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Genetic Modification of T Cells with IL-21 Enhances Antigen Presentation and Generation of Central Memory Tumor-Specific Cytotoxic T Lymphocytes

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Abstract

An optimized antigen-presenting cell (APC) for tumor immunotherapy should produce a robust antigen specific cytotoxic T cell (CTL) response to tumor-associated antigens, which can persist in vivo and expand on antigen re-encounter. IL-21 synergizes with other γ -chain cytokines to enhance the frequency and cytotoxicity of antigen-specific CTL. Since T cells themselves may serve as effective APCs (TAPC) and may be useful in vivo as cellular vaccines, we examined whether CD8⁺ T cells genetically modified to produce IL-21 could induce immune responses to tumor associated antigen peptides in healthy HLA-A2+ donors. We found that IL-21 modified TAPC enhanced both the proliferation and survival of MART-1 specific CD8⁺ T cells, which were enriched by >8-fold over cultures with control non-transgenic TAPC. MART-1-specific CTL produced IFN- γ in response to cognate peptide antigen, and killed primary tumor cells expressing MART-1 in an MHC restricted manner. IL-21 modified TAPC similarly enhanced generation of functional CTL against melanoma antigen gp100 and the B-CLL associated RHAMM antigen. Antigen-specific CTL generated using IL-21 gene-modified TAPC had a central memory phenotype characterized by CD45RA[−], CD44^{high}, CD27^{high}, CD28^{high}, CD62L^{high} and IL-7 receptor- α ^{high}, contrasting with the terminal effector phenotype of CTL generated in the absence of IL-21. Thus, TAPC stimulation in the presences of IL-21 enhances proliferation of tumor antigen-specific T cells and favors induction of a central memory phenotype, which may improve proliferation, survival and efficacy of T cell based therapies for the treatment of cancer.

Keywords

IL-21; T-antigen presenting cell; CTL; adoptive immunotherapy

INTRODUCTION

Adoptive cell therapy (ACT) using antigen-specific cytotoxic T lymphocytes (CTL) has produced complete clinical responses and sustained remissions in Epstein-Barr virus (EBV)-associated lymphoma^{1,2} and solid tumors.³ While remarkable regression of inoperable and

drug resistant tumors have been observed following CTL therapy, it is evident that the vast majority of infused CTL (10^7 to 10^{11} highly tumor-specific T cells) fail to survive and persist within the host following infusion.^{4–8} The precise mechanism of this deficiency is not clear and may be attributable to a poor homeostatic environment or lack of CD4+ T cell help,^{9,10} but recent focus has been on the status of the infused cells themselves. Both clinical trials and murine ACT studies have suggested that extensive in vitro expansion of CTL and tumor infiltrating lymphocytes can adversely affect persistence and efficacy,^{11–15} and that protocols to limit in vitro expansion result in improved clinical outcomes.^{3,16}

T cells can be categorized into distinct states of differentiation based on phenotypic and functional attributes, where antigen-dependent proliferation results in the differentiation of naïve and memory T cells into effector T cells.^{17,18} During the acquisition of effector function (e.g. cytotoxicity, cytokine production), T cells concomitantly lose expression T cell survival (e.g. CD27, CD28 and IL-7R α) and migration (e.g. CD62L and CCR7) to lymphoid organs. Importantly, expression of both CD27 and CD28 have been shown to be associated with proliferation and survival of adoptively transferred T cells.^{12,19–21} Likewise, IL-7R α is critical for homeostatic T cell survival,²² whereas CD62L-dependent migration enhances CTL function by allowing interaction with professional APC within the lymph nodes.^{13,14} These studies suggest that early effector T cells may have improved survival and persistence compared to late effector CTL, and that culture conditions and techniques aimed at preserving this state may improve the anti-tumor effect of CTL therapy.

Interleukin-21 (IL-21), a member of the γ -chain family of cytokines which is produced by activated CD4+ T cells and natural killer T (NKT) cells,^{23–25} was recently shown to enhance the expansion, lymphoid homing and antitumor effects in the pmel ACT mouse model.²⁶ In addition, this study demonstrated that pmel CD8+ T cells exposed to IL-21 exhibit a gene expression profile distinct from that of IL-2 and IL-15, indicating that IL-21 suppressed or altered T cell differentiation following stimulation.²⁶ Similar trends were observed in human T cells, where IL-21 exposure maintained expression of CD28 and enhanced cell survival, proliferation, antigen affinity and production of cytokines following antigen stimulation.^{27, 28} These studies suggest that use of IL-21 might be used to control effector differentiation to make T cells more effective as anti-cancer agents.

IL-21 appears to inhibit the maturation and function of dendritic cells (DC), which are commonly used for in vitro generation of tumor-specific CTL.²⁹ Ansen and colleagues recently demonstrated that artificial APC (gene-modified K562) may be used instead of DC, which allows specific expansion of tumor-specific T cells.²⁸ We and others have previously demonstrated that activated T cells, which upregulate MHC I and II, and the costimulatory molecules CD80, CD83 and CD86 can be used as APC (T antigen presenting cells; TAPC), and that TAPC may serve as a potent cellular vaccine for the treatment of cancer, apart from their utility as APC for in vitro generation of CTL.^{30,31} Indeed, the use of TAPC as a cellular vaccine has recently been demonstrated for the treatment of melanoma.³² To this end, we demonstrate that TAPC, a source of MHC-matched APC that are genetically modified to secrete IL-21 are potent APC capable of generating tumor-specific CD8+ CTL from healthy donors. Furthermore, CTL generated with IL-21 producing TAPC possess phenotypic and functional characteristics of early effector memory T cells.

MATERIALS AND METHODS

Blood donors and cell lines

After informed consent, peripheral blood was obtained from healthy HLA-A2+ donors on a protocol approved by the Baylor College of Medicine Institutional Review Board. CTL and TAPC cultures were maintained in 45% RPMI 1640 (GIBCO), 45% Click (Eagle Ham amino

acids; Irvine Scientific, Santa Anna, CA), 2 mM GlutaMAX-1 (Invitrogen, Carlsbad, CA), 5% human AB serum (Valley Biomed), referred to hereafter as T cell media (TCM-AB). Senma (HLA-A2+), Mel-1143 (HLA-A2-), K562, CEM.T2 and HSB-2 tumor cell lines were maintained in RPMI supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 2 mM GlutaMax-1.

Construction and production of IL-21 lentivirus

To clone human IL-21, CD4⁺ T cells were isolated from PBMC by MACS selection using CD4 microbeads (Miltenyi Biotech, Auburn, CA) and activated for 48 hours on non-tissue culture treated plates coated with 1 µg/ml anti-CD3 (OKT3; Ortho Pharmaceuticals, Raritan, NJ) and 1 µg/ml anti-CD28 antibody (Pharmingen, San Diego, CA). Following activation, RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and used as a template for One-Step RT-PCR (Qiagen) using gene-specific primers. The IL-21 forward PCR primer included a CACC tag (5'-CCACATGGGATCCAGTCCTGGCAACA-3') for directional insertion into pENTR/D-TOPO (Invitrogen, Carlsbad, CA), while the reverse PCR primer was made without the TGA stop codon (5'-TCAGGAATCTTCACTTCCGTGTGTTCTA-3') to allow translation of IL-21 with the fluorescent Lumio tag. Following insertion into pENTR/D-TOPO, the expression cassette was then transferred by LR recombination into the pLenti6.2/C-Lumio/V5-DEST plasmid per the manufacturer's protocol (Invitrogen), to create the lentiviral expression plasmids pLenti-6.2/IL21-C-Lumio/V5 (hereafter called pLenti-IL21-Lumio (Fig 1a). Lentiviral supernatant was produced by transiently transfecting 293FT cells using Invitrogen's ViraPower Expression System (utilizing vesicular stomatitis virus G glycoprotein (VSVG) packaging pseudotype). As a control, pLenti6.2/C-Lumio/*lacZ* (pLenti-*lacZ*) was used. Transient supernatant was harvested at 48 and 72 hrs after transfection, snap-frozen and stored at -80°C until use.

Generation of IL-21-producing T cells

To examine the effects of IL-21, CD8⁺ T cells were selected from PBMC and activated on CD3/CD28-coated plates for 48 hours in TCM-AB supplemented with 100 U/ml IL-2 (Proleukin; Chiron, Emeryville, CA). To transduce with lentivirus, activated CD8⁺ T cells, non-tissue culture treated plates were first coated with 4 µg/ml Retronectin (TaKaRa, Otsu, Japan) at 4°C overnight, washed with phosphate buffered saline (PBS) and then coated with lentiviral supernatant. Activated T cells were then plated at 5×10^5 cells/well in supernatant supplemented with 100 U/ml IL-2 and incubated at 37°C/5% CO₂ for 3 days. After incubation, TAPC were harvested, washed and subsequently expanded in TCM-AB plus IL-2. After expansion for a week, the cells were once again CD8 selected by MACS (Miltenyi) to eliminate confounding IL-21 production by residual CD4⁺ cells.

Analysis of IL-21 secretion by gene-modified T cells

Initial detection of transgenic IL-21 in T cells was performed using lentivirus generated from the pLenti-IL21-Lumio construct, where the IL-21 protein includes the 6 amino acid Lumio tag allowing fluorescent detection of recombinant protein within the cell (Fig 1a). Briefly, mock-transduced or T cells transduced with pLenti-IL21-Lumio virus were activated for 48 hours on CD3/CD28 plates in TCM-AB supplemented with IL-2. After activation, T cells were harvested and cultured for 24 hours at 37°C in Opti-MEM Reduced Serum Medium (Invitrogen) to reduce Lumio background. T cells were then washed in Hank's Buffered Saline Solution (HBSS) and then incubated in 1 µM Lumio Green Labeling Reagent (Invitrogen) for 15 minutes at room temperature in the dark. After incubation, cells were washed in HBSS and incubated with Disperse Blue 3 and analyzed by fluorescent microscopy and flow cytometry. IL-21 secretion was subsequently confirmed by ELISA. 1×10^6 IL-21 and *lacZ* gene-modified T cells were activated on CD3/CD28 plates and on days 0 (prior to activation), 3, 5 and 7, cells

were harvested and replated in 24-well plates and incubated in fresh media for 24 hours at 37°C. ELISA plates were prepared by incubating 96-well protein binding plates (Immuno Plate Maxisorp, Nalge Nunc, Rochester, NY) with 2 µg/ml purified mouse monoclonal anti-IL-21 capture antibody (BD Pharmingen) overnight at room temperature. Plates were washed and then incubated with supernatants from activated gene-modified T cells and compared to a standard curve of serial diluted recombinant IL-21 protein (Biosource, Camarillo, CA). Plates were incubated for 24 hours at 4°C, washed and then incubated with biotinylated mouse anti-IL-21 antibody (BD Pharmingen) for 2 hours at room temperature. The ELISA was then developed using streptavidin, substrate and stop solution (all from R&D Systems, Minneapolis, MN). The optical density of each well was then determined at 450 nm using a microplate reader and concentration of IL-21 per 1×10^6 cells per 24 hours calculated from the standard curve.

Generation of tumor-specific CTL

All CTL experiments used cells from HLA-A2+ donors. Activated IL-21 gene-modified and non-modified TAPC were resuspended at 1×10^6 cells/mL of TCM-AB media and pulsed with a single HLA-A2 restricted peptide. These peptides were derived from two melanoma antigens: MelanA/MART-1 (ELAGIGILTV) and gp100 (IMDQVPFSV); or from a B-CLL antigen: RHAMM (ILSLELMKL)³³ and were custom synthesized by Genemed Synthesis (San Francisco). During peptide loading of TAPC, each peptide was pulsed at 10 µg/mL of TAPC in the presence of $\beta 2$ microglobulin at 3 µg/mL, for 2 hrs at 37°C with frequent agitation. Prior to use, TAPC were washed in TCM-AB and irradiated to 30 Gy. Autologous CD8+ cells were used as responders at a 1:2 stimulator to responder ratio (1×10^6 TAPC: 2×10^6 responder T cells in 2 ml in each well of a 24-well plate) in TCM-AB media. The CTL cultures were also supplemented with exogenous cytokines- rhIL-7 at 10 ng/mL and rhIL-12 at 10 ng/mL (both from R&D Systems) on day 0 to augment priming immune responses. The responder CD8+ cells were stimulated weekly with irradiated peptide pulsed TAPC for up to 3 stimulations, using the protocol described above. After the second stimulation the CTL cultures were supplemented with IL-2 at 50 U/ml twice weekly to enhance CTL expansion.

Phenotyping

To determine antigen-specific T cell frequency and phenotype, cells were analyzed by flow cytometry (FACS Calibur; BD) using peptide pentamers for MART-1 (ELA) and gp100 (IMD) in combination with APC-labeled FluoroTag (Proimmune, Springfield, VA) and FITC, PE and PerCP conjugated antibodies for CD3, CD8, CD27, CD28, CD44, CD45RA, CD62L, CCR7 and CD127 (IL-7R α) (all from BD). To determine the percentage of cells capable of secreting IFN- γ following activation, we performed intracellular cytokine flow cytometry (CFC). CTL generated were stimulated for 2 weeks with TAPC-lacZ or TAPC-IL21 and then rested for 1 week with media supplemented with 100 U/ml IL-2. Cells were harvested and resuspended in TCM-AB, and then non-specifically stimulated with 25 ng/ml phorbol myristate acetate (PMA; Sigma, St. Louis, MO) and 1 µg/ml ionomycin (Sigma). After incubation for 1 hour at 37°C, 10 µg/ml Brefeldin A (Sigma) was added and the cells were cultured for another 5 hours. Following incubation, cells were washed, fixed in 1% paraformaldehyde and permeabilized with 0.1% saponin (Sigma) in PBS. Cells were then incubated with antibodies to CD3, CD8 and IFN- γ (all BD) and analyzed by flow cytometry.

ELISpot and cytotoxicity assays

Determination of frequency and function of tumor-specific CTL was determined using IFN- γ ELISpot and 51Chromium (Cr51)-release assays. For IFN- γ ELISpot analysis, 1×10^5 cells were resuspended in TCM-AB and plated in triplicate on Multiscreen 96-well plates (Millipore, Bedford, MA) coated with mouse anti-human IFN- γ antibody (Mabtech, Cincinnati, OH). CTL were stimulated for with cognate tumor peptide at 500 ng/ml diluted in TCM-AB and compared

to CTL stimulated with an irrelevant control peptide, or CTL in media alone. After incubating the plate for 20 hours at 37°C, the plate was developed as previously described.³⁴ To measure antigen-specific cytotoxicity, CTL were assayed against Cr51-labeled target cells in a standard 4 hour release assay. MART-1 (ELA)-specific CTL were tested for cytotoxicity against two different tumor cell lines, mel1143 (HLA-A2+; MART-1+) and Senma (HLA-A2-; MART-1+). To determine the cytotoxic potential of CTL, CTL were FACS sorted following labeling with ELA pentamer (>95%) and then used as effector cells against CEM.T2 cells pulsed with ELA peptide. CTL were also compared for cytotoxicity against K562 and HSB-2 cell lines for natural killer (NK) cell-mediated killing. Specific lysis was calculated as $([\text{experimental release} - \text{spontaneous release}] / [\text{maximum release} - \text{spontaneous release}]) \times 100$.

Cell death/apoptosis assays

To determine whether IL-21 has an effect on cell survival, we examined cell death and apoptosis of T cells exposed to IL-21 compared to control T cells by staining cells with Annexin-V and propidium iodide (PI; BD) followed by FACS analysis. Here, 1×10^6 T cells were plated in media alone or supplemented with 100 U/ml IL-2, 10 ng/ml IL-21 or both cytokines. Cells were analyzed for PI and Annexin staining by FACS analysis on days 0, 3, and 7.

RT-PCR

To measure expression of Tcf7 and Lef1 gene expression in CTL exposed to IL-21, we performed RT-PCR. Tumor-specific CTL expanded with TAPC-*lacZ* or TAPC-IL21 were harvested after 14 days in culture and RNA was isolated and used as template for One-Step RT-PCR (Qiagen) with PCR primers for Tcf7, Lef1 and GAPDH (SABiosciences, Frederick, MD).

Statistics

Paired, two-tailed Student's T-test was used to determine statistical differences between experimental groups. A *P*-value of less or equal to .05 was considered statistically significant.

RESULTS

CD8+ TAPC can be genetically modified to secrete IL-21

To determine if transgenic IL-21 could enhance generation of antigen-specific CTL using TAPC as stimulator cells, we genetically modified CD3/CD28 stimulated CD8 selected T cells with pLenti-IL21-Lumio or pLenti-*lacZ*-Lumio, or mock transduced (Retronectin only) activated T cells. To confirm transgene expression and transduction efficiency, we assayed mock-transduced and pLenti-IL21-Lumio transduced T cells using the Lumio In Cell labeling reagent. Gene-modified T cells expressed high levels (52% transduction efficiency over background Lumio fluorescence) of the Lumio tag compared to mock transduced T cells when analyzed by fluorescent microscopy and FACS analysis (Fig. 1a & b). To confirm that transduced T cells could secrete IL-21, we analyzed supernatant from IL-21 and mock transduced T cells by ELISA and found that only T cells modified with pLenti-IL21-Lumio produced detectable quantities (>1 ng/ml) of IL-21 (Fig. 1c). IL-21 secretion was dependent on activation, where IL-21 secretion peaked 3 days post-CD3/CD28 stimulation and was no longer detectable at 7 days post stimulation. As observed with transgenes under the control of long terminal repeat (LTR) promoters, secretion of IL-21 was correlated to T cell activation^{35,36}. Importantly, restimulation of previously activated T cells could induce further IL-21 production to levels similar to the initial stimulation (Fig. 1d). This shows that CD8+ T cells, which do not have the capacity to endogenously produce IL-21, can be modified to secrete the cytokine in an activation dependent manner.

Phenotype and proliferation of IL-21 expressing TAPC

Previously, we demonstrated that TAPC expressing IL-7 and IL-12 could be rapidly produced from peripheral blood T cells, and that activation of gene-modified TAPC upregulated expression of MHC class I and II, as well as costimulatory molecules,³⁰ however the effects of IL-21 secretion on TAPC were not known. TAPC were transduced with either IL-21 or *lacZ* and measured for proliferation post CD3/CD28 stimulation. Both IL-21 and control TAPC expanded rapidly, over 150-fold (range, 150–350-fold) by 14 days suggesting that IL-21 did not enhance or suppress proliferation during primary stimulation (Fig. 2a). To examine whether IL-21 affected the phenotype of activated TAPC, IL-21 and *lacZ* expressing cells were stimulated for 48 hours with CD3/CD28 antibodies and analyzed by flow cytometry. Both IL-21 and *lacZ* modified TAPC expressed high levels of MHC class I and II, as well as the costimulatory molecules CD80, CD86 and CD83 (Fig. 2b). However, as observed previously,²⁷ IL-21 affected expression of CD28. These data show that like IL-7/IL-12 gene-modified TAPC, IL-21 transduction does not affect the expression of antigen presentation molecule or proliferation, indicating that production of IL-21 secreting TAPC is possible.

IL-21 gene-modified TAPC enhance selection and expansion of MART-1-specific CTL

Because activated CD4⁺ T cells produce IL-21, we performed our experiments using highly purified CD8⁺ T cells as both TAPC and responder cells, thereby avoiding any confounding effects from endogenous IL-21. To test whether IL-21 promoted the generation of tumor-specific CTL, we performed initial experiments using the MART-1, HLA-A2–restricted peptide ELAGIGILTV, which has a single amino acid substitution to increase its immunogenicity.³⁷ Control or IL-21-secreting TAPC were activated for 48 hours to increase MHC and costimulatory molecule expression, then pulsed with ELA peptide and used to stimulate autologous CD8⁺ T cells in the presence of IL-7 and IL-12, as previously described.³⁰ Pentamer staining showed that stimulation of autologous T cells with ELA peptide loaded, IL-21 secreting TAPC markedly enhanced ELA-specific CTL generation after two weekly stimulations (Fig. 3a). Similar results were obtained in 5 additional HLA-A2⁺ donors where mean ELA pentamer frequency was 8.5% (range, 1.1% to 18.3%) using TAPC-IL21 versus a mean of 2.0% (range, 0.12% to 5.8%) ($P=.04$) (Fig. 3b). In addition, ELA-specific CTL stimulated with TAPC-IL21 expanded 3.7-fold more (mean=1149-fold; range, 275 to 2400-fold) than CTL generated from TAPC-*lacZ* (mean=314-fold; range, 30 to 750-fold) ($P=.02$) (Fig. 3c). A similar trend was observed by ELISpot assays, where CTL generated from TAPC-IL21 showed an increase in IFN- γ producing cells following peptide stimulation ($P=.02$) (Fig. 3d). It is important to note, that IL-21 alone was not sufficient to generate TAA-specific CTL, but required IL-7 and IL-12 during the priming phase (1st stimulation) (Fig. 3e).

In addition to producing IFN- γ in response to peptide stimulation, MART-1 specific CTL generated from TAPC-IL21 also killed HLA-A2⁺, MART-1 expressing melanoma tumor cells (Senma), but not HLA-A2[–], MART-1 tumor cells (Mel-1143) (Fig. 4a). This improvement in cytotoxicity appeared to be due to the increased frequency of ELA-specific CTL generated by IL-21 secreting TAPC rather than enhanced cytotoxic function on a per cell basis, where CTL FACS sorted following ELA-pentamer labeling showed equivalent killing against ELA peptide loaded CEM.T2 cells (Fig. 4b).

To confirm that the enhanced generation of ELA-specific CTL observed using TAPC-IL21 was not confined to the MART-1 heteroclitic peptide, CTL lines were generated against gp100 and RHAMM. As found with ELA, gp100 showed enrichment of IMD-specific CTL when stimulated with IL-21 secreting, peptide-loaded TAPC compared to TAPC alone (Fig. 5a). When assessed by IFN- γ ELISpot assays, both gp100 and RHAMM-specific CTL generated by TAPC-IL21 showed greater antigen-specific frequency compared to TAPC-*lacZ* (Fig. 5b &

c). Together, these data demonstrated that CD8⁺ CTL generated against a immunogenic tumor peptide are more efficiently generated in the presence of an IL-21 secreting APC.

IL-21 enhances generation of antigen-specific T cells with a central memory phenotype

CD4⁺ T cell help during primary infection is essential for the programming a functional CD8⁺ + memory response capable of a rapid secondary expansion upon antigen rechallenge.^{38,39} Since IL-21 is produced by activated CD4⁺ T cells²³ and has previously been shown to alter the gene expression program and function of murine T cells,^{26,27} we examined whether stimulation with IL-21 expressing TAPC could produced phenotypic and functional differences in responding tumor-specific CTL. First, we examined the phenotype of bulk CTL cultures for expression of receptors that distinguish between naïve, central and effector memory T cells.^{40,41} Analysis of CTL primed with IL-7/IL-12, and then stimulated with TAPC or IL-21 secreting TAPC showed an increase in CD62L and CCR7 expression, as well as a marked increase in expression level of both CD27 and CD28 (Fig. 6a). To examine the phenotype of tumor-specific CTL, MART-1-specific T cells were gated following ELA pentamer labeling and then analyzed for surface expression. We observed that MART-1-specific T cells showed a significant increase in CD27 and CD62L expression ($P < .05$), whereas CD28 was moderately elevated (Fig. 6b & c). Interestingly, CTL cultured in the presence of IL-21 showed more homogeneous expression of these receptors, whereas in the absence of IL-21, bimodal expression was seen (Fig. 6b). Subsequently, we examined whether IL-21 affected the gene expression program as previously observed in murine splenocytes.²⁶ CTL cultured with IL-7, IL-12 and IL-21 were compared to control (IL-7 and IL-12, but no IL-21) T cells and examined for expression of Tcf7 and Lef1. Here, we observed that human T cells exposed to IL-21 during cell expansion also showed increased expression of Tcf7 and Lef1 transcripts, as compared to the housekeeping gene, GAPDH (Fig. 7a). In addition, to a central memory phenotype and expression of genes associated with central memory T cells, CTL stimulated with IL-21 producing TAPC also showed improved survival in the absence of IL-2 as determined by Annexin-V/PI staining (Fig. 7b). Furthermore, central memory T cells have also been shown to produce elevated levels of proinflammatory cytokines compared to effector memory T cells following activation.²⁸ To test whether IL-21 could influence cytokine secretion, we examined IFN- γ production using intracellular staining (which allows analysis of frequency of IFN- γ producing T cells) following PMA/Ionomycin stimulation. We observed that in 5 donors, exposure to IL-21 enhanced the percentage of T cells capable of secreting IFN- γ (Fig. 7c). Together with enhanced antigen-specific proliferation (Fig. 3d), CTL generated with IL-21 show a central memory phenotype (CD27^{high}CD28^{high}CD62L^{high}CCR7^{low}), improved survival in the absence of IL-2, enhance IFN- γ production and expression of genes associated with immature effector T cells²⁶ suggesting that IL-21 stimulated tumor-specific T cells may possess improved function in adoptive therapy studies.

DISCUSSION

We previously reported the genetic modification of TAPC with IL-7 and IL-12 were capable of selecting and expanding MART-1 and MAGE-3-specific CTL from healthy donors.³⁰ Because IL-21 has been shown to inhibit DC function,²⁹ we chose to evaluate the effects of IL-21 for CTL generation using an approach similar to Ansen et al,²⁸ where non-professional APC genetically modified to secrete IL-21 were examined for their capacity to stimulate tumor-specific CTL. Using a model antigen (MART-1), we show that IL-21 genetically modified CD8⁺ TAPC, in conjunction with IL-7 and IL-12, significantly enhanced the reactivation and expansion of peptide-specific CD8⁺ CTL by >8-fold compared to stimulation with TAPC plus IL-7 and IL-12 alone. In addition to increasing antigen-specific CTL frequency, CTL generated with the IL-7/IL-12/IL-21 combination were fully functional, producing IFN- γ in response to cognate antigen stimulation and retaining cytotoxicity towards HLA-A2 matched, MART-1

expressing tumor cells. Importantly, the requirement for IL-7 and IL-12 could not be overcome by addition of IL-21 alone (data not shown). Nevertheless, TAPC-producing IL-21 were superior APC for the enrichment and expansion of CTL specific for a variety of tumor associated antigens (e.g. MART-1, gp100, RHAMM).

Li and colleagues previously showed that tumor-specific CTL generated during IL-21 exposure showed enhanced proliferation and peptide specificity, while retaining high expression of the costimulatory receptor CD28.²⁷ Experiments performed using IL-21 secreting artificial APC (K562 cells modified to express HLA-A2, CD80, CD83 and IL-21) produced CTL with improved functional qualities (e.g. expansion and cytokine secretion).²⁸ In the murine system, exposure of gp100-specific CD8+ T cells to IL-21 induced a unique differentiation program compared to IL-2 and IL-15.²⁶ Our results also support these studies showing that stimulation with IL-21 secreting TAPC promote the generation of tumor-specific CD8+ CTL with a central memory phenotype, as characterized by the expression of CD27, CD28, CD62L, CCR7 and IL-7R α . This was different from the MART-1 specific CTL generated in the absence of IL-21 which had a terminal effector phenotype characterized by CD44^{high}, CD62L^{low}, CD27^{low}, CD28^{low}, IL-7 receptor- α ^{low}. It is important to note that while IL-21 maintained tumor-specific CTL with a central memory phenotype for more than 4 weeks (>1000-fold expansion), CTL eventually reverted to an effector memory phenotype, with loss of CD62L and CCR7 during long-term expansion (>8 weeks, data not shown). While IL-21 may be necessary for central memory formation, it is likely that other co-stimulatory molecules, such as 41BBL, may be required for long-term central memory persistence during continued TCR stimulation.⁴²

Since CD4+ help is required for generation of long-lived CD8+ memory cells capable of long-term persistence and expansion in vivo,³⁸ our data suggest that IL-21 produced by activated CD4+ cells may be one of the signals responsible for programming of memory. Multiple studies have shown that antigen-specific CTL with a memory phenotype, expressing high levels of co-stimulatory molecules (CD27, CD28) have longer telomere lengths, proliferate better on antigenic exposure and persist longer in vivo in both murine models and melanoma patients, resulting in more effective adoptive immunotherapy on a per cell basis.^{14,21} Concordant with the programming of a memory phenotype, we have also shown that T cells activated by CD3/28 stimulation in the presence of IL-21 have improved proliferation and survival as compared to T-cells activated in the absence of IL-21. Our data suggest that the proliferative and anti-apoptotic effects of IL-21 on antigen-activated T cells are most prominent between days 9–12 after activation. These findings support previous data showing that in contrast to IL-2 which promotes activation-induced cell death in the clonal contraction phase of the immune response, IL-21 promotes long-term survival of antigen-specific cells while maintaining effector activity.⁴³

These data suggest that TAPC modified to secrete cytokines such as IL-7, IL-12 and IL-21, may be useful as autologous or allogeneic cellular vaccines. The combination of these cytokines enhances the generation of tumor-specific CTL and when IL-21 is included, promotes the generation of CTL with improved survival and proliferation antigen reencounter. Additionally, IL-21 appears to suppress the inhibitory effects of regulatory T cells,^{44,45} which may be required to induce sufficient antigen-specific immunity during vaccination in patients with cancer. As activated T cells are readily gene modified, TAPC may serve as an unlimited source of APC expressing appropriate MHC molecules which may be used as an in vivo cellular vaccine, and which may be particularly potent when secreting a variety of prostimulatory cytokines such as IL-7, IL-12 and IL-21.

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Figure 1. Gene-modification of TAPC with IL-21

A) IL-21 was cloned by PCR and subcloned into the pLenti6.2/C-Lumio/DEST vector by LR recombination, which allows a IL-21 fusion protein expressing the Lumio tag. The plasmid pLenti6.2/*LacZ*-V5 was used as a control plasmid. **B)** TAPC were transduced with pLenti-IL21-Lumio or mock transduced labeled with 1 μ M Lumio Green and analyzed by fluorescent microscopy or **(C)** by flow cytometry, where >50% of pLenti-IL21-Lumio TAPC (black line) were positive for Lumio Green compared to mock transduced (gray line). **D)** TAPC transduced with either IL-21 or *lacZ* lentivirus were assayed by ELISA for IL-21 production following CD3/CD28 stimulation. **E)** TAPC cultured for 10 days were reactivated with CD3/CD28 for 24 hours and tested for IL-21 by ELISA demonstrating that IL-21 production can be restored in pLenti-IL21-Lumio transduced T cells.

Figure 2. Proliferation and phenotype of IL-21 transduced TAPC

A) TAPC transduced with IL-21 or lacZ were enumerated for 14 days following CD3/CD28 stimulation demonstrating that IL-21 did not affect proliferation of non-specific T cell expansion. **B)** The phenotype of IL-21 and control TAPC was analyzed by flow cytometry for the expression of CD28, CD80, CD83, CD86, HLA-ABC and HLA-DR demonstrating increased CD28 expression in TAPC-IL21.

Figure 3. Generation of ELA-specific CD8+ CTL using TAPC-IL21

A) CTL cultures stimulated weekly with ELA peptide-pulsed TAPC-IL21 or TAPC-*lacZ* were assayed for ELA frequency by pentamer analysis. **B)** ELA-pentamer frequency was measured on day 14 for 6 donors demonstrating a significant increase in ELA-specific CTL when stimulated with TAPC-IL21 compared to TAPC-*lacZ* (* $P < .05$). **C)** Analysis of CTL cultures generated from IL21 or control TAPC was assessed by IFN- γ ELISPOT showing a significant increase in ELA specificity in IL-21 stimulated cultures (* $P < .05$). **D)** Expansion of ELA-specific CTL was calculated by multiplying the frequency of ELA pentamer-specific T cells by overall culture growth. Fold-expansion was significantly greater in IL-21 stimulated cultures (* $P < .05$). **E)** IL-21 alone was insufficient for the generation of ELA-specific CTL, and required the presence of IL-7 and IL-12 during priming. Frequency of ELA-specific CTL as determined by pentamer staining shown at day 0 (top) compared to TAPC-IL21 alone, or TAPC-*lacZ* plus rhIL-7 and rhIL-12, or with TAPC-IL21 plus rhIL-7 and rhIL-12.

Figure 4. Cytotoxicity of ELA-specific CTL generated by TAPC-IL21

A) ELA-specific CTL generated by both IL-21 and *lacZ* transduced TAPC showed specific killing of HLA-A2⁺ (matched), MART-1+ tumor cells (Senma) but not HLA-A2⁻, MART-1 + tumor cells (Mel-1143) demonstrating that cytotoxic effector function was not adversely affected by IL-21. **B)** To determine if IL-21 enhanced cytotoxicity on a cell per cell basis, ELA-specific CTL were FACS sorted after ELA pentamer labeling (>95%) and used as effector cells against CEM.T2 (T2) cells pulsed with ELA peptide. Both CTL generated in the presence of IL-7 and IL-12, and stimulated with either TAPC-*LacZ* or TAPC-IL21 showed equivalent killing to peptide-bearing target cells.

Figure 5. Generation of gp100 and RHAMM-specific CTL using TAPC-IL21

A) To demonstrate that TAPC-IL21 have the capacity to stimulate CTL specific for other tumor antigens, CTL cultures were initiated using IMD (gp100) peptide-pulsed TAPC and subsequently analyzed for IMD-specific T cell frequency using pentamer analysis. **B)** IFN- γ ELISpot analysis for IMD frequency was elevated in 3 normal donors when TAPC-IL21 were used as APC compared to TAPC-*lacZ*. **C)** Similarly, CTL generated with RHAMM peptide-pulsed TAPC-IL21 showed increased RHAMM-specific CTL when compared to cultures stimulated with TAPC-*lacZ*.

Figure 6. Central memory phenotype in ELA-specific CTL stimulated with TAPC-IL21
A) Flow cytometric analysis of bulk culture CTL stimulated with IL-21 TAPC showed increased expression of CD27, CD28, CD62L and CCR7 compared to control TAPC. IL-21 producing TAPC also enhanced the generation of CD27^{high}CD28^{high} CTL (inset box). **B)** To examine the phenotype of ELA-specific CTL, cultures stimulated with either IL-21 or control TAPC were labeled with ELA pentamer APC, and subsequently labeled with CD62L FITC and IL-7Rα. During analysis, CTL cultures were gated on ELA pentamer positive cells and analyzed for CD62L and IL-7Rα expression, which showed a marked increase in IL-7Rα expression in IL-21 treated CTL cultures. **C)** ELA pentamer gated CTL were analyzed for expression of CD27, CD28 and CD62L following stimulation with control of IL-21 producing TAPC. This analysis showed that IL-21 treatment enhanced expression of all 3 receptors whereas control TAPC produced a large number of cells (bimodal expression) that lacked CD27, CD28 and CD62L expression. **D)** Analysis of ELA-specific CTL lines generated from 6 donors showed significantly higher expression of CD27⁺, CD27⁺CD28⁺ and CD62L⁺ CTL with IL-21 compared to control TAPC (**P*<.05).

Figure 7. Unique gene expression program and functional characteristics of ELA-specific CTL generated with TAPC-IL21

A) Gene expression of Tcf7 and Lef1 were compared by RT-PCR from ELA-specific (MART-1) CTL generated from 3 donors with either IL-21 or control TAPC. Tcf7 was upregulated in 3/3 CTL generated from IL-21 producing TAPC, while Lef1 was upregulated in 2/3 CTL lines, when compared to the internal housekeeping control GAPDH. **B)** Survival of IL-21 or control treated CTL was analyzed by Annexin V/PI staining following stimulation with CD3/CD28 antibodies and subsequent culture in IL-2 containing media, or media alone. IL-21 stimulated CTL lines showed increased survival during IL-2 withdrawal. **C)** IL-21 enhanced the frequency of IFN- γ producing CTL. CTL stimulated with and without IL-21 were subsequently activated with PMA/ionomycin. In 5 donors, IFN- γ production was significantly increased in IL-21 treated CTL (* $P < .05$).