

IL-21 Influences the Frequency, Phenotype, and Affinity of the Antigen-Specific CD8 T Cell Response¹

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IL-21, a newly described cytokine belonging to the IL-2 γ -chain receptor cytokine family (that includes IL-2, IL-7, and IL-15), has been described as an important regulator of the cellular immune response. In this study, the role of IL-21 in the generation of a human Ag-specific CD8⁺ T cell response is characterized by tracking a rare, but measurable population of self-Ag-specific T cells *in vitro*. Autologous dendritic cells pulsed with the melanoma antigen recognized T cells 1 self-peptide were used to stimulate CD8⁺ T cells from HLA-A2⁺ healthy donors and melanoma patients. We demonstrate that exposure to IL-21 increased the total number of MART-1-specific CD8⁺ T cells that could be elicited by >20-fold and, at the clonal level, enriched for a population of high-affinity CD8⁺ T cells with a peptide dose requirement more than 1 log₁₀-fold less than their untreated counterparts. Phenotypic analysis of T cells from IL-21-treated cultures revealed a unique population of CD45RO⁺CD28^{high}CD8⁺ T cells, a phenotype that was stable for at least 4 wk after IL-21 exposure. These CD28^{high}CD8⁺ T cells produced IL-2 upon Ag stimulation and represent potential helper-independent CTLs. Our studies demonstrate a significant role for IL-21 in the primary Ag-specific human CTL response and support the use of IL-21 in the *ex vivo* generation of potent Ag-specific CTLs for adoptive therapy or as an adjuvant cytokine during *in vivo* immunization against tumor Ags. *The Journal of Immunology*, 2005, 175: 2261–2269.

Interleukin-21 is a newly described cytokine belonging to the IL-2 γ -chain receptor cytokine family (IL-2, IL-7, IL-15) (1–3) and appears to share many of the properties associated with T and NK cell activation and differentiation. In murine studies, IL-21 potentiates the maturation and effector function of NK cells and promotes T cell activation in response to alloantigen (4). As a cytokine which limits NK cell expansion and promotes activation of murine CD8 T cells, IL-21 is believed to play a role in the transition from innate to adaptive immunity (4). Among CD4 T cells, IL-21 has been described as both a Th1 cytokine which up-regulates the expression of genes associated with innate immunity (5) as well as a Th2 cytokine that inhibits the differentiation of naive Th cells into IFN- γ -producing Th1 cells (6). The effects of IL-21 in the development of innate immunity and CD4 Th responses are well characterized (5, 7), but its role in the priming of an Ag-specific CD8⁺ T cell response, particularly in humans, has not been fully explored. This is due in part to limitations in detecting and tracking low-frequency Ag-specific responses among naive T cells in a nontransgenic model. Although responses to most self-Ags (such as gp100 and NY-ESO-1) can provide examples of a naive response, they often exist in very low frequency; the most accessible population is represented by melanoma antigen A/melanoma antigen recognized by T cells (MART-1)³ specific

CTLs which, in the healthy unprimed individual, has been characterized phenotypically and functionally as naive T cells (8, 9).

In this study, we characterize the role of IL-21 in the induction of Ag-specific human CD8⁺ T cell responses using peptide-MHC tetramers to track a rare but measurable population of CTL precursors recognizing a self-Ag (9). We find a potent effect of IL-21 in generating a high-affinity Ag-specific CTL response that exceeds by >20-fold, that which can be achieved in control cultures. Induction of a high-affinity CD8 response against self-Ags, which are represented increasingly as potential immune targets in cancer immunotherapy predict a significant role for IL-21 in Ag-specific antitumor strategies.

Materials and Methods

Cell lines and reagents

Melanoma cell lines A375 (gift from S. Rosenberg, NCI, Bethesda, MD) and Mel 526 (gift from M. Lotze, University of Pittsburgh, Pittsburgh, PA) were maintained in RPMI 1640 with 25 mM HEPES, 4 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, 10 mM sodium pyruvate, 1 mM nonessential amino acids, and 10% FBS (HyClone). Both lines express the HLA-A2 allele, but only Mel 526 expresses the MART-1 Ag. The T2 cell line is a TAP-deficient T-B cell hybrid expressing the HLA-A2 allele. EBV-lymphoblastoid cell lines (LCL) are EBV-transformed lymphoblastoid cell lines established in our laboratory.

Induction of human Ag-specific CD8⁺ T cells

Melanoma M27-35 (AAGIGILTV) peptide-specific T cells were generated in a manner similar to that previously described (10–12). Donor blood was typed by the HLA Typing Laboratory at the Puget Sound Blood Center (Seattle, WA). CD8⁺ T cells were first isolated by a CD8-positive isolation kit (Dynabeads; Dynal) from leukapheresis PBMCs, suspended in CTL medium consisting of RPMI 1640, 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin (Invitrogen Life Technologies), and 10% human serum from normal donors, and then placed in 6-well tissue culture dishes (Costar) at 6×10^6 cell/well. Mature dendritic cells (DCs) were harvested and pulsed with 40 μ g/ml synthesized peptides at 2×10^6 cell/ml in the presence of 3 μ g/ml β_2 -microglobulin (The Scripps Laboratory, San Diego, CA) in PBS with 1% human serum albumin (Invitrogen Life Technologies) for 4 h at room temperature. After washing three times with sterile PBS (Invitrogen Life Technologies), DCs were mixed with purified CD8 T cells at 3×10^5 cells/well in 6-well plates. Cytokines, IL-15 (10 ng/ml; R&D Systems), IL-2 (10 U/ml; Chiron), IL-7

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³Abbreviations used in this paper: MART-1, melanoma antigen recognized by T cells 1; DC, dendritic cell; LCL, lymphoblastoid cell line.

(10 ng/ml; R&D Systems), or IL-21 (30 ng/ml; Zymogenetics) were added individually to each well immediately after the culture was initiated. IL-2 (50 IU/ml) and IL-7 (10 ng/ml) were added 1 day after the second stimulation to further facilitate expansion of activated Ag-specific T cells.

DCs were generated as previously described (13) by exposing adherent PBMCs to IL-4 (500 U/ml; R&D Systems) and GM-CSF (800 U/ml; Amgen) in AIM-V medium (Invitrogen Life Technologies) followed by maturation using IL-1 β at 2 ng/ml, IL-6 at 1000 U/ml, TNF- α at 10 ng/ml (R&D Systems), and PGE₂ at 1 μ g/ml (Sigma-Aldrich) for an additional 2 days. The mature DC population contained >90% CD83⁺ DCs on day 8 as determined by FACS analysis.

Ab plus peptide-MHC tetramer staining of T cells

PE- or allophycocyanin-labeled M27-MHC-tetramer and G154-MHC-tetramers were produced in the immune monitoring laboratory at the Fred Hutchinson Cancer Center based on previously described protocols (14). For sample analysis, 0.5×10^6 cells in 25 μ l of 2% FCS/PBS were first stained with peptide tetramer-PE or allophycocyanin (final concentration of 20 μ g MHC/ml) for 1 h at room temperature, followed by anti-CD28-allophycocyanin (BD Pharmingen) or anti-CD28-FITC (Caltag Laboratories), anti-CCR7-PE, and anti-CD45RO or anti-CD45RA-FITC (BD Pharmingen) staining for 20 min at 4°C. After washing with PBS, cells were resuspended in PBS containing 2% FBS and 4',6'-diamidino-2-phenylindole was added. Data were acquired using a FACSCalibur flow cytometer and CellQuest (BD Biosciences) and analyzed using FlowJo software (TreeStar).

Enrichment for naive and memory subsets

T cells were purified from human PBMCs by the sequential application of a combination of magnetic beads and an AutoMACS Magnetic Sorter (Miltenyi Biotec). CD8⁺ cells were isolated using negative selection with the CD8 isolation kit II. Subsequent naive (CD8⁺CD45RO⁻CD45RA⁺CD62L⁺) cell selection involved depletion of memory CD8 cells using a CD45RO bead, followed by positive selection of CD62L-positive cells by staining with PE-conjugated CD62L Ab (BD Pharmingen) and incubation with an anti-PE bead. Memory cell isolation (CD8⁺CD45RA⁻CD45RO⁺) involved depletion of the naive population with a CD45RA⁺ bead. Typical purities assessed by FACS were in excess of 95%.

Cloning and expansion of Ag-specific CTLs

The cloning and expansion procedures were identical to that previously described (10, 15). Tetramer-positive sorted T cells were plated at limiting dilution in 96-well round-bottom plates (Nalge Nunc International) in the presence of irradiated feeder cells (PBLs and LCLs) at a responder:stimulator ratio of 1:50,000 along with anti-CD3 mAb (OKT3; Ortho Tech) and 50 U IL-2/ml in 0.2 ml of CTL medium. Wells positive for clonal growth were identified 10–14 days after plating and screened in a microcytotoxicity assay. Peptide-specific clones were transferred to 25-cm² flasks (Costar), restimulated with anti-CD3 mAb, and irradiated allogeneic PBLs

and LCLs were added as feeder cells for rapid expansion. The cultures were fed with IL-2 at 50 U/ml 24 h after restimulation and then every 3 days. After 14 days, cells were used for further analyses or cryopreserved.

In vitro cytotoxicity assay

Target cells (375, 526 melanoma cell lines, or T2 cells) were labeled with 100 μ Ci of ⁵¹Cr and cocultured with effector cells for 4 h at 37°C plus 5% CO₂. For peptide dose titration studies, T2 were pulsed with a peptide at concentrations ranging from 10¹ to 10⁷ pg/ml for 1 h and then washed before ⁵¹Cr labeling. Released ⁵¹Cr was measured with a gamma scintillation counter and percent specific lysis was determined by using the formula: percent specific release = experimental release – spontaneous release/total release. Spontaneous release was <10% of the total release in all assays.

MHC/peptide dissociation assay to identify high- and low-affinity CTL clones

CTL clones were stained with allophycocyanin-tetramer (20 μ g/ml) for 1 h at room temperature and washed once with cold PBS to eliminate unbound tetramer. Cells were incubated in the presence of an excess (100 μ g/ml) of PE-labeled tetramer to prevent rebinding of allophycocyanin-tetramer after their dissociation from TCR. During this period, aliquots of cells were collected at different time points and fixed in 1% paraformaldehyde for flow cytometry analysis. The rate of APC tetramer dissociation is inversely correlated with TCR affinity (16).

Results

IL-21 augments the frequency of Ag-specific CD8⁺ T cells generated following primary in vitro stimulation

A model system for primary in vitro stimulation of Ag-specific T cells was established by isolating CD8⁺ T cells from PBMCs of HLA A2⁺ healthy donors and coculturing with autologous mature DCs pulsed with immunogenic epitopes of the tumor-associated self-Ag, MART-1 (M27-35 peptide). Cultures were grown with no added cytokine or with increasing doses of IL-21 (0.1–100 ng/ml). Following in vitro stimulation, IL-21-mediated augmentation of Ag-specific CTL was not observed below a concentration of 10 ng/ml, was optimal at 30 ng/ml, and inhibitory at 100 ng/ml (Fig. 1). The reason for the inhibition at 100 ng/ml is not clear, but at this concentration, IL-21 may have a counterregulatory effect associated with its role as a Th2 cytokine (6). A concentration of 30 ng/ml was used in subsequent studies.

The use of other cytokines belonging to the common γ -chain cytokine receptor family was also compared with IL-21. IL-2, IL-7, and IL-15 at their respective optimal concentrations produced no added effect on the frequency of MART-1-specific CD8⁺

FIGURE 1. The effect of IL-21 on CD8⁺ T cells is dose dependent. CD8⁺ T cells from a healthy HLA-A2⁺ donor was stimulated in vitro with autologous mature DCs pulsed with the MART-1, M27 peptide as described in *Materials and Methods*. Varying concentrations of IL-21 (1.0–100 ng/ml) were added at time of stimulation. On day 7 after the second stimulation, 10⁶ cells from each experiment group were harvested and stained with 20 μ g/ml peptide/MHC tetramer-PE. Data are expressed as percentage of tetramer-positive cells among gated lymphocytes (purified CD8⁺ cells). Data are representative of cultures from three HLA-A2⁺ donors.

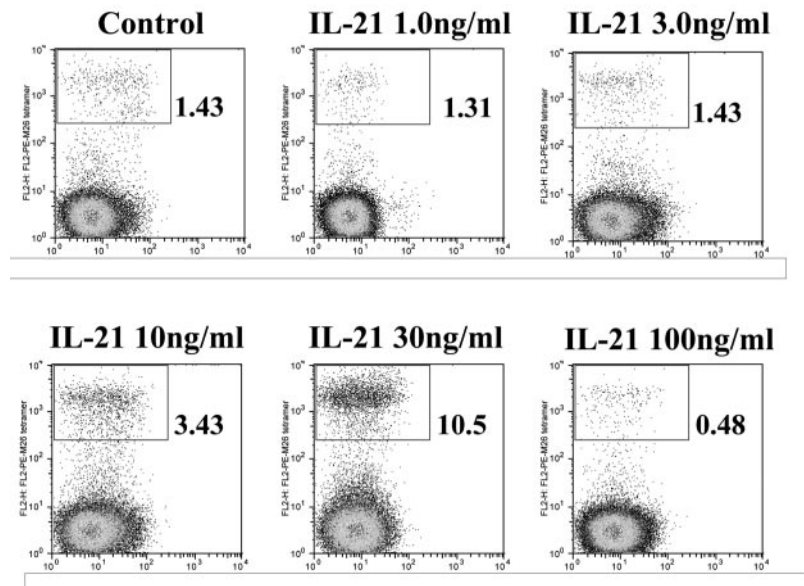
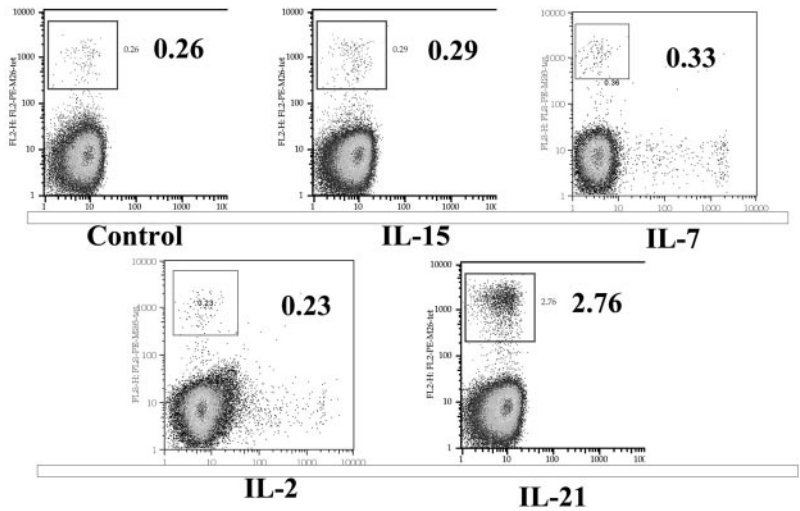


FIGURE 2. Effect of γ -chain receptor cytokines during primary in vitro stimulation of CTLs. CD8⁺ T cells from a healthy HLA-A2⁺ donor was stimulated in vitro with autologous mature DCs pulsed with the MART-1, M27 peptide as described in *Materials and Methods*. Optimal concentrations of IL-15 (30 ng/ml), IL-7 (10 ng/ml), IL-2 (20 U/ml), and IL-21 (30 ng/ml) were added at the time of primary stimulation. On day 7, 10⁶ cells from each experiment group were harvested and stained with 20 μ g/ml peptide/MHC tetramer-PE. Data are expressed as percentage of tetramer-positive cells among gated lymphocytes (purified CD8⁺ cells).



T cells compared with no cytokine control cultures during primary in vitro stimulation (Fig. 2).

To evaluate more rigorously, the role of IL-21 in augmenting the CD8 T cell response, T cell cultures were grown with no added cytokine or with IL-21, and the frequency of MART-1-specific CD8 T cell responses was evaluated 7 days after stimulation by tetramer staining (Fig. 3A). In representative healthy donors (donors CG, NE, and LD), a 16- to 20-fold increase in MART-1-

specific CD8⁺ T cell frequency was observed in IL-21-exposed cultures compared with no cytokine control cultures (0.12 vs 2.26%, 0.12 vs 1.95, and 0.11 vs 2.2%, respectively) following one cycle of in vitro stimulation (Fig. 3A). The absolute numbers of Ag-specific T cells generated in IL-21-treated cultures exceeded control cultures by >20- to 30-fold (Fig. 3B).

The addition of IL-2 and IL-7, however, does promote the ex vivo expansion of previously primed, Ag-experienced T cells as

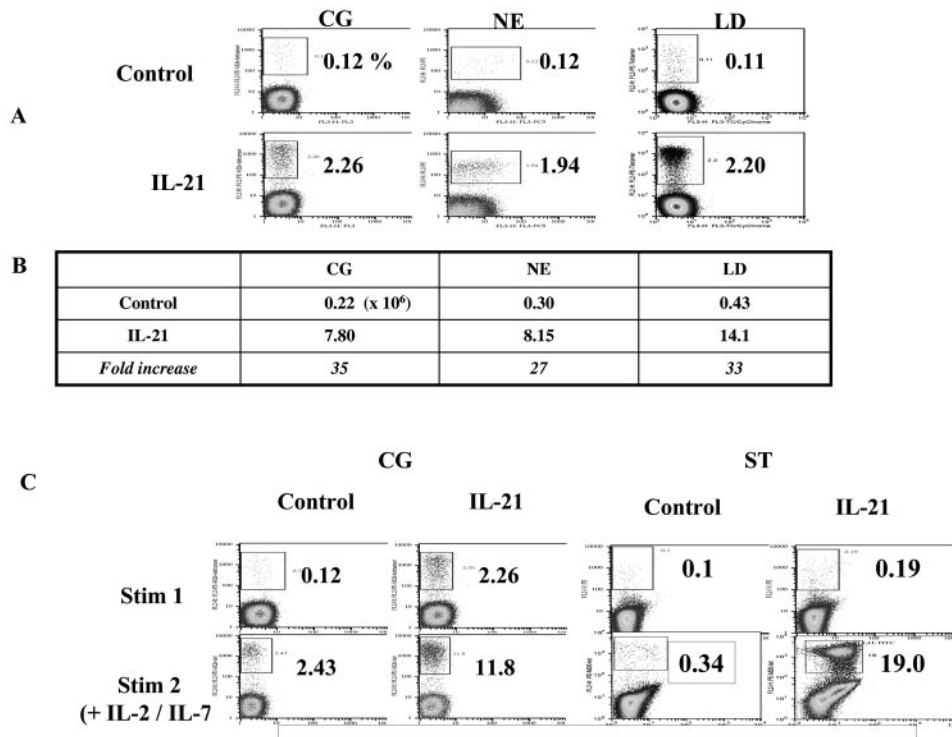


FIGURE 3. IL-21 enhances the generation of MART-1-specific CTLs. CD8⁺ T cells from healthy HLA-A2⁺ donors CG, NE, and LD were stimulated in vitro with autologous mature DCs pulsed with the MART-1, M27 peptide as described in *Materials and Methods*. *A*, On day 7 after stimulation, 10⁶ cells from each experimental group were harvested and stained with 20 μ g/ml peptide/MHC tetramer (PE, vertical axis) and a vital dye (propidium iodide or 4'6'-diamidino-2-phenylindole, horizontal axis). Data are expressed as percentage of tetramer-positive cells among gated lymphocytes (purified CD8⁺ cells). *B*, The absolute number in millions of tetramer-positive cells corresponding to untreated and IL-21-treated cultures from donors CG, NE, and LD depicted in *A* and the fold increase in absolute numbers of IL-21-treated to untreated cultures. *C*, Cultures from a normal healthy donor, CG, and a patient with metastatic melanoma, ST, were analyzed on day 7 after the first (Stim 1) and second (Stim 2) stimulation in the presence or absence of IL-21 during stimulation 1. IL-2 and IL-7 were added after stimulation 2. Data are expressed as percentage of tetramer-positive cells among gated lymphocytes (purified CD8⁺ cells). Results above are representative of three separate experiments for each donor.

demonstrated by our group and others. When added to cultures following a second in vitro stimulation, IL-2 (10 U/ml) and IL-7 (10 ng/ml) produced a further increase in the magnitude of the MART-1-specific CD8 T cell population among IL-21-treated (11.8%) over untreated cultures (2.43%; Fig. 3C, donor CG).

One practical outcome of these studies would be the capacity to augment tumor-associated Ag-specific CTL responses in patients with melanoma, a tumor which shares expression of MART-1. In a representative patient, the frequency of MART-1-specific CTLs generated in IL-21-treated compared with untreated cultures after two cycles of in vitro stimulation demonstrate a 40-fold increase when IL-21 was added compared with untreated controls (19.0 vs 0.34%; Fig. 3C, patient ST).

To evaluate whether the increase in frequency and absolute numbers of Ag-specific CD8 T cells generated among IL-21-treated cultures was due to enhanced proliferation and/or enhanced survival, naive CD8 T cells were labeled with CFSE, stimulated in vitro with MART-1 peptide-pulsed autologous DCs and, at day 7, evaluated for fraction of dividing cells (as determined by quantum decreases in CFSE staining accompanying each cell division) and apoptosis (annexin V staining). For CFSE staining, analyses performed on the tetramer-positive (MART-1-specific) T cell population demonstrate a substantially greater fraction of nondividing cells (rightmost compartment) among untreated cultures (44%) than IL-21-treated cultures (18%; Fig. 4). In fact, the ratio of rapidly dividing (leftmost compartment) to nondividing Ag-specific T cells is >3-fold greater among the IL-21-treated compared with the untreated cultures (63 (18%) vs 36 (44%)). That the effect of IL-21 on T cell proliferation is Ag specific is demonstrated by the

large fraction of tetramer-negative (non-Ag specific) T cells remaining in the nondividing phase (95.6 and 87.9%).

Annexin V staining of tetramer-positive T cells on day 7 reveals a modest decrease in the fraction of apoptotic (annexin V-positive) Ag-specific T cells among IL-21-treated cultures compared with untreated cultures (10.4 vs 5.4% of tetramer-positive T cells, respectively, Fig. 4). Taken together, these results suggest that the increase in frequency and absolute numbers of Ag-specific CD8 T cells generated among IL-21-treated cultures was due predominantly to enhanced Ag-specific cellular proliferation and in minor part to increased survival or decreased apoptosis.

IL-21 enhances Ag-specific T cell response among a predominantly naive CTL population

The capacity of IL-21 to enhance the generation of Ag-specific CD8⁺ T cells was evaluated separately among naive and memory T cells. Purified populations of naive (>98% CD45RA⁺CD62L⁺) CD8⁺ T cells were compared with memory (100% CD45RO⁺) CD8⁺ T cells from both a healthy normal donor (donor CG) (Fig. 5) and an individual with metastatic melanoma (patient ST). Whereas IL-21 exerts minimal effect on the frequency of MART-1-specific cells generated from memory CD8⁺ T cells (0.10–0.15% and 0.05–0.037%), a 12- to 90-fold increase is observed among naive CD8 T cells following IL-21 exposure (0.94–12.5% and 0.08–7.08%), suggesting that IL-21 influences primarily naive T cells.

CTL generated from IL-21-treated cultures represent a population of high-affinity Ag-specific T cells with enhanced tumor reactivity

To further characterize the function of Ag-specific T cell populations generated under the influence of IL-21 at the clonal level, tetramer-positive CD8⁺ T cells from both a healthy donor (donor CG) and melanoma patient (patient ST) were sorted on day 7 and cloned at limiting dilution into 96-well plates. MART-1-specific clones identified by microcytotoxicity assays were expanded and tested for 1) the peptide concentration required for 50% maximal lysis (P₅₀) of peptide-pulsed T2 cells and 2) the ability to lyse

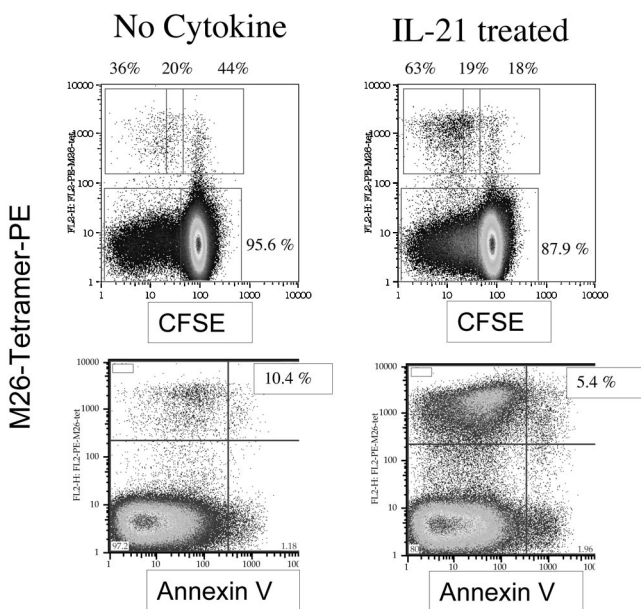


FIGURE 4. IL-21-treated cells undergo increased proliferation and decreased apoptosis during primary in vitro stimulation. Purified populations of naive (CD45RA⁺CD62L⁺) lymphocytes were preincubated with CFSE and stimulated with MART-1 peptide in the absence (No Cytokine) or presence of IL-21 (IL-21 treated). On day 7, cells were stained with MART-1 peptide-MHC tetramer-PE and analyzed for the fraction of dividing cells (CFSE) or cells undergoing apoptosis (annexin V). CFSE-stained cells lose fluorescence intensity with successive divisions. Results are expressed as percentage of nondividing (*rightmost box*), dividing (*center box*), and rapidly dividing (*leftmost box*) tetramer + cells. For apoptotic cells, annexin V staining cells are represented as a percentage of tetramer-positive cells in the *right upper quadrant*.

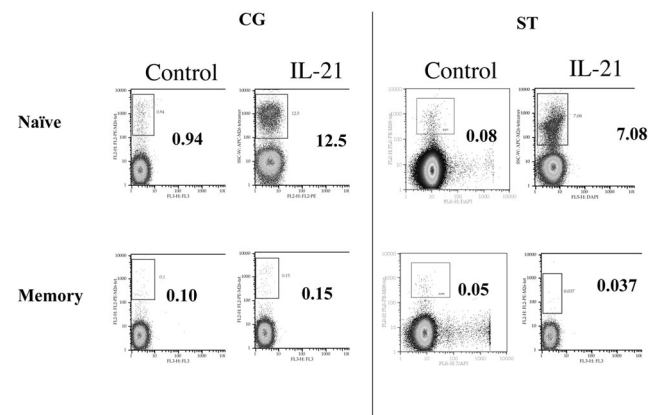


FIGURE 5. IL-21 influences primarily naive vs memory CD8⁺ T cells. Highly purified populations of naive (CD45RA⁺CD62L⁺) or memory (CD45RO⁺) T cells from a healthy normal donor (CG) and a donor with melanoma (ST) were evaluated for induction of Ag-specific responses to MART-1 peptide in the absence or presence of IL-21. The percentage of MART-1-specific CTLs are expressed as percentage of tetramer-positive cells among total gated lymphocytes (CD8⁺ purified cells) after two cycles of in vitro stimulation. Results are representative of experiments performed on two other normal healthy donors (donors NE and LD) and two other patient donors (donors AM and RE).

Ag-positive melanoma targets. For evaluating P_{50} , the HLA-A2-transfected EBV B cell line T2 was titrated with peptide concentrations ranging from 10^7 to 10^2 pM. Results are presented as the peptide dose requirement (nanomolar) for 50% lysis (P_{50}). CTL clones generated from IL-21-treated cultures required a >1 log lower peptide dose requirement than their untreated counterparts, mean 3 nM (range, 0.6–30 nM) vs mean 80 nM (range, 16–500 nM), respectively (Fig. 6A). A similar effect of IL-21 was seen for CTL clones generated from melanoma patient S.T. (Fig. 6B).

At an E:T ratio of 10:1, T cell clones isolated following stimulation in the presence of IL-21 displayed much higher specific lytic activity against the MART-1-positive 526 melanoma cell line (35–45%) than those isolated in the absence of IL-21 (Fig. 6, C and D). For each individual clone, increased tumor reactivity was coincident with decreased peptide dose requirement, suggesting that CTLs generated in the presence of IL-21 exhibited a higher avidity interaction with its cognate target.

That the increased tumor avidity is attributable to a higher affinity TCR and not other accessory factors can be demonstrated using tetramer-based TCR staining assays. Although the intensity of tetramer staining can generally be correlated with TCR affinity (11, 17), a more precise definition of TCR affinity can be obtained based on the rate of tetramer dissociation, K_d , from its specific TCR ligand (16). In this assay, the K_d of the TCR-peptide-MHC interaction or TCR affinity is inversely correlated with the fraction of bound tetramer remaining over time in the presence of an excess of unlabeled tetramer. CTL clones elicited from IL-21-treated or untreated cultures were stained with M27 peptide-tetramer-PE and incubated with excess unlabeled M27-tetramer. The fraction of tetramer-bound CTLs was determined by flow cytometry at specified time points (2–60 min). TCR/tetramer-peptide off rates were found to be significantly faster for clones isolated from untreated cultures compared with clones generated in IL-21-treated cultures (Fig. 7). Taken together, these results demonstrate

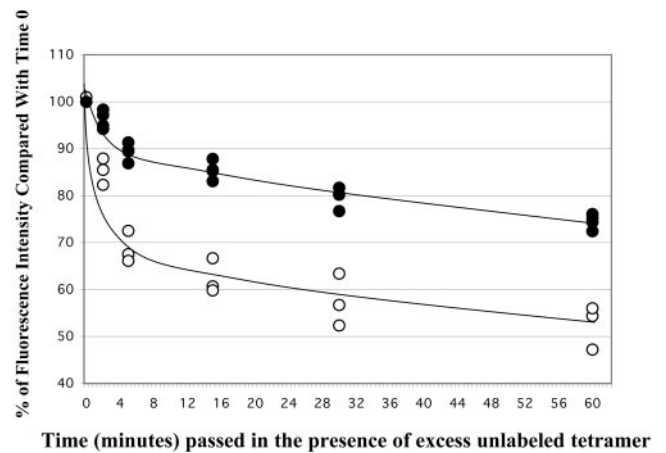


FIGURE 7. IL-21-treated cultures enriched for CTLs expressing higher affinity TCR: tetramer dissociation assay. The tetramer dissociation assay, which depicts the fraction of bound tetramer remaining over time in the presence of an excess of unlabeled tetramer, was used as a surrogate measure of the TCR dissociation rate or TCR affinity of individual clones. The fraction of PE-tetramer-labeled MART-1-specific CTL clones from IL-21-treated cultures (●) was compared with clones from untreated cultures (○) over time (2–60 min). The rate of decrease in the percent fluorescence intensity of tetramer staining from time 0 is inversely correlated with TCR affinity. Results are representative of four donors.

that IL-21 treatment leads to the generation of T cell clones expressing high-affinity TCR.

To demonstrate whether IL-21-mediated enrichment for high-affinity T cells was due to oligoclonal expansion of a limited number of Ag-specific T cells or represented a broader effect on the T cell repertoire, we examined TCR $V\beta$ expression among the cohort

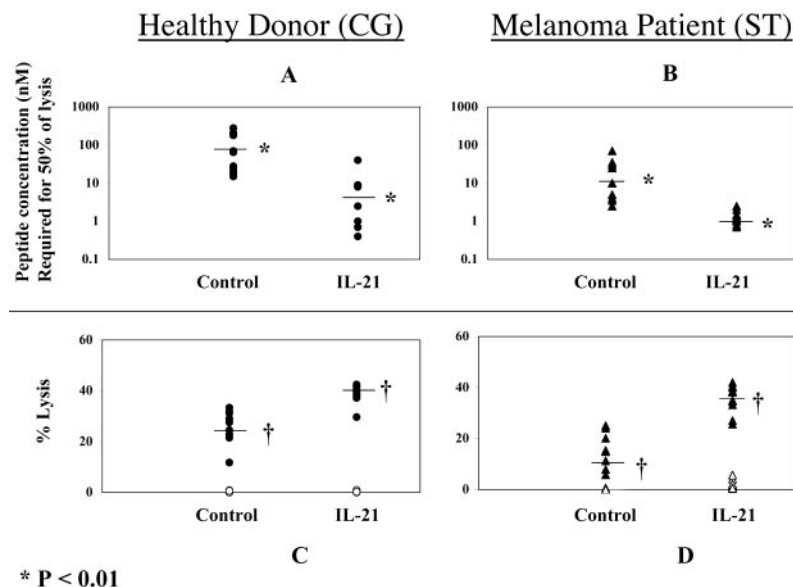


FIGURE 6. IL-21 preferentially induces the generation of high-avidity Ag-specific CTLs. Individual MART-1-specific $CD8^+$ T cell clones were isolated from cultures stimulated in the absence (Control) or presence (IL-21) of IL-21. Eight to 12 representative CTL clones from each experimental condition were evaluated for target affinity in a chromium release assay using T2 cells pulsed with decreasing concentrations of M27 peptide (peptide dose titration analysis). Data are expressed as the concentration (nanomolar) required to reach 50% maximal lysis of target cells. On average, CTL clones generated by IL-21 demonstrated a decreased peptide dose requirement for specific lysis compared with CTL clones generated in no cytokine control (*, $p < 0.01$). A, Healthy donor CG (●); B, melanoma patient ST (Δ). These same clones were evaluated for specific reactivity to a MART-1⁺ tumor cell line (526) at E:T ratio of 10:1 in a standard 4-h ^{51}Cr release assay. Significantly greater lysis of Ag-positive tumor (526: ● and ▲) with background lysis of Ag-negative tumor (375: ○ and △) was observed in CTL clones isolated from IL-21-treated culture than those isolated from no cytokine control. (†, $p < 0.01$). C, Healthy donor CG (● and ○); D, melanoma patient ST (Δ and ▲). Results are representative of three normal healthy donors and three patient donors.

Table I. TCR V β usage among high- and low-affinity clones^a

| | V β usage |
|---------------|-----------------|
| Low affinity | |
| Clone 2 | V β 21.3 |
| Clone 3 | V β 13.1 |
| Clone 4 | V β 13.2 |
| Clone 5 | V β 13.1 |
| Clone 7 | V β ?? |
| Clone 8 | V β 7.2 |
| Clone 9 | V β 23 |
| Clone 10 | V β 14 |
| Clone 11 | V β 13.1 |
| High affinity | |
| Clone 1 | V β 5.3 |
| Clone 2 | V β 20 |
| Clone 3 | V β 3 |
| Clone 5 | V β 17 |
| Clone 7 | V β 14 |
| Clone 8 | V β 20 |
| Clone 9 | V β 17 |
| Clone 10 | V β 13.1 |
| Clone 12 | V β 3 |

^a Individual high- and low-affinity clones were stained with a panel of anti-TCR-V β Abs. Unknown TCR-V β expression designated "??". Results are shown for clones obtained from one HLA-A2⁺ donor and representative of clones obtained from three other donors.

of high- and low-affinity T cell clones using a panel of anti-V β Abs. For example, for patient C.G., among nine high-affinity T cell clones, seven expressed unique V β chains (only two shared V β expression) and a similarly diverse TCR repertoire was observed among the group of low-affinity T cell clones in this patient (among 10 different low-affinity clones, only 2 shared the same V β), suggesting that the effect of IL-21 was not due merely to the expansion of an oligoclonal population of high-affinity T cell clones in vitro (Table I).

IL-21 enriches for a population of CD45RO⁺, CCR7⁻, CD28⁺CD8⁺ T cells following in vitro stimulation

CTLs recognizing the self-Ag MART-1 are present at very low frequency (0.0–0.5% by tetramer staining) in the peripheral blood of donors and are usually characterized by a naive phenotype expressing CD45RA⁺(CD45RO⁻), CCR7⁺, and CD28^{int} (9) (Fig. 8, Pre-Stimulation). About one-half of these precursors (42%) ex-

press intermediate levels of CD28 and nearly 50% are CCR7⁺. We examined the differentiation phenotype of this rare but measurable population of naive T cells under the influence of IL-21. In IL-21-treated or untreated cultures, in vitro Ag-specific stimulation results in a shift from CD45RA⁺ to predominantly CD45RO⁺ expression (Fig. 8, Post-Stimulation). As expected, Ag stimulation is also accompanied by a decrease in CCR7 expression. In IL-21-treated cells however, the level of CD28 expression remained high compared with untreated control cells and this CD45RO⁺CD28^{high} phenotype among IL-21-treated CD8⁺ T cells persisted at least 4 wk after primary in vitro stimulation (Fig. 8). This up-regulation of CD28 expression was observed in both naive healthy donors and melanoma patients for both MART-1- and gp100-specific CTLs.

To evaluate whether up-regulated CD28 expression led to a functionally competent signal, we analyzed Ag-driven IL-2 production among IL-2- treated (CD28^{high}) vs untreated (CD28^{low}) CD8⁺ T cells. Tetramer-positive CD8⁺ T cells from day 27 cultures of IL-21-treated and untreated cultures were stimulated with B7⁺ T2 cells with and without M27 peptide pulsing. After cocultivation for 48 h, supernatants were collected and analyzed for cytokine production. IL-2 production was significantly elevated among IL-21-treated Ag-specific T cells (Fig. 9) and inhibited by addition of CTLA4 Ig (0.5 μ g/ml), suggesting that IL-2 production in these cells was B7-CD28 dependent.

To extend these findings to T cells recognizing other self-Ags, the influence of IL-21 on CD8⁺ T cells was evaluated in similar fashion using two other tumor-associated self-Ags, the melanosomal Ag gp100 (G154 peptide) and the cancer-testis Ag NY-ESO-1 (NY157). Although responses to NY-ESO-1 and, in particular, gp100 are represented in the peripheral blood of unprimed individuals by lower T cell frequencies than responses to MART-1, a similar increase in the population of Ag-specific CTLs among cultures receiving IL-21 is observed. For the gp100 (G154 epitope) and NY-ESO-1 (NY157 epitope), a 7- to 10-fold increase in Ag-specific T cell frequency is observed accompanied by a 15- to 20-fold increase in absolute numbers of Ag-specific CTLs when IL-21 is added (Fig. 10A). Significant up-regulation of CD28 expression among IL-21-treated Ag-specific CTLs is also noted (Fig. 10B). NY-ESO-1- and gp100-specific CTL clones derived from IL-21-treated cultures were also represented by high-affinity interaction with their respective targets (P₅₀ < 1 nM) in a similar manner to IL-21-treated MART-1-specific CTLs (data not shown).

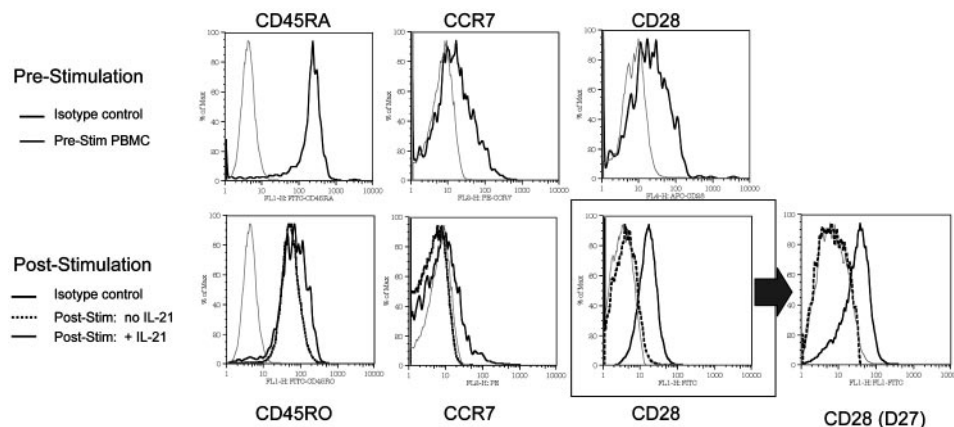


FIGURE 8. IL-21-treated cultures yield a population of CD28^{high} Ag-specific CTLs. Cells were collected from prestimulation (Pre-Stim) PBMCs and then 7 days after stimulation (Post-Stim) with MART-1 peptide-pulsed autologous DCs in the absence or presence of IL-21. Cells were stained for MART-1-tetramer and simultaneously with either CD45RA or CD45RO, CD28 and CCR7 (Fig. 5A). Histogram analysis for individual phenotypic markers was performed on gated MART-1-tetramer staining cells. CD28 expression on gated tetramer-staining cells is also shown for culture from day 27 after stimulation. These results are representative of cultures from three donors.

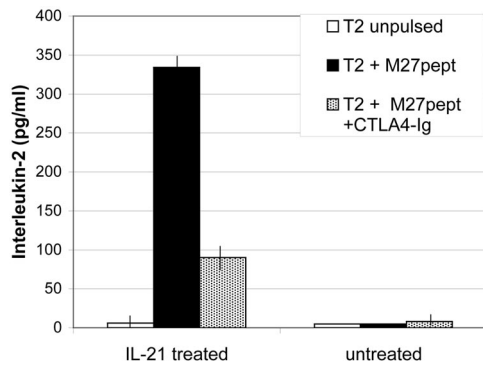


FIGURE 9. IL-2 production following Ag-specific stimulation of CD28^{high} and CD28^{low}-expressing CTLs. MART-1 tetramer-positive CD8⁺ T cells from IL-21-treated (CD28^{high}) or untreated (CD28^{low}) cultures were sorted and cocultivated with either unpulsed T2 cells, MART-1 (M27) peptide-pulsed T2 cells alone, or with CTLA4-Ig (0.5 μg/ml) to block B7-CD28 engagement. Supernatants were collected 48 h later and analyzed for IL-2 by ELISA. Specific induction of IL-2 production among CD28^{high}-expressing MART-1-specific CTLs following Ag stimulation and inhibition by CTLA4-Ig is observed, suggesting that IL-2 induction among IL-21-treated cells is CD28 dependent. Results are the mean of triplicate assays, error bars as shown.

Discussion

In our study of both human healthy donors and melanoma patients, we demonstrate a positive regulatory role for IL-21 in the induction of a primary Ag-specific human CD8 T cell response.

Using peptide-MHC tetramers to track a rare but measurable naive T cell population recognizing a normal self-Ag (MART-1), we demonstrate that, in the presence of IL-21, the frequency and absolute numbers of MART-1-specific CD8 T cells that could be elicited increased by >20-fold compared with cultures grown in the absence of IL-21. The enhanced generation of an Ag-specific T cell response appears to be specific to this γ-chain receptor cytokine since the addition of IL-2, IL-7, or IL-15 during initial priming had no added effect over cultures that received no cytokine. IL-21-exposed and Ag-primed T cells retained the capacity to respond to growth-promoting cytokines, such as IL-2 and IL-7 and could be readily isolated and expanded.

We found that the effect of IL-21 to augment the Ag-specific response was limited to the naive and not memory T cell population using preselected responder T cells. One explanation may be that a larger number of MART-1-specific T cells reside among the naive population (9). However, measurable frequencies of MART-1-specific T cells can also be detected among the memory population (Fig. 3 and Ref. 18) and yet these failed to expand when IL-21 was added. Since equivalent levels of IL-21 receptor (as determined by quantitative RT-PCR) are expressed in naive and memory CD8⁺ T cells (our unpublished data), downstream signaling events may account for this difference. In the case of patients with melanoma, a previous encounter with Ag-bearing tumor cells may lead to defective signaling among memory T cells rendering them unresponsive to IL-21 mediated proliferation in vitro (19, 20).

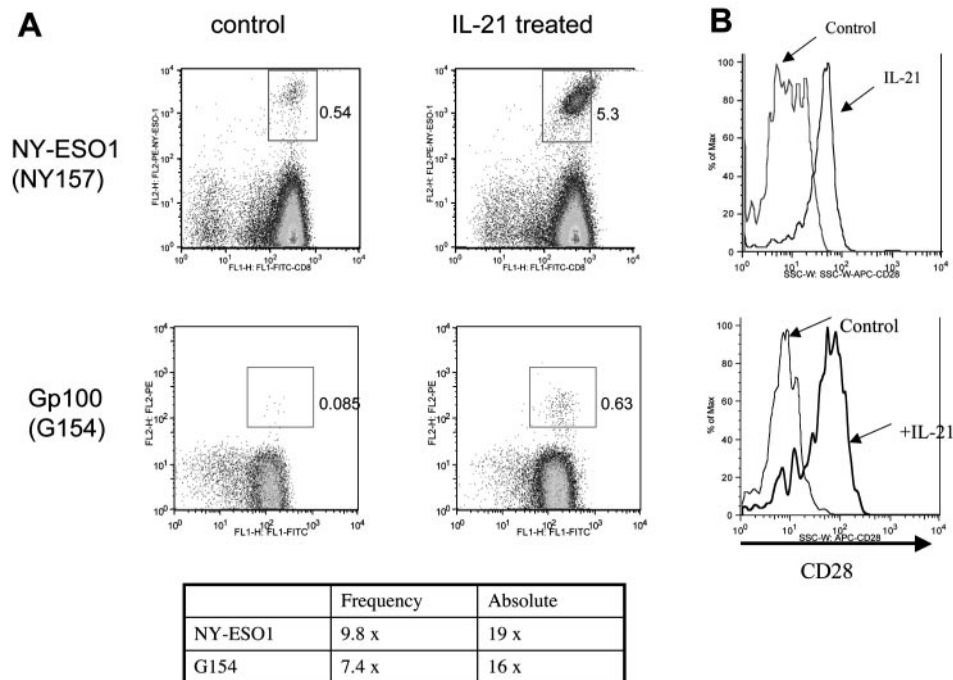


FIGURE 10. IL-21 influences the CD8 T cell response to gp100 and NY-ESO-1 Ag. CD8⁺ T cells were stimulated in vitro with autologous DCs pulsed with NY-ESO-1 (NY157) or gp100 (G154) peptide. IL-21 (30 ng/ml) was added to IL-21-treated cultures. Six days after primary in vitro stimulation, cultures were analyzed for Ag specificity and surface phenotype by tetramer staining and multiparametric analysis on flow cytometry. Experiments were performed as described in *Materials and Methods*. *A*, NY-ESO-1- and G154-specific CTL frequency are shown as percentage of all CD8⁺ T cells next to the boxed gates. For example, the fold increase in NY-ESO-1-specific CTLs was 9.8-fold greater among IL-21-treated cells over control (5.3%:0.54%). The fold increase in absolute numbers of NY-ESO-1-specific CTLs was calculated based on numbers of cells in respective cultures and for NY-ESO-1 was found to be almost 20-fold greater among IL-21-treated cells. *B*, Gated tetramer-positive cells from control or IL-21-treated cultures were analyzed for CD8 expression. All cells were CD45RO⁺, CCR7⁻. Histogram analysis for CD28 expression among NY-ESO-1- or gp100-specific CTLs was found to be significantly up-regulated among IL-21-treated cultures compared to controls. These results are representative of six separate experiments from three HLA-2⁺ individuals.

Although the molecular events responsible for an enhanced Ag-specific T cell response have yet to be defined, Ag-primed T cells do undergo increased proliferation and decreased apoptosis when exposed to IL-21 compared with their untreated counterparts. IL-21 treatment led to up-regulated CD28 expression and enriched for a population of T cells expressing a stable unique phenotype, CD45RO⁺, CD28^{high}, CCR7⁻CD8⁺, that may be characterized as intermediate between a naive (CD45RO⁻CD28⁺CCR7⁺) and memory (CD45RO⁺CD28⁻CCR7^{-/+}) T cell (21). Enhanced proliferation and survival secondary to CD28 costimulation may have contributed to the greater numbers and prolonged survival of Ag-specific CTLs elicited during the primary response among IL-21-treated cultures. Since helper-independent, Ag-driven CD28⁺CD8⁺ T cells represent potentially more effective CTLs for adoptive immunotherapy, understanding the mechanism of IL-21-mediated up-regulation of CD28 in human CD8 T cells may allow us to manipulate and expand a population of helper-independent T cell clones for adoptive immunotherapy without the requirement for genetic modification (22).

Exposure to IL-21 during primary in vitro stimulation also led to the generation of Ag-specific T cell clones of uniformly higher affinity and target cell avidity. These clones were represented by diverse TCR V β s, suggesting that this was not likely the result of an expanded population of a few high-affinity clones, but a more global effect on the T cell repertoire. Since the IL-21 receptor is expressed on both DCs used for stimulation and the responder T cells, it is unclear which cell type is responsible for this phenomenon. Previous studies have shown an increased probability of isolating higher affinity T cell clones when cytokines such as IL-10, that down-regulate the stimulatory capacity of APCs, are used in culture (23). In this case, a dampened stimulatory capacity may enrich for a population of higher affinity CTLs. For IL-21, Brandt et al. (24) have shown recently that IL-21 leads to maturational arrest among murine DCs, resulting in reduced MHC expression and decreased stimulatory capacity for T cell activation. However, in our studies, IL-21 was added to human DCs that had already undergone full maturation. In preliminary studies, we found that the addition of IL-21 to mature DCs did not affect surface expression of MHC class I, HLA-DR, CD80, CD83, or CD86 compared with untreated DCs, suggesting that dampened expression of surface stimulatory molecules is not likely an explanation for the enhanced generation of high-affinity T cells in vitro. Preincubation of mature human DCs with IL-21 also had no effect on the frequency or affinity of CD8⁺ tetramer-positive T cells that could be generated (data not shown). Methods that prevent IL-21 engagement on either T cells or DCs, for example, preincubation with anti-IL-21 receptor Abs as these become available or the use of receptor-negative APCs may shed some light on the cell type responsible for this observation.

The use of IL-21 in augmenting an Ag-specific CD8 T cell response has been explored in mouse models and found to be highly effective in eradicating aggressive tumors (25–27). The selective effect of IL-21 in our study on naive vs memory T cells suggests a greater influence during priming, and, in fact, murine studies demonstrate a strong priming effect characterized by a slow rejection response and induction of prolonged antitumor memory. IL-21 appeared to promote long-term survival of previously activated Ag-specific CD8 T cells in vivo as a result of reduced apoptosis through an indeterminate mechanism possibly involving STAT3 phosphorylation or induction of a central memory phenotype (28). In our studies, some of these effects may be attributable to CD28 up-regulation among IL-21-treated CD8 T cells.

In conclusion, our data indicate that IL-21 enhances the generation of human Ag-specific CD8⁺ T cells characterized by CD28

up-regulation and expression of high-affinity TCR resulting in Ag-driven helper-independent IL-2 production, increased target avidity, and augmented Ag-specific tumor killing. The results of our in vitro studies suggest that IL-21 plays positive role in the induction of a human Ag-specific CD8 T cell response and support the use of this cytokine in immunotherapeutic strategies.

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Disclosures

The authors have no financial conflict of interest.

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