[38, 39] There was no significant production of anti-inflammatory Th2 cytokines IL-4, IL-5, and IL-13, and there was only a small increase in IL-10 production from baseline (**Figure 7A**). In contrast, IL-1Ra, IL-6, and IL-17 were significantly secreted by γδ T cells and have roles together for IL-17 inflammatory responses important γδ T cells in killing OvCa (**Figure 7B**).[31, 40] Moreover, pro-inflammatory Th1 cytokines IL-2, IL-12 (p70), interferon-γ (IFNγ), and tumor necrosis factor-α (TNFα) were all significantly produced by γδ T cells when TCR was stimulated compared to mock stimulated controls (**Figure 7C**). Extremely high expression of chemokines CCL3 (macrophage inflammatory protein-1α; MIP1α), CCL4 (MIP1β), and CCL5 (regulated and normal T cell expressed and secreted; RANTES) were also detected (**Figure 7D**). In aggregate, TCR stimulation in γδ T cells led to a largely pro-inflammatory response desired for cell-based OvCa therapy.

It is of interest to employ γδ T cells for adoptive T cell therapy because they have less risk for graft- versus-host disease (GvHD). γδ T cells did not proliferate or produce IFNγ in response to healthy donor B cells (selected for their relative abundance in PBMC and ability to function as APC) nor did they have any significant killing of normal cells (**Figure 8; upper left**). Proliferation, IFNγ production, and killing were observed in positive controls, which attests to their functional capacity while leaving healthy cells untouched (data not shown). In addition to having fewer responses to normal cells, γδ T cells displayed an inherent ability to kill ovarian cancer. All OvCa cell lines tested in standard 4-hour chromium release assays were lysed by γδ T cells but healthy B cells were not killed (**Figure 8**). The order of killing tumor cell lines was cell line and donor dependent, which could not be directly correlated to a particular Vδ subset of deference to a certain combination

of Vδ lineages (**Figure 8**). In aggregate, γδ T cells showed broad anti-tumor effects with limited reactivity to healthy cells.

Lastly, the ability of γδ T cells to target and eliminate established OvCa xenografts *in vivo* was evaluated. NSG mice were used for their ability to accept human tumor xenografts well and were injected with CAOV3-*ffLuc*-mKate tumor cells i.p. then randomized into treatment groups. Stable disease was established after 8 days of engraftment and either PBS (negative control) or γδ T cells (escalating doses) were administered i.p. to the mice. Tumor burden was monitored during the experiment with non-invasive bioluminescence imaging (BLI) following D-luciferin administration (**Figure 9A**). γδ T cells significantly ($p = 0.0005$) eliminated established CAOV3 tumors during the course of the experiment whereas mice treated with vehicle (PBS) retained high tumor burden (**Figure 9B-C**). Persistent OvCa disease was evident in the PBS group by (i) flux maintenance to day 43 (**Figure 9B**), (ii) increase in flux to day 79 (**Figure 9D**), and (iii) death of mice (n=3). In contrast, mice treated with γδ T cells displayed significantly lower CAOV3-*ffLuc*-mKate flux after 79 days post-engraftment compared to the day prior to treatment (day 7 post-engraftment) and no mice died in this group (**Figure 9D**). Thus, polyclonal γδ T cells were effective in treating OvCa *in vivo* and represent an attractive approach to cell-based OvCa treatment.

<u>Specific Aim #3: To examine the role of endogenous co-stimulation for pro-inflammatory γδ T cell expansion in both normoxia and hypoxia.</u> Normal donor PBMC will be sorted for γδ T cells (as in Aim#2) and expanded in parallel in 1% O_2 and 20% O_2 on aAPC with aAPC expressing differing co-stimulatory ligands: (i) none, (ii) CD70, (iii) CD86, (iv) CD137L (4-1BBL), or (v) CD275 (ICOSL). Gene expression was profiled using nCounter platform to identify candidate genes crucial for expansion in hypoxia. Polarization towards IL-17 or IFNγ producing lineages was evaluated following expansion by flow cytometry and intracellular cytokine staining. Cytolytic potency of cells expanded on different co-stimulatory ligands will be evaluated by CRA when targeted to ovarian cancer cell lines in both hypoxia and normoxia.

Progress Report Specific Aim#3.. In order to maximize the anti-tumor efficacy of γδ T cells for OvCa therapy, it is important to distinguish what molecules on aAPC drive their proliferation. The aAPC discussed in Aims #1 and #2 co- expressed CD86 and 4-1BBL co-stimulatory molecules, which were high value targets for these studies. It was also advantageous to examine IL-17/IFNγ polarization and growth in hypoxia in the same

studies. Reports in αβ T cells show that CD70 and CD86 led to IFNγ production but ICOS-L correlated with IL-17. Whether the same is true for γδ T cells is not currently known nor is their ability to proliferate in hypoxia with aAPC as a stimulus. Thus, aAPC were re-engineered on the K562 parental background to express on the following co- stimulatory molecules: (i) none, (ii) CD70, (iii) CD86, (iv) 4-1BBL (CD137L), or (v) ICOS-L (CD275). PBMC were sorted on paramagnetic columns (TCRγ/δ isolation kit) to isolate γδ T cells that were then stimulated with one of the above aAPC for 9 days at 37^{O}C in either in normoxia or hypoxia. Co-cultures were either given no cytokine, IL-2, IL-21, or both IL-2 and IL-21. After stimulation, cells were (i) counted for proliferation using trypan blue exclusion and (ii) tested for IFNγ/IL-17 polarization. For the latter scenario, expanded cells were either mock activated or stimulated with leukocyte activation cocktail (LAC; PMA/Ionomycin) in the presence of secretory pathway inhibitor GlogiPlug for 6 hours at 37^{O}C in normoxia or hypoxia then stained for CD3, TCRγδ, IFNγ, and IL-17 and analyzed by flow cytometry. As anticipated, the γδ T cells did not appreciably expand in the absence of cytokine or aAPC. However, the addition of both IL-21 and IL-2/IL-21 led to γδ T-cell numeric expansion especially when aAPC expressed 4-1BBL and with a combination of CD86 and 4-1BBL (**Figure 10A and 10C**). It was also interesting that there was proliferative synergy between CD86 and 4-1BBL. Signficantly, robust expansion of γδ T cells was observed in hypoxia in multiple scenarios, which corroborated the approach to expand hypoxia-sensitive γδ T cells. Culture conditions could be adapted to result in emergence of T cells that produced IL-17 and IFNγ (**Figure 10B and 10D**). The most IL-17 was observed with IL-21/4-1BBL in hypoxia and with IL-2/IL-21/4-1BBL in normoxia. The same conditions led to IFNγ production with the additional condition of IL-2/IL-21/CD86/4-1BBL in normoxia. It is also interesting that the absence of oxygen or lack of IL-2 abrogated the functionality of cells stimulated with CD86/4-1BBL. Moreover, IL-17 production in normoxia with IL-2/IL-21 was halted with the addition of CD86 but had no effect on IFNγ (**Figure 10D, left side, top and bottom panels**). It appears that 4-1BBL is the crucial molecule on aAPC to drive γδ T-cell proliferation and to yield IL-17 producing lineages, especially in normoxia. It was also unexpected that ICOS-L neither induced considerable growth nor led to IL-17 production. Nonetheless, the culture system can be adapted to generate γδ T cells that can produce IFNγ, IL-17, or both.

The long-term objective of this study is to create novel T cell-based treatments of disease-resistant ovarian cancer that can function in the hostile tumor environment with minimal toxicity to normal tissues.

KEY RESEARCH ACCOMPLISHMENTS

Major advances have been made on this study and the project is poised for timely completion.

Specifically, the key research accomplishments are:

1. There has been significant headway in targeting OvCa with CAR^+ T cells
2. There has been significant progress in targeting OvCa with polyclonal γδ T cells
3. ROR1 expression was detected on OvCa and was determined to be superior to cMet as a target
4. ROR1 expression was shown to be retained or increased in hypoxia (refractory areas of OvCa)
5. We show evidence that γδ T cells have inherent anti-tumor cytotoxicity towards OvCa
6. γδ T cells can proliferate with similar kinetics as in normoxia
7. γδ T cells produce both IFNγ and IL-17

In sum, this progress report gives two cell-based methods to treat OvCa with evidence of efficacy in hypoxia.

REPORTABLE OUTCOMES

The pre-clinical data for ROR1-specific T cells (described in "Progress Report Specific Aim #1") has not been published but is being compiled into a manuscript at present and is anticipated to be completed by the end of 2013. Clinical trials are underway at MD Anderson Cancer Center using aAPC, SB, and CARs for cancer treatment, and ROR1-specific CAR trial was approved by the Recombinant DNA Advisory Committee (RAC) for the treatment of chronic lymphocytic leukemia (CLL). We anticipate that a future clinical trial can be written for women with relapsed OvCa in the coming year. A manuscript detailing the utility of CAR^+ γδ T cells for leukemia treatment was published in *Molecular Therapy*, but is not directly applicable to OvCa.[37] A second paper about γδ T cells targeting OvCa (detailed in "Progress Report Part 3") has been written and is awaiting submission to *Journal of Experimental Medicine*.

CONCLUSION

We provide two T cell-based approaches for OvCa treatment that are both directly applicable to the clinic. First, ROR1 was shown to be expressed on OvCa in both hypoxia and normoxia, and CAR^+ T cells re-directed to ROR1 could specifically and efficiently lyse OvCa targets. Second, polyclonal γδ T cells were expanded to clinically-relevant numbers on aAPC, could lyse many OvCa cell lines, and eliminated OvCa xenografts *in vivo*. Thus, translation of these T cell therapies will give women with advanced OvCa novel options in their treatment.

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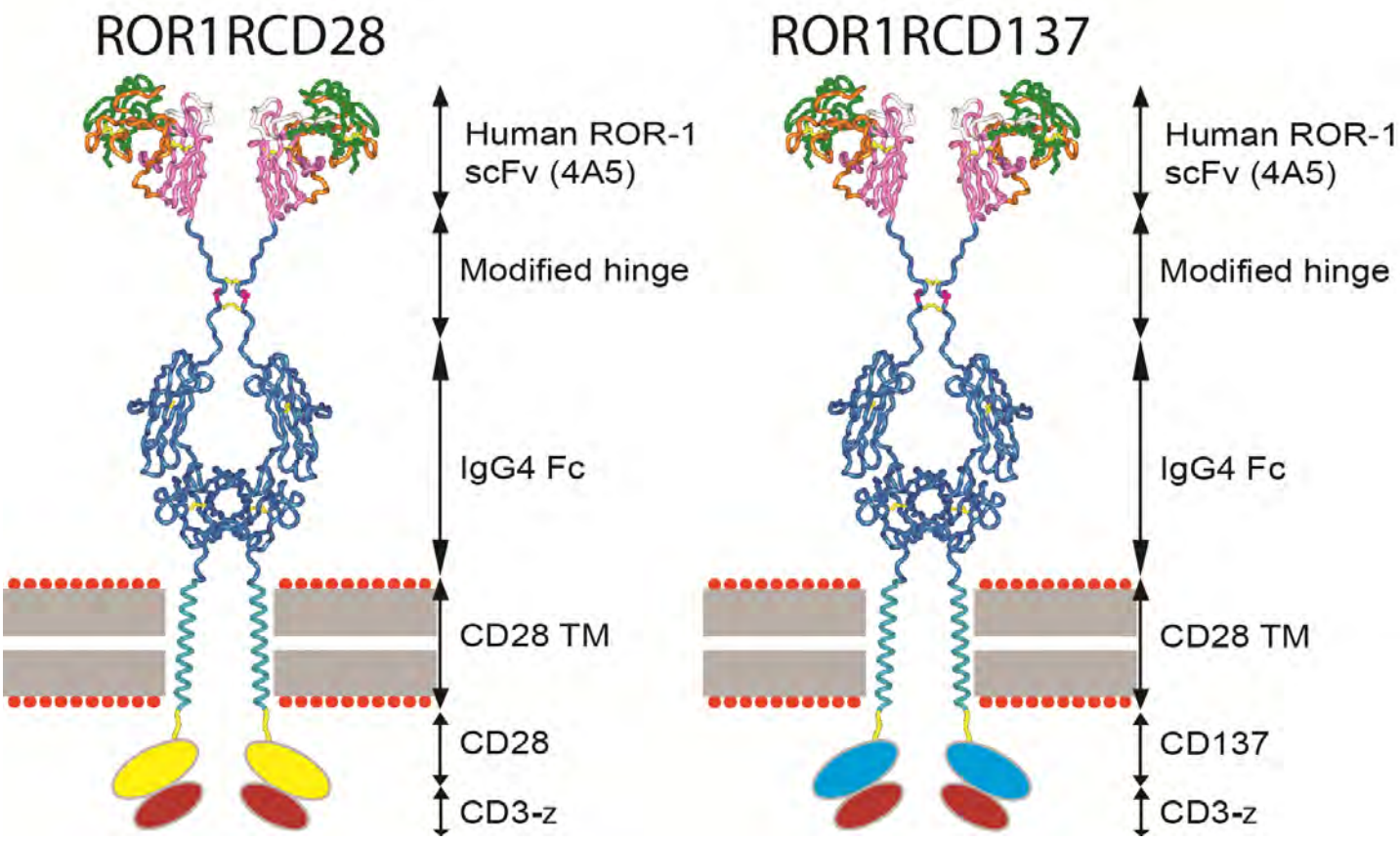
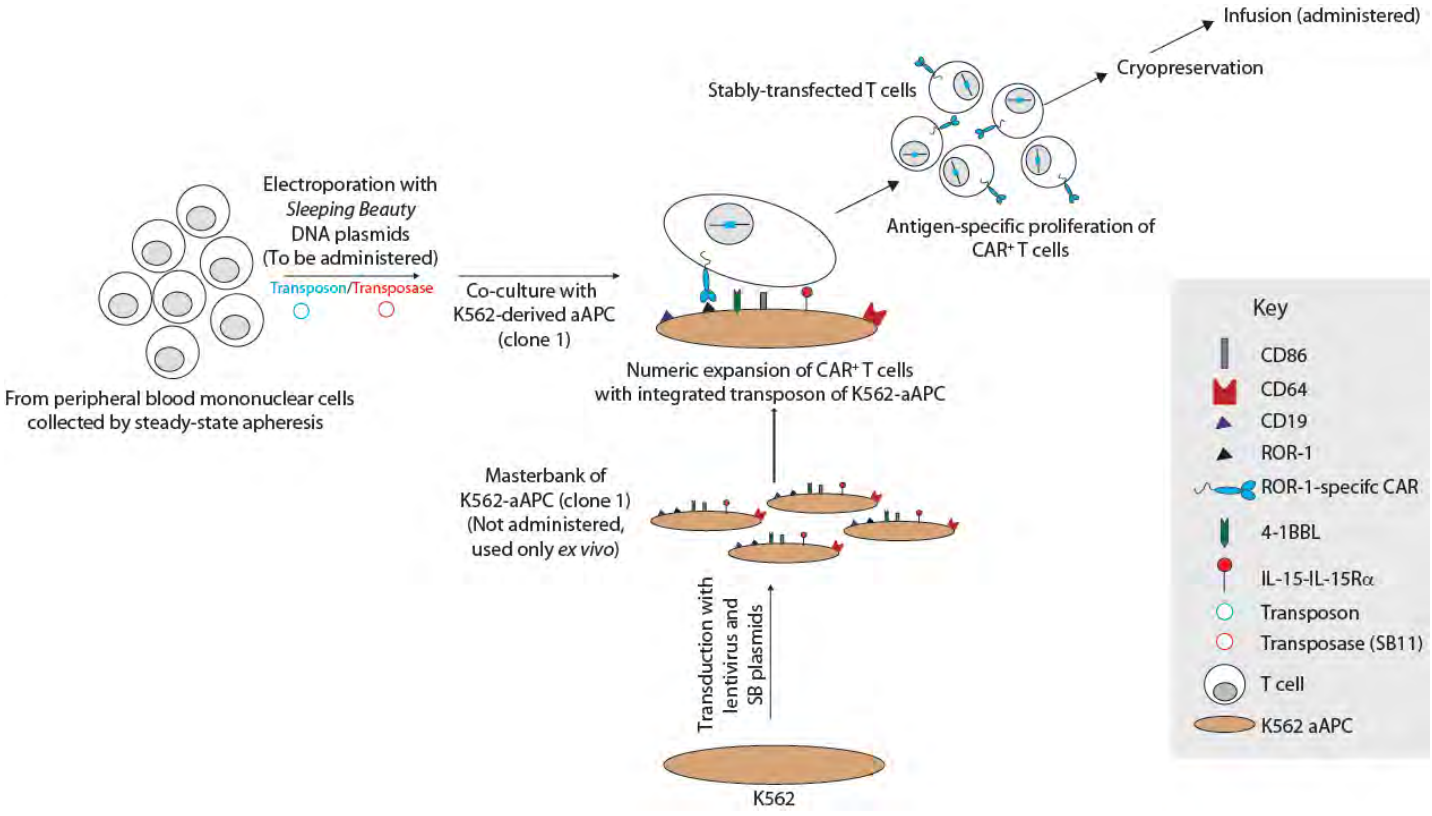
APPENDICES

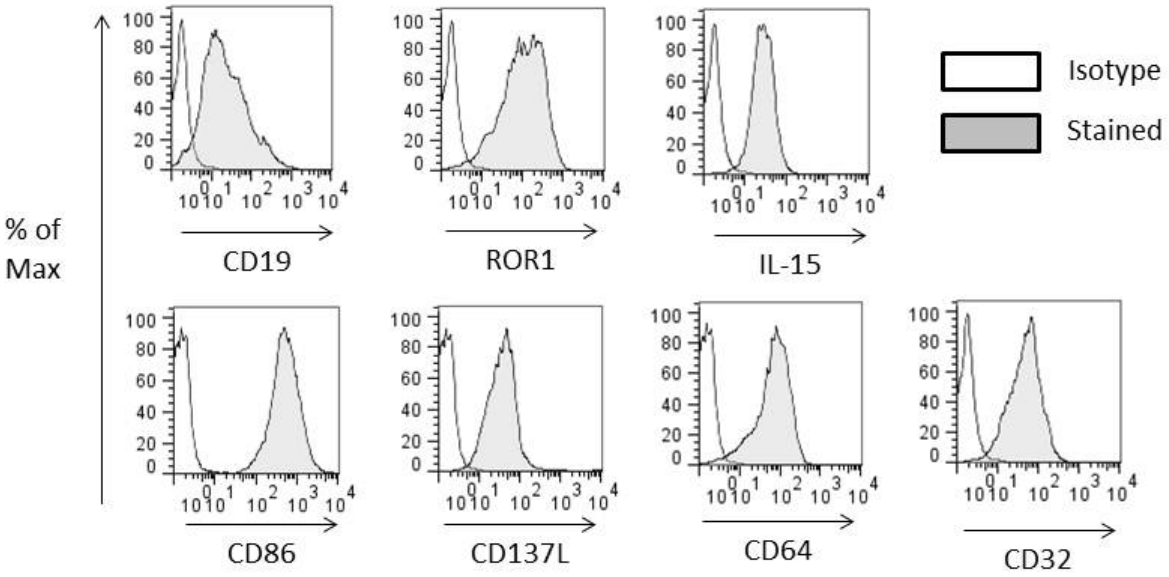
Appendix 1. Schematics of CARs used in studies. Chimeric antigen receptors (CAR) are fusion proteins of (i) antigen-specific binding regions of monoclonal antibodies constructed into single chain variable fragments (scFv), (ii) a hinge region, (iii) an IgG4 constant region (Fe) stalk, (iv) CD28 transmembrane (TM) domain, (v) co-stimulation domain (either CD28 (yellow) or CD137 (blue)), and CD3-zeta T cell signaling domains. ROR1- specific CARs signaling through CD28 (ROR1RCD28; Left) and CD137 (ROR1RCD137; Right).



Appendix 2. Schematic for propagation of CAR+ T cells. Peripheral blood mononuclear cells (PBMC) were electroporated with *Sleeping Beauty* (SB) plasmids expressing SB transposon (CAR) and SB transposase by Amaxa nucleofection. Co-culture of CAR+ T cells with artificial antigen presenting cells (aAPC; "Cione1") led to numeric expansion of CAR+ T cells and enforced CAR expression through ROR1.



Appendix 3. Phenotype of Clone1 aAPC used for propagation of ROR1-specific CAR^{+} T cells. Flow cytometry was used to assess recombinant expression of antigens (CD19 and ROR1), membrane-bound cytokines (IL-15 fused to IL-15Rα), co-stimulatory molecules (CD86 and CD137L), and Fc receptors (CD32 and CD64) on Clone1 aAPC. Isotype staining represented in open plots and filled plots represent antigen-specific staining.



SUPPORTING DATA

Figure 1. ROR1 expression on OvCa in hypoxia and normoxia. OvCa cell lines were grown in normoxia (20% O_2) or hypoxia (1% O_2) for three days at 37^{o}C and were then stained for ROR1 expression with 4A5 monoclonal antibody specific for ROR1.[12] (A) Representative flow plots from A2780, EFO27, and OC314 cell lines where black lines are normoxia, red lines are hypoxia, dashes are isotype controls, and solid lines are ROR1 staining. (B) Normalized ROR1 ($ROR1_{normal}$) mean fluorescence intensity (MFI) in OvCa cell lines in normoxia (open bars) and hypoxia (closed bars). MFI was calculated by: $MFI_{ROR1} - MFI_{isotype}$.

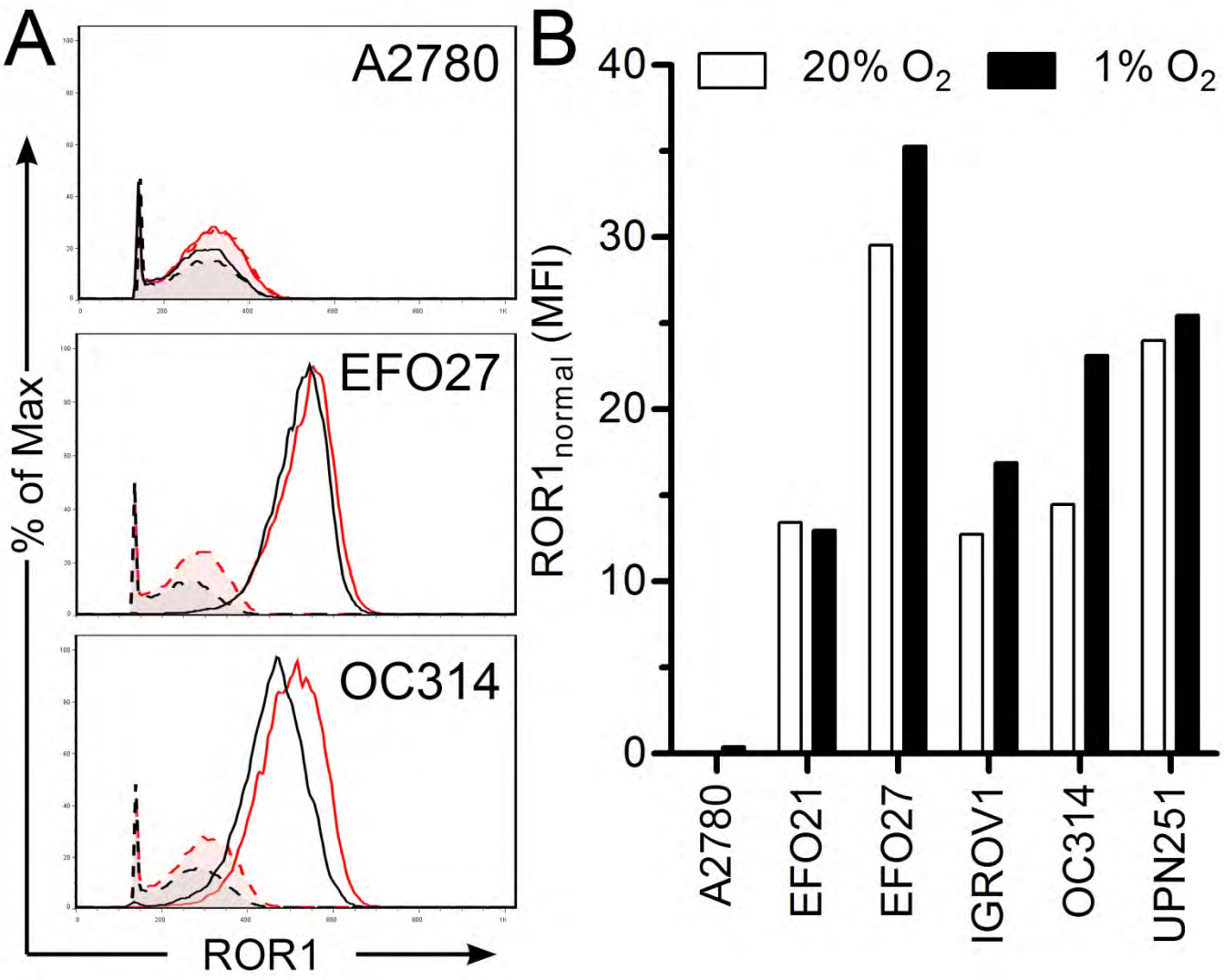


Figure 2. Expansion of CAR+ T cells on ROR1+ aAPC. PBMC were electroporated with ROR1-specific CARs signaling through CD28 (ROR1RCD28; closed squares) or CD137 (ROR1RCD137; open sqares) and stimulated five times (each point on graphs) with ROR1+ aAPC along with IL-2 and IL-21. (A) Total cell counts during culture period. (B) CAR+ T cell counts during culture period.

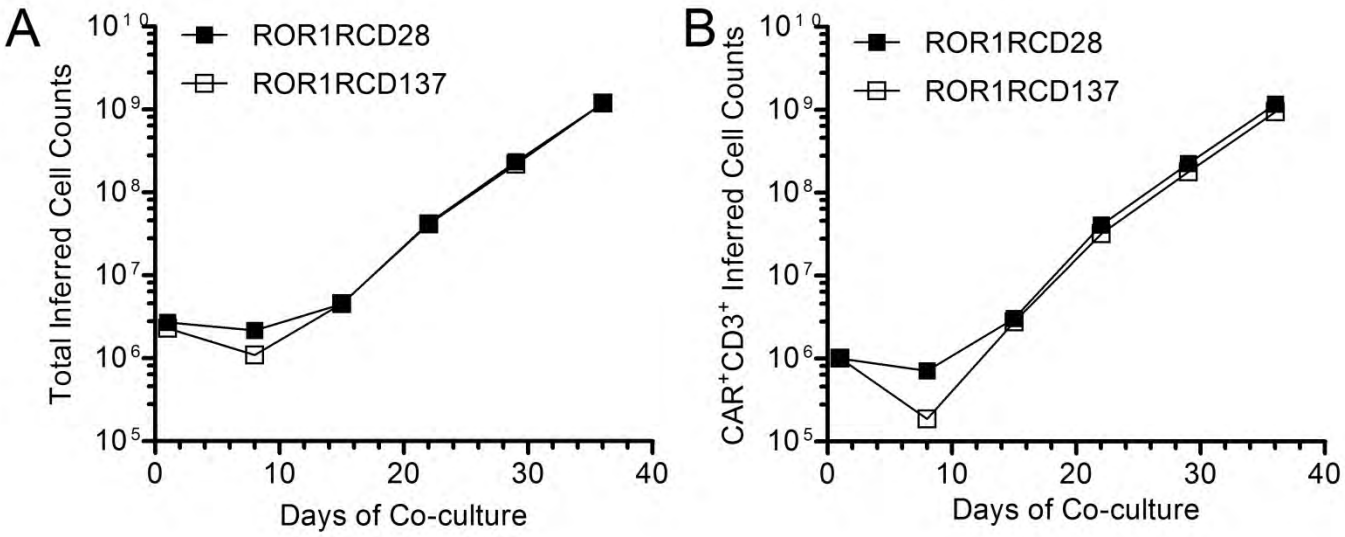


Figure 3. CAR expression after expansion on ROR1+ aAPC. Stable CAR expression after 29 days of co- culture on ROR1+ aAPC. Sham electroporated "No DNA" cells were stimulated with OKT3-loaded aAPC for negative controls. Gate frequencies are displayed in upper right corners.

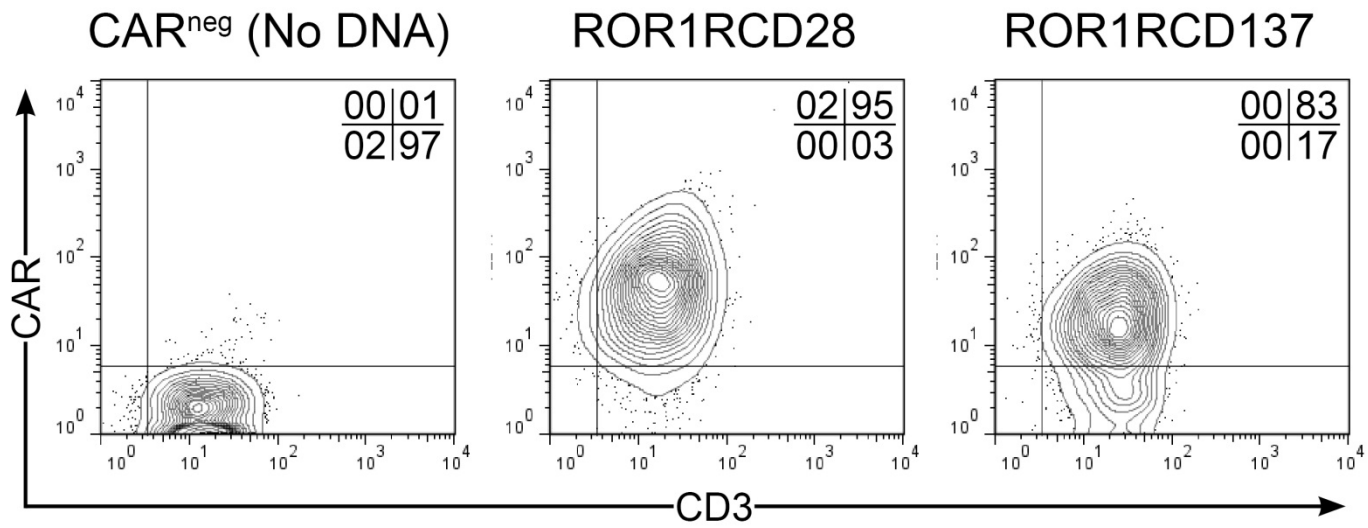


Figure 4. Specific killing of ROR1^{+} tumor cells by CAR^{+} T cells. Standard 4-hour chromium release assays were performed after 35 days of expansion on aAPC with CAR^{neg} (triangles), ROR1RCD28 (circles), or ROR1RCD137 (squares) T cells against (A) EL4-ROR1^{neg}, (B) EL4-ROR1^{+}, (C) A2780 (ROR1^{neg} OvCa), and (D) EFO27 (ROR1^{+} OvCa) cell lines. Two-way ANOVA with Bonferroni's post-tests used for statistical analyses on triplicate measurements where *p<0.05 and ***p<0.001.

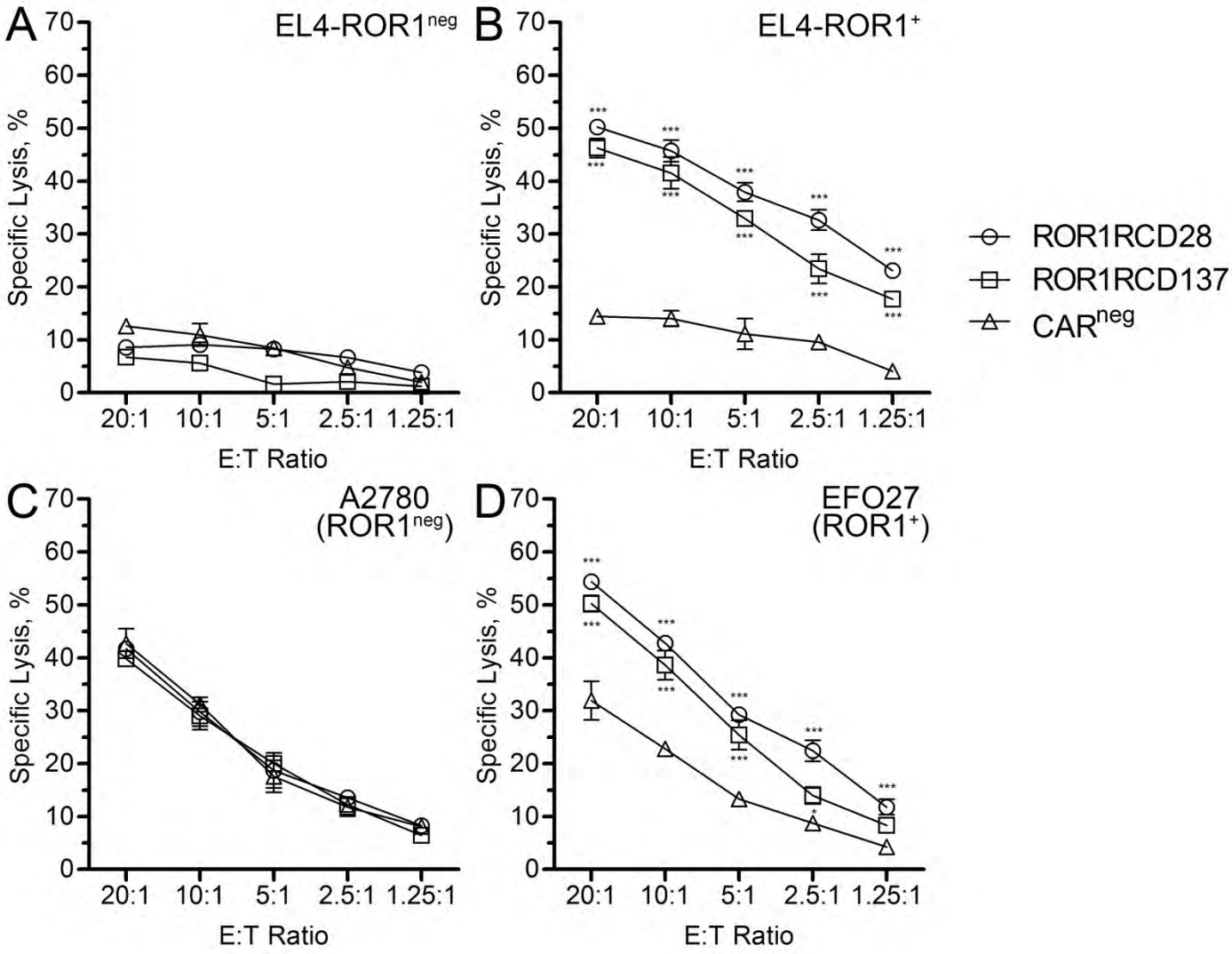


Figure 5. Expansion of γδ T cells on aAPC. (A) Flow cytometry of CD3 (y-axis) and TCRγδ (x-axis) expression in PBMC prior to paramagnetic bead sorting at Day 0 and of T cell cultures after 22 days of co-culture on aAPC. One representative donor of four healthy donors is shown and quadrant gate frequencies are displayed in the upper right corners of flow plots. (B) Total inferred cell counts of viable cells during co-culture period. (C) Fold expansion of cells in co-culture during co-culture period. Black lines are mean ± SD from 4 healthy donors, gray lines are individual donors, arrows represent stimulations with aAPC, and data are pooled from two independent experiments.

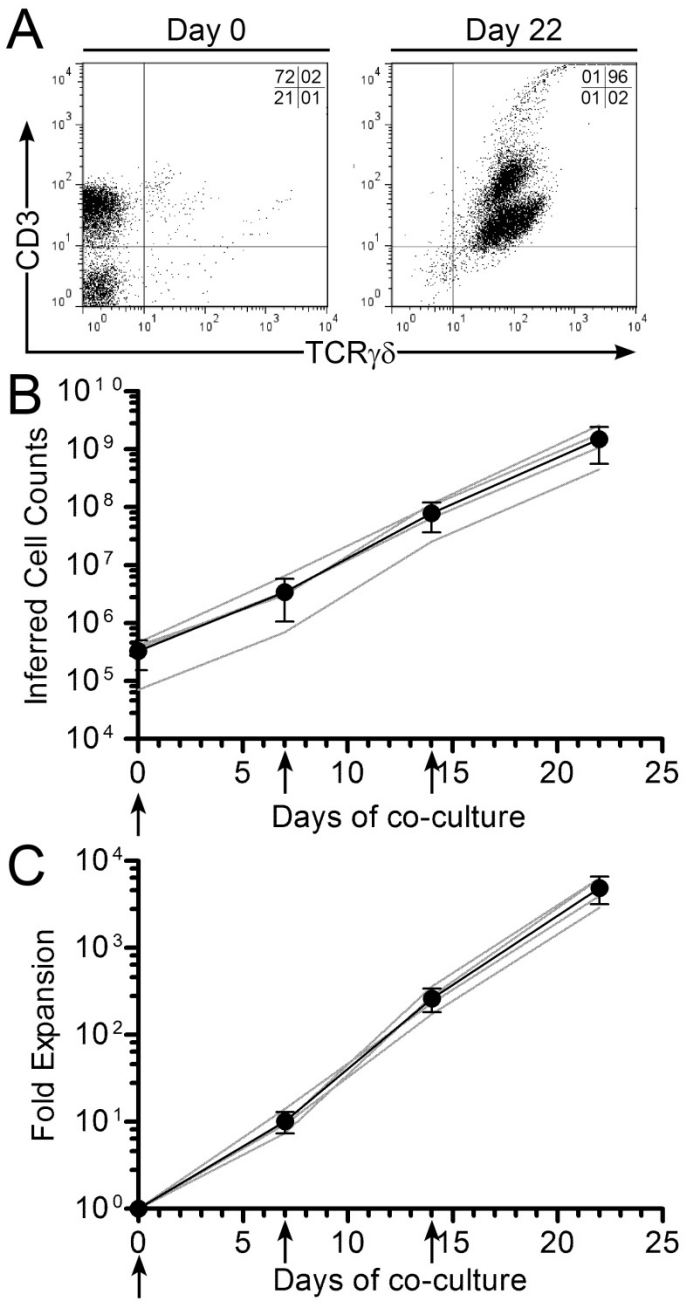


Figure 6. TCRγδ expression on aAPC-expanded γδ T cells. (A) Flow cytometry of TCRδ2 and TCRδ1 expression in γδ T cells at day 22 of co-culture. (B) Flow cytometry of TCRδ2 and TCRγ9 expression in γδ T cells at day 22 of co-culture. Numbers in lower right corners correlate with donor numbers in (A) and (B) where quadrant frequencies are displayed in upper right corners. (C) Direct TCR expression array "DTEA" detection of Vδ mRNA alleles in γδ T cells at day 22 of co-culture where Vδ1*01, Vδ2*02, and Vδ3*01 alleles are in graphs from left to right, respectively, and each of the four donors are numbered on x-axes. (D) DTEA detection of Vγ allele mRNA expression in γδ T cells at day 22. Lines are mean ± SD where each circle represents an individual healthy donor grown in two independent experiments.

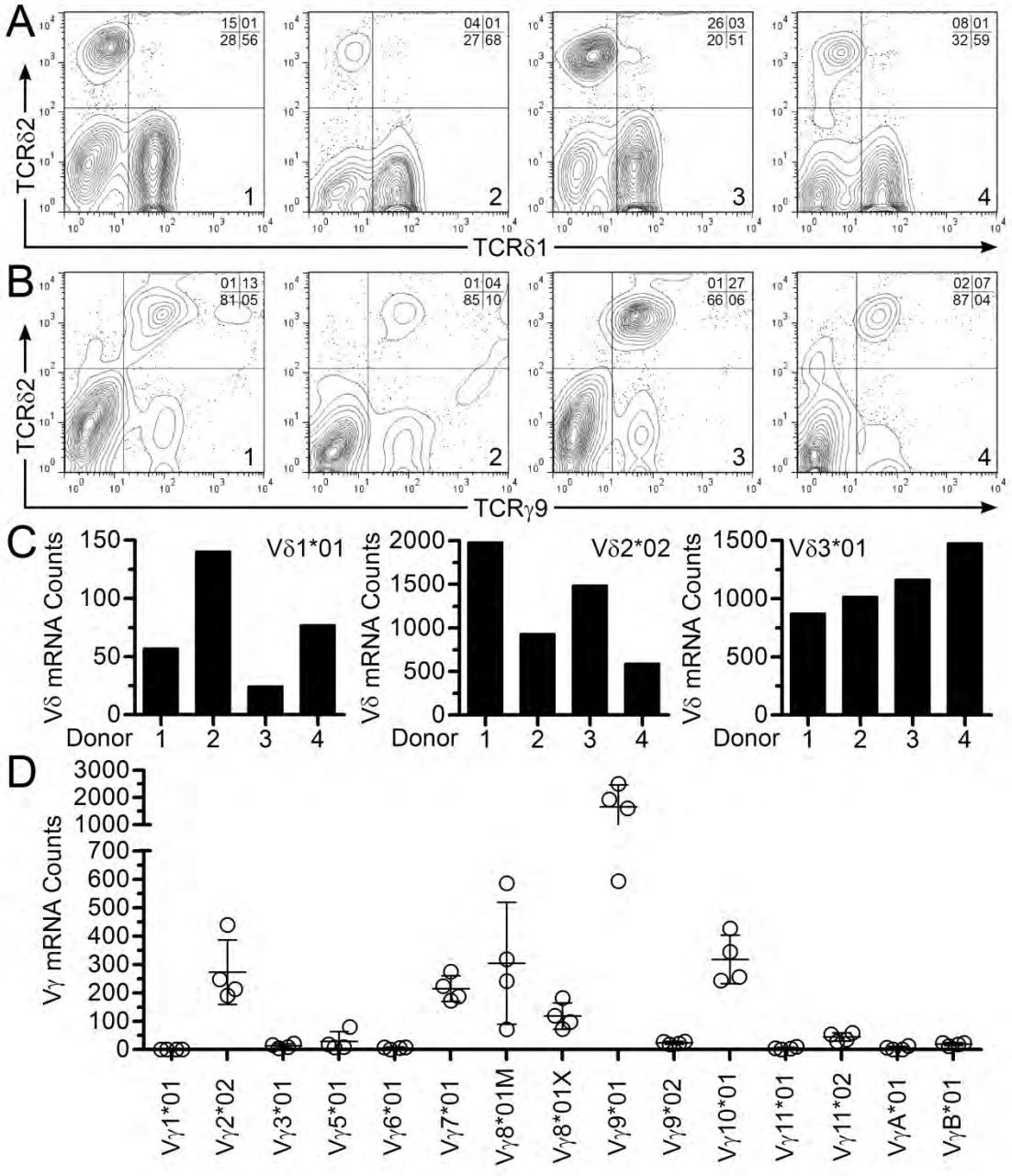


Figure 7. Cytokines and chemokines secreted by γδ T cells expanded on aAPC. Cells at day 22 were co-cultured with a mock activation cocktail (complete media) or leukocyte activation cocktail (LAC; PMA/Ionomycin) for 6 hours at 37^{o}C. Conditioned media was interrogated on 27-Plex Luminex array to detect cytokines and chemokines. A, Th2 cytokines. B, Th17 cytokines. C, Th1 cytokines. D, Chemokines. Data are mean ± SD from 4 healthy donors. Student's t-test performed for statistical analysis between mock and LAC groups for each molecule. *p<0.05, **p<0.01, and ***p<0.001.

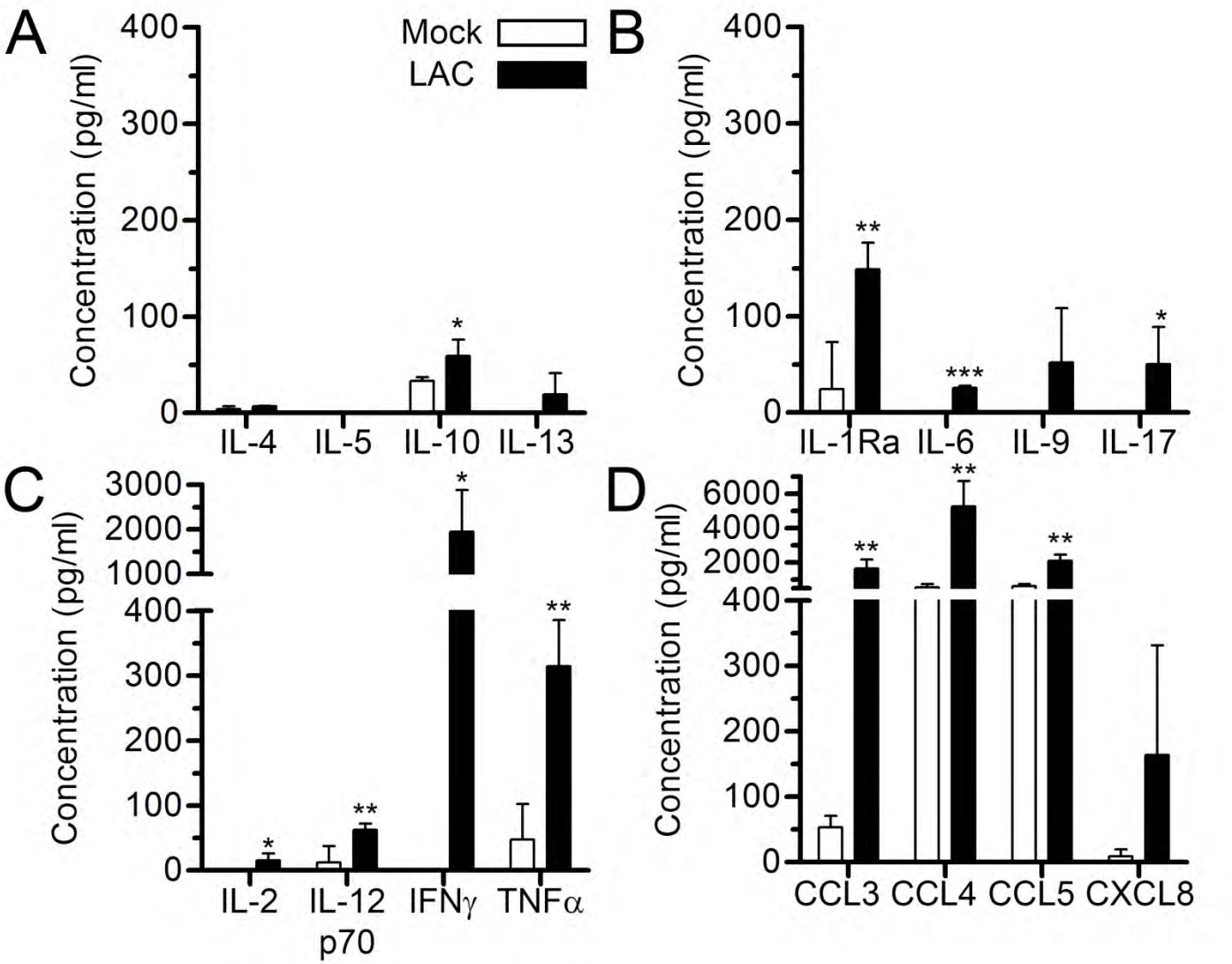


Figure 8. *In vitro* cytolysis of tumor cells by γδ T cells. Standard 4-hour chromium release assays were performed with increasing effector (γδ T cells) to target (E:T) ratios against healthy B cells from an allogeneic donor (top left; one of four representative donors) and OvCa cell lines A2780, EF021, EF027, IGROV1, OC314, UPN251, and CAOV3. Each line represents an individual effector where data is mean ± SO (n = 3 wells per assay) from two independent experiments.

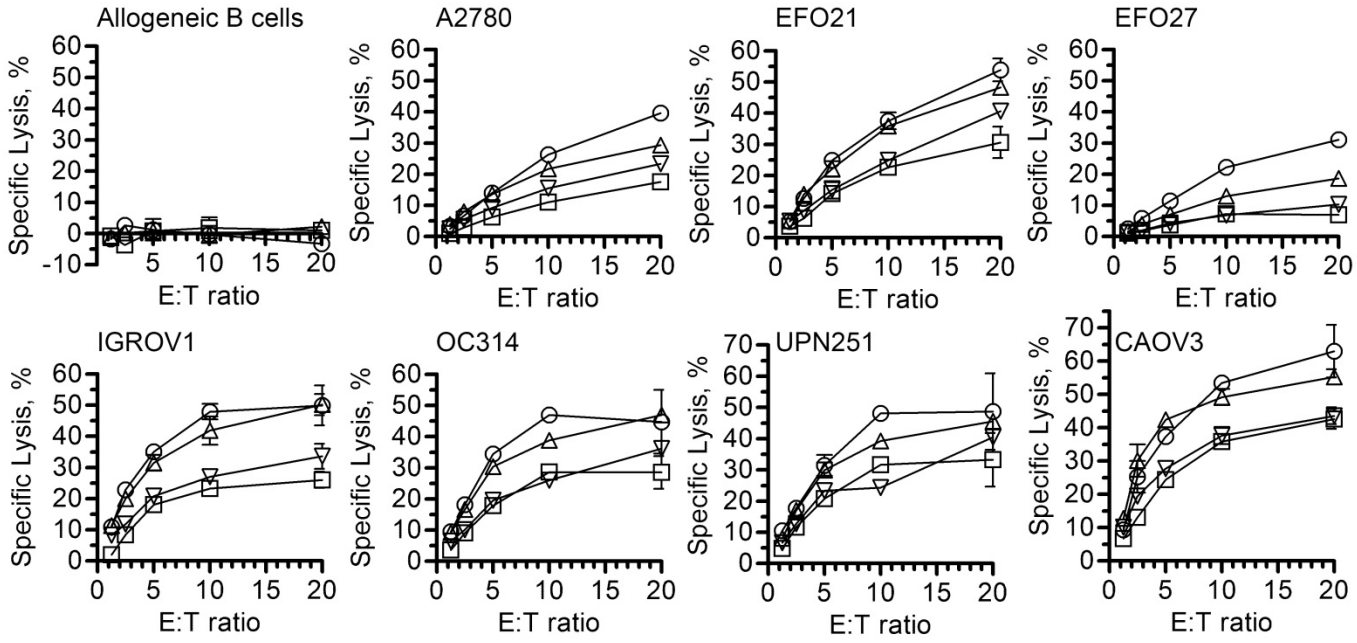


Figure 9. OvCa tumor clearance by polyclonal γδ T cells *in vivo*. (A) Schematic of experiment with legend to right. (B) Kinetics of bioluminescent flux of CAOV3-*ffLuc* xenografts from mock treated (closed squares) and γδ T cell treated (open squares) mice during experiment. Two-way ANOVA with Bonferroni's post-tests was used for statistical analysis where n = 10, *p<0.05, and ***p<0.001. (C) Representative images 43 days after engraftment and treated with PBS (top panels) or γδ T cells (bottom panels). (D) Long-term BLI flux comparison between day prior to treatment (Day 7; squares) and 79 days (circles) post-engraftment. Student's t-test performed between time points for each group with p-values above comparisons.

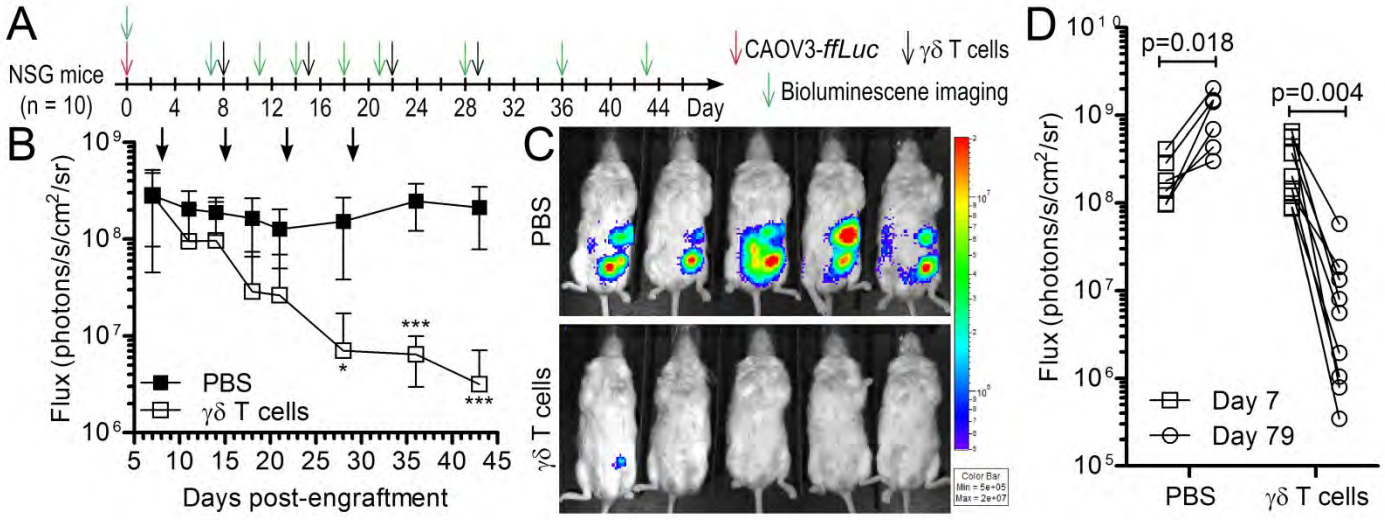


Figure 10. γδ **T cells expanded on aAPC in normoxia and hypoxia and polarized towards IFNγ and IL-17 production.** PBMC were sorted on TCRγ/δ+ isolation kit and 10^4 purified γδ T cells were then given a single stimulation with either IL-21 (A and B) or IL-2 and IL-21 (C and D) along with no aAPC or $2x10^4$ aAPC with one of the following co-stimulatory molecules: (i) none, (ii) CD70, (iii) CD86, (iv) 4-1BBL, (v) ICOS-L, or (vi) CD86 and 4-1BBL. Duplicate cultures were then placed in 20% O_2 or 1% O_2 for nine days at 37^oC with humidified conditions. (A and C) After the 9 day incubation, cells were counted using trypan blue exclusion. Numbers above hatched bars represent fold changes of cytokine-treated cells compared to cultures without cytokines (bars not hatched). (B and D) Remaining cells were then mock activated (black line) or activated with leukocyte activation cocktail (LAC, PMA/Ionomycin, red line) for 6 hours in the presence of secretory inhibitor GolgiPlug at the same oxygen concentration as the 9 day culture. Cells were then stained for CD3, TCRγδ, IFNγ, and IL-17 and analyzed by flow cytometry. Data shown are histograms gated on $CD3^+TCR\gamma\delta^+$ cells where cytokine detected (x-axis) is in upper right corner and y-axis is % of maximum value. Top panels are 4-1BBL cultures and bottom panels are CD86/4-1BBL cultures. Left panels are 20% O_2 and right panels are 1% O_2.