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Amino-Terminal Extended Peptide Single-Chain Trimers Are Potent Synthetic Agonists for Memory Human CD8+ T Cells

Beatriz M. Carreno,* Michelle Becker-Hapak,* Megan Chan,* Wen-Rong Lie,† Xiaoli Wang,‡ Ted H. Hansen,‡ and Gerald P. Linette*

Upon Ag exposure, most memory T cells undergo restimulation-induced cell death. In this article, we describe a novel synthetic agonist, an N-terminal extended decamer peptide expressed as a single-chain trimer, the amino-terminal extended peptide MHC class I single-chain trimer (AT-SCT), which preferentially promotes the growth of memory human CD8+ T cells with minimal restimulation-induced cell death. Using CMV pp65 and melanoma gp100 Ags, we observe the in vitro numerical expansion of a clonally diverse polyfunctional population of Ag-specific CD8+ T cells from healthy individuals and vaccinated melanoma patients, respectively. Memory CD8+ T cells stimulated with AT-SCT presented on MHC class I/II-null cells show reduced cytokine production, slower kinetics of TCR downregulation, and decreased cell death compared with native nonamer MHC class I single-chain trimer (SCT)-activated T cells. However, both ERK phosphorylation and cell cycle kinetics are identical in AT-SCT- and SCT-activated T cells. Probing of SCT and AT-SCT peptide–MHC complexes using fluorochrome-conjugated TCR multimers suggests that nonamer- and decamer-linked peptides may be anchored differently to the HLA-A2 peptide-binding groove. Our findings demonstrate that modified peptide–MHC structures, such as AT-SCT, can be engineered as T cell agonists to promote the growth and expansion of memory human CD8+ T cells. The Journal of Immunology, 2012, 188: 000–000.

The specificity of T cell activation is largely dictated by the interaction of the TCR with peptide–MHC (p-MHC) (1). TCR–p-MHC interactions can lead to a spectrum of responses in mature T cells ranging from full activation, resulting in clonal expansion (or cell death), to a state of anergy, resulting in unresponsiveness (2–4). These distinct outcomes have been attributed, in part, to differences in binding affinity or off-rates of the interaction between TCR and p-MHC (5–8). Single amino acid substitutions can create structural variants of antigenic peptide ligands, referred to as altered peptide ligands (APL), which allow manipulation of the strength of the TCR–p-MHC interaction. APL can induce a complete range of T cell functions (agonist) or a subset of T cell functions (partial agonist) (9, 10); in a few instances, it can be more potent (superagonist) than the native ligand (11, 12). It is also well established that memory T cells display a lower threshold for activation (13, 14) and are more sensitive to cell death compared with naive resting T cells upon Ag exposure (15–17).

MHC class I single-chain trimers (SCT) are fusion proteins, which consist of an antigenic peptide–flexible linker–β2-microglobulin (β2m)-flexible linker–I H chain polypeptide (18). The essential properties of SCT are multifold and include preservation of the native structure of peptide/β2m/MHC class I, as detected by CD8+ T cells and confomational-dependent mAbs, such as 25-D1.16 (19). Crystallographic data with the SIINFEKL/β2m/Kb SCT confirm structural equivalence with free peptide bound to Kb (20) and, importantly, SCT encoding SIINFEKL/β2m/Kb stimulates IL-2 secretion and vigorous proliferation of primed CD8+ OT-1 transgenic T cells (21). Additionally, several independent studies demonstrated that, as DNA vaccines, SCT can elicit robust CD8+ T cell responses to well-defined Ags in murine models (19).

Recently, we reported on the biochemical stability of HLA class I-containing SCT and their serological recognition by conformational-dependent Abs, as well as demonstrated their recognition by Ag-specific T cells (22). In the current study, we examine whether SCT, expressed in the MHC class I/II-null cell line K562, may represent a p-MHC entity for ex vivo stimulation of memory (Ag-primed) human CD8+ T cells. From a series of SCT constructs generated, a new p-MHC entity, the amino-terminal extended peptide MHC class I single-chain trimer (AT-SCT), was identified as a potent inducer of human CD8+ T cell expansion and growth. AT-SCT for two well-characterized Ags are described in detail: the CMV pp65 antigenic peptide (NLVPMVATV) and the melanoma gp100 G209-2M antigenic peptide (IMDVQVFFSV). AT-SCT delivers a TCR signal distinct from conventional native SCT, resulting in >400-fold expansion of Ag-specific human CD8+ T cells from healthy donors, as well as melanoma patients.

Materials and Methods

Generation of SCT-expressing K562 transfectants

HLA-A*0201 SCT were generated, as previously described (22). For AT-SCT generation, synthetic DNA oligonucleotides (Integrated DNA Tech-
nologies, Coralville, IA) encoding 10-mer antigenic peptides extended at the N terminus of peptide in AT-SCT) or amino acids at N terminus of peptide in AT-SCT) or amino acids with bulky nonpolar (YNLV) and uncharged polar (NNLV) R groups, as well as nonbulky uncharged polar (GNLV) R group. The ANLV construct was poorly expressed on the cell surface and was not studied further; all other AT-SCT constructs were expressed on the cell surface at levels similar to the SCT. For IMD (G209-2M), constructs containing the naturally occurring amino acid residue (TMDM) were made; for comparison with the CMV system, an AT-SCT construct expressing the amino acid with a nonbulky charged polar (NIMD) R group was made. A construct containing human β2m and HLA-A*0201 with no covalently linked peptide, referred to as a single-chain dimer (SCD), was also generated.

Generation of disulfide trap SCT (SCT dt) molecules was performed, as previously described (22). All constructs were verified by DNA sequencing.

K562 cells (American Type Culture Collection, Manassas, VA) were transfected with constructs using Nucleofector kit 5 (Lonza, Basel, Switzerland), and stable cell lines were selected and maintained in 0.5 μg/ml Geneticin (Life Technologies, Grand Island, NY). Transfectants were assessed for HLA-A2 expression using the mAb BB7.2 (Serotec, Kidlington, U.K.). Expression of NLV (CMV pp65)/A*0201 and IMD (G209-2M)/A*0201 peptide complexes on the surface of K562 transfectants was assessed using PE-conjugated TCR multimers (Altos Bioscience, Miramar, FL) (23), as per the manufacturer’s instructions.

Human donors

Leukapheresis was performed to obtain peripheral blood leukocytes from normal donors through the Barnes-Jewish Hospital blood bank. Donors were tested for CMV serology and HLA class I typed. Leukapheresis was also performed from melanoma patients enrolled in a phase I clinical trial (NCT00683670; Dendritic Cell Vaccination for Advanced Melanoma). Written informed consent was obtained from all healthy donors and melanoma patients prior to any study procedures, and both studies were approved by the Siteman Cancer Center Protocol Review and Monitoring Committee and the Washington University Human Research Protocol Organization.

Generation and expansion of Ag-specific T cells

Dendritic cells (DC) were generated from monocyte cultures in GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) and matured using CD40L and IFN-γ (100 U/ml), as previously described (24). CD8+ T cells were isolated from PBMCs using a CD8+ negative-selection kit (Miltenyi Biotec, Auburn, CA); purity was 85–90% (data not shown). Purified CD8+ T cells (0.5 × 10^6/ml) were cultured at a 20:1 ratio with irradiated (2500 rad) autologous monocyte-derived DC (mDC) pulsed with peptide (2 μg/ml/mDC/ml) in 48-well trays in Stemline media (S1694; Sigma-Aldrich, St. Louis, MO) supplemented with 5% pooled human sera (Stemline-5). Peptides used were CMV pp65 (NLVPMMATV) and melanoma human gp100-derived peptide G209-2M (IMDQVPFSV). Human IL-2 (10–50 U/ml; Chiron, Emeryville, CA) was added every 2 d, starting 48 h after culture initiation. Fourteen days after DC stimulation, T cell cultures were harvested, characterized for Ag-specific frequencies using HLA/peptide tetramers (see below), and restimulated with irradiated (10,000 rad) SCT- or AT-SCT–expressing K562 cells at a 1:1 ratio. Cultures were initiated in either six-well plates (10^6 each T and SCT or AT-SCT) or T25 flask (5 × 10^5 each) using Stemline-5. Twenty-four hours after stimulation, cultures were supplemented with IL-2 (500 U/ml), and viable cell counts were performed daily. Cell concentrations were maintained at 5 × 10^6/ml throughout the culture period. For large-scale expansion, T cells were cultured in gas-permeable Lifecell bags (Nexell Therapeutics, Emeryville, CA). On days 10–14 of secondary stimulation, the percentage of tetramer+ cells and the number of viable cells were used to determine tetramer yields and tetramer folds, as described previously (24) and in the legend of Fig. 1. T cells from >15 healthy donors and 4 melanoma patients were analyzed during the course of this study.

Tetramer analysis, phenotypic analysis, and cytokine production

Tetramer analysis was done as previously described (24). Flow cytometry analysis was done using the following fluorochrome-conjugated anti-human mAbs: CD25, CD27, CD45RO (Invitrogen, Carlsbad, CA), CD28, CCR7 (BD, Franklin Lakes, NJ), CD57, CD69, CCR5 (BioLegend, San Diego, CA), and PD-1 (eBioscience, San Diego, CA). For assessment of cytokine/chemokine granule content, cells were fix/permeabilized with Cytofix/Cytoperm (BD), according to the manufacturer’s instructions, and stained with Abs to perforin, granzyme A (BioLegend), and granzyme B (Invitrogen). Polyfunctionality of expanded Ag-specific T cells was determined as follows: 14 d after SCT or AT-SCT stimulation, T cells were restimulated with SCT at 1:1 ratio in RPMI 1640 supplemented with 5% pooled human sera (RPMI-5) and brefeldin A (10 μg/ml; eBioscience). As controls, cells were cultured in media in the absence of stimulation. Sixteen hours after stimulation, cells were harvested, washed, fix/permeabilized with Cytofix/Cytoperm, and stained with pretreated Abs specific for IFN-γ (BioLegend), TNF-α, IL-2 (eBioscience), and MIP-1β (BD). Cells were then washed, fixed with 2% paraformaldehyde, and analyzed by polychromatic flow cytometry. Functional complexity of Ag-specific responses was assessed as described (25). The proportion of CD8+ T cells contributing to each response pattern was determined, grouped, and color-coded according to the number of positive functions. Slices in the pie charts represent the proportion of responding CD8+ T cells that have produced all four cytokines or any combination of three, two, or one cytokines. For analysis of cytokines secreted by T cells upon SCT activation, cultures were activated, as described above, in the absence of brefeldin A, and supernatants were collected 24 h after activation and characterized using a Milliplex cytokine kit (Millipore, Billerica, MA), as per the manufacturer’s instructions. For characterization of TCR Vβ usage by Ag-specific CD8+ T cells after AT-SCT expansion, T cells were purified using a cell sorter (98% tetramer+CD8+), and total RNA was extracted (RNeasy, Qiagen, Valencia, CA), followed by a one-step cDNA synthesis using nested primers specific for each of the 22 TCR Vβ family members (BioMed Immunootech, Tampa, FL). TCR clonality was determined using a 4% high-resolution gel, as per the manufacturer’s instructions (BioMed Immunootech). Flow cytometry was performed using a BD Bioscience (San Jose, CA) FACSscan flow cytometer (upgraded by Cytek Development [Freemont, CA] to three lasers and 16 fluorescent parameters plus two scanners). Data were acquired and analyzed using FlowJo software (Ashland, OR).

Apoptosis and CFSE

For apoptosis determination, CMV pp65 (NLV)-specific T cells (90% tetramer+) were stimulated with irradiated SCT or AT-SCT at a ratio of 1:1 in RPMI-5 for the indicated time, harvested, and stained with FITC-conjugated Annexin-V using binding buffer, followed by addition of 7-amino-actinomycin D (7AAD) and analysis by flow cytometry. A total of 2.5 × 10^6 events was collected in the side scatter (SSC)/forward light scatter (FSC) gate. For CFSE analysis, cells were labeled using the CellTrace CFSE kit (Invitrogen), as per the manufacturer’s instructions, and stimulated as described above. At the indicated time, cells were harvested and analyzed by flow cytometry.

Determination of ERK phosphorylation

To assess ERK phosphorylation upon SCT or AT-SCT stimulation, NLV-specific T cells (94% tetramer+) were allowed to equilibrate for 1 h at 37°C in RPMI-5 before stimulators were added (1:10 T/SCT ratio); they were spun down to pellet cells and incubated for the indicated time at 37°C, followed by fixation in 4% paraformaldehyde, permeabilization with 90% methanol, and staining with anti-phospho ERK Ab (clone 20A; Santa Cruz Biotechnology, Santa Cruz, CA). Cells were then washed, fixed with 2% paraformaldehyde, and analyzed by flow cytometry. Determination of ERK phosphorylation was done using a 4% high-resolution gel, as per the manufacturer’s instructions (BioMed Immunootech). Flow cytometry was performed using a BD Bioscience (San Jose, CA) FACSscan flow cytometer (upgraded by Cytek Development [Freemont, CA] to three lasers and 16 fluorescent parameters plus two scanners). Data were acquired and analyzed using FlowJo software (Ashland, OR).

 δ^3^Cr-release and peptide-competition assays

Protocols for chromium-release assays to measure specific lysis were described (24, 26). For direct recognition by NLV-specific CTL, AT-SCT– and SCT-expressing K562 cells were labeled with 25 μCi [Cr] for 1 h, washed, and used in a standard 4-h assay. For recognition of CMV-infected cells, T98G (HLA-A*0201+) glioblastoma cells were infected using supernatants derived from human foreskin fibroblast cells expressing a bacterial artificial chromosome containing full-length AD169 for the indicated time and labeled with 25 μCi [Cr] for 1 h prior to a standard 4-h assay (27). For direct recognition by G209-2M–specific CTL, melanoma cell lines DM6 (HLA-A2/gp100+) and A375 (HLA-A2/gp100+) were labeled with 25 μCi [Cr] in the presence or absence of G209-2M peptide (5 μg/ml) for 1 h, washed, and used in a standard 4-h assay. For peptide-competition assays (22), SCT, AT-SCT, and HLA-A2+CD8+ T cells were incubated for 2 h with irradiated SCT-expressing K562 cells, G209-2M peptide, followed by labeling with 25 μCi [Cr] for 1 h. After incubation, targets were washed twice and tested against a G209-2M-specific CTL line (26) in a standard 4-h chromium-release assay.
Results

Amino-terminal extended SCT promotes expansion of memory (Ag-primed) human CD8+ T cells

To evaluate ex vivo proliferative responses of memory (Ag-primed) CD8+ T cells upon restimulation, we chose to study the HLA-A*0201–restricted/CMV pp65–specific NLV 495–503 peptide system in healthy CMV-seropositive donors (28). To generate memory CD8+ T cells, purified resting peripheral blood CD8+ T cells were stimulated in vitro with NLV peptide-pulsed autologous mDC for 14 d (24). As shown in Fig. 1A (left panel), a representative healthy individual had, at baseline, CMV pp65–specific (as determined using NLV/A*0201 tetramer) CD8+ T cell frequencies of 1.11%. After a 14-d stimulation with peptide-pulsed autologous mDC, the frequency of NLV-specific CD8+ T cells increased significantly to >40% (Fig. 1A, middle panel); this memory CD8+ T cell population (Supplemental Fig. 1) (29) was used for restimulation assays, using SCT, as depicted in Fig. 1A and as described in this study.

To examine whether SCT may represent a p-MHC entity for ex vivo stimulation of memory human CD8+ T cells, we elected to express the SCT constructs (Fig. 1B) in K562 cells, a human leukemia cell line void of HLA class I/II molecules (Supplemental Fig. 1A).
Fig. 1). Previous work demonstrated the usefulness of K562, as an APC, to expand human CD4+ and CD8+ T cells in vitro (30–33). The parental K562 cells used for our experiments were selected for expression of CD54, CD58, CD80, and CD86 (Supplemental Fig. 1). NLV-specific memory CD8+ T cells were restimulated with SCT-expressing K562 cells at a 1:1 ratio and cultured for 12–14 d in media supplemented with IL-2. No other cells or cytokines were added to the cultures.

Upon restimulation, nonamer (NLVPMVATV) peptide expressed as SCT failed to significantly increase the Ag-specific frequency (Fig. 1A, top right panel), and it modestly increased Ag-specific T cell yields (Fig. 1C, see below). We interpreted the previous finding as evidence that SCT, expressed in K562 cells, provide an activation signal leading to restimulation-induced cell death (RICD), reminiscent of the observation that Ag stimulation can promote clonal deletion of mature memory T cells (2, 16). We postulated that modification of the peptide to create a partial agonist would lead to reduced RICD (34). To test this hypothesis, a series of SCT constructs was generated with various peptide modifications, including N-terminal extensions of the agonist peptide sequence (Fig. 1B). We tested a variety of constructs (data not shown) and found that N-terminal extended decamer peptides in the SCT format, referred to as AT-SCT, were most stimulatory for NLV-specific T cells. Restimulation of memory CD8+ T cells with soluble peptides (nonamer or decamer) did not lead to the numerical expansion of Ag-specific T cells obtained with AT-SCT (Supplemental Fig. 2). Additional experiments were performed comparing memory CD8+ T cell growth upon restimulation with SCT and AT-SCT; representative results using NLV-specific T cells are shown in Fig. 1A and 1C. In this example, 0.5 × 10^6 (0.22 × 10^6 NLV-specific) CD8+ memory T cells were stimulated with SCT- or AT-SCT–expressing cells, and cultures were continued for 14 d, as described in Materials and Methods. Live cell growth kinetics were determined by manual counting using trypan blue exclusion; at the termination of culture, surviving cells were stained with NLV/A*0201 tetramer and anti-CD8 mAb to assess the frequency of Ag-specific CD8+ T cells by flow cytometry (Fig. 1A) and calculate Ag-specific T cell yields (Fig. 1C). As shown, AT-SCT stimulation elicited a large expansion of T cells (2.4 × 10^8 T cells), with

**FIGURE 2.** Recognition of SCT and AT-SCT by soluble TCR multimers. (A) SCD were stained with mAbs to HLA-A2 (left panels) or pulsed with soluble 9-mer or 10-mer peptide and stained with HLA-A*0201/CMV pp65 NLV-specific TCR multimers (right panels). (B) NLV nonamers expressed by SCT and SCT dt molecules are equally recognized by TCR multimer (C), whereas NNLV decamer in AT-SCT or AT-SCT dt are not recognized by this TCR reagent. Dot plots are representative of cell populations expressing similar levels HLA-A2.
enrichment of the Ag-specific CD8+ T cell population (75.4% tetramer+, 1.8 × 10^5 NLV-specific T cells). In contrast, the growth of the Ag-specific CD8+ T cell population was modest after SCT stimulation (37.4% tetramer+, 1.6 × 10^5 NLV-specific T cells) (Fig. 1A, 1C).

To further expand this observation, memory CD8+ T cells obtained from eight healthy CMV-seropositive donors (after NLV-pulsed autologous mDC stimulation) were restimulated, in large-scale cultures, with SCT- or AT-SCT–expressing K562 cells; Ag-specific yields and frequencies are shown in Fig. 1D and 1E. In all cases, AT-SCT–expressing cells were the superior agonist to stimulate NLV-specific CD8+ T cell growth (>10-fold increases in Ag-specific T cell yields over SCT, p = 0.0371, Fig. 1D). In addition, the frequency of Ag-specific CD8+ T cells (82.3% versus 63.8%, p = 0.0054, Fig. 1E) and fold increases in Ag-specific T cells (563 ± 160 versus 65 ± 17, p = 0.0015, Fig. 1F) at day 14 were significantly increased in the AT-SCT–stimulated culture compared with the SCT culture.

Additional AT-SCT constructs containing an amino acid residue other than asparagine were tested in proliferation assays for their ability to stimulate NLV-specific memory CD8+ T cells (see Materials and Methods). Independent of the amino acid (Asn, Gly, or Tyr) identity, higher levels of proliferation were observed when memory NLV-specific T cells were stimulated with AT-SCT compared with SCT (Supplemental Fig. 2). Altogether, these results demonstrate that N-terminal extended NLV-peptide in the SCT format (AT-SCT) promotes the numerical expansion (>500-fold) of memory CD8+ T cells upon restimulation.

**TCR multimers differ in their recognition of SCT and AT-SCT**

To confirm the presentation of an amino terminal extended (decamer) peptide by AT-SCT, HLA-A*0201 molecules were isolated by immunoprecipitation from SCT- and AT-SCT–expressing K562 cells and subjected to amino acid sequencing by Edman degradation. The amino acid analysis confirms that the SCT-expressing cells presented the NLV nonamer sequence, whereas the AT-SCT cells presented the NNLV decamer peptide (Supplemental Fig. 3). To further characterize p-MHC complexes, SCT and AT-SCT constructs expressed by K562 cells were probed using TCR multimers specific for HLA-A*0201/CMV pp65 NLV (23). Cell surface expression of the SCT and AT-SCT constructs was determined using the HLA-A2–specific mAb (BB7.2) (Fig. 2, dot plots labeled HLA-A2). The HLA-A2/CMV pp65 TCR multimer stained 9-mer and 10-mer peptide-pulsed SCD-expressing cells (Fig. 2A), as well as SCT-expressing cells (Fig. 2B). Surprisingly, this CMV pp65 TCR multimer failed to stain the AT-SCT–expressing cells (Fig. 2C). Multiple factors, including peptide instability, could explain the lack of TCR multimer binding to AT-SCT. Next, we examined SCT dt constructs, because the disulfide bond secures the peptide on SCT, making these molecules unsusceptible to peptide exchange (22). As shown in Fig. 2C, disulfide trap AT-SCT (AT-SCT dt) are not recognized by the TCR multimer, suggesting that NNLV instability is unlikely to account for this lack of recognition. We previously demonstrated that peptide-competition assays allow one to examine occupancy of the MHC peptide-binding groove (18, 22). As shown in Fig. 3A, exogenous competitor peptide could displace SCT (nonamer) and AT-SCT (decamer) peptides at similar concentrations, suggesting that linked peptides may display similar MHC occupancy. Importantly, and in accordance with proliferation results, NLV-specific CD8+ T cells could recognize, equally well, in a standard [51Cr]-release assay, cells expressing SCT and AT-SCT (Fig. 3B) and SCT dt and AT-SCT dt (Fig. 3C), used as targets. Altogether, these data strongly support the notion that p-MHC complexes in the AT-SCT format are structurally distinct from those on SCT. These changes may result in TCR-activation signals that promote the numerical expansion of memory CD8+ T cells.

**SCT and AT-SCT are ligands with distinct potencies**

We investigated the early activation events of memory T cells when stimulated by SCT- and AT-SCT–expressing cells. Upon TCR activation, memory CD8+ T cells upregulate CD69 and CD25 cell surface molecules, downregulate TCR expression, secrete IFN-γ (and other cytokines), and undergo cell division. As shown in Fig. 4A, the kinetics of CD69 and CD25 upregulation were similar in SCT- and AT-SCT–stimulated T cells, suggesting that early events associated with TCR triggering by SCT and AT-SCT may be similar in these T cell populations. Importantly, upon activation with either SCT or AT-SCT, these T cells displayed
identical kinetics of ERK phosphorylation (Fig. 4B). In contrast, 24 h after activation, ∼80% of SCT-stimulated (Ag-specific) CD8+ T cells downregulate TCR (CD3) expression, whereas only 42% of AT-SCT–stimulated (Ag-specific) CD8+ T cells downregulate cell surface TCR (Fig. 4C). Interestingly, the percentage of IFN-γ–secreting cells was greater in cultures stimulated with SCT compared with AT-SCT at each time point studied. As confirmation, we tested and found higher levels of IFN-γ and other cytokines in supernatants obtained from SCT-stimulated T cells (Fig. 4D). The diminished TCR downregulation and IFN-γ response after AT-SCT stimulation were consistently observed in multiple experiments using memory NLV-specific CD8+ T cells derived from multiple individuals (Fig. 4E, data not shown). Altogether, these results suggest that TCR signals transmitted by SCT and AT-SCT may be quantitatively different.

**AT-SCT promotes proliferation with decreased cell death**

We considered whether the striking numerical expansion of AT-SCT–stimulated T cells could be explained by either increased cell proliferation or decreased cell death. To this end, NLV-specific T cells were purified by cell sorting 14 d after mDC stimulation (98.8% NLV/A*0201 tetramer+) and stained with CFSE to monitor cell division. As shown in Fig. 5A, T cells from SCT- and AT-SCT–stimulated cultures enter mitosis at identical rates, as determined by CFSE content. In contrast, cell number and viability were very different, as determined by manual cell counts using trypan blue (Fig. 5B). Although cell numbers decreased in SCT-stimulated T cell cultures, AT-SCT–stimulated T cells increased in number, suggesting that the increased T cell growth observed in these cultures may result from decreased apoptosis. To examine the fraction of T cells undergoing apoptosis during the proliferative response, NLV-specific T cells were stimulated with SCT or AT-SCT. At various time points after stimulation, T cells were stained with 7AAD and Annexin V to monitor cell viability. At 48 h postactivation, 79% of the SCT-stimulated T cells stained positive with annexin (16.4% annexin+/7AAD+), consistent with apoptotic cell death (Fig. 5C). In contrast, just 23% of the AT-SCT–stimulated T cells stained positive with annexin (6.1% annexin+/7AAD+). Therefore, despite similar proliferative rates, a smaller fraction of T cells undergo apoptosis in AT-SCT–stimulated cultures compared with SCT-stimulated T cells, leading to significantly higher yields of Ag-specific T cells 14 d after restimulation (Fig. 1).
AT-SCT–stimulated T cells display “early” effector CD8+ characteristics

Large-scale cultures of AT-SCT–stimulated T cells, obtained from five of the eight donors shown in Fig. 1E and 1F, were studied to assess functional and phenotypic traits (25, 29). Cytokine (IFN-γ, IL-2, TNF-α, MIP-1α) production by AT-SCT–stimulated NLV-specific T cells was evaluated by intracellular staining using polychromatic flow cytometry. In T cell cultures obtained from five healthy individuals, 80% of NLV-specific CD8+ T cells were polyfunctional (produced more than one cytokine), and 25% of these cells were positive for all four functions (Fig. 6A). Phenotypically, 80% of the expanded cells expressed CD28, and 22–46% were positive for CD27 (Fig. 6B). All cells were positive for CD45RO and effector molecules granzyme A, granzyme B, and perforin (Fig. 6B). Thus, despite the large numerical expansion undergone by AT-SCT–stimulated cells, these retain an “early” effector memory phenotype (Supplemental Fig. 4). Expanded T cells displayed high avidity for Ag (EC50, < 100 pM) when tested in a standard 4-h [51Cr] assay using peptide-pulsed T2 target cells (Fig. 6C), and they recognized CMV-infected HLA-A*0201 target cells (Fig. 6D). Finally, to determine whether AT-SCT stimulation resulted in the preferential expansion of selected clones, the TCR repertoire was characterized using a Vβ clonality-detection assay. As shown in Fig. 6E and 6F, AT-SCT–expanded NLV-specific T cells used a diverse set of TCR Vβ regions, including Vβ subsets (BV8, BV13, BV14, BV20) frequently identified in healthy CMV-seropositive individuals (35). Collectively, these results show that stimulation of memory human CD8+ T cells with AT-SCT results in a population of polyfunctional and high avidity Ag-specific CD8+ T cells.

Melanoma-specific T cells are efficiently expanded with AT-SCT ligands

To investigate whether AT-SCT ligands in other antigenic systems share the structural and functional features described in the CMV NLV-peptide system, the melanoma gp100-derived antigenic peptide G209-2M was studied (36). An HLA-A*0201/G209-2M–specific TCR multimer reagent, similar to the one used in the NLV-peptide system, was used to probe the p-MHC complexes expressed by SCT (IMD nonamer), as well as two AT-SCT

FIGURE 5. Decreased cell death rates in AT-SCT–stimulated T cells. (A) NLV-specific T cells (98.8% NLV/A*0201 tetramer+) were loaded with CFSE and restimulated with SCT- or AT-SCT–expressing cells; at the indicated times, they were harvested, counted, and analyzed by flow cytometry. The x-axis is the CFSE-staining intensity, whereas the y-axis represents the percentage of maximum cell number (% of Max). Equal numbers of events were collected on FSC/SSC-gated populations. Results are representative of two experiments performed. (B) Manual cell counts using trypan blue exclusion were performed to determine viability in cultures shown in (A). Bars represent cell counts obtained at the indicated time point from SCT- or AT-SCT–stimulated cultures. Results are representative of two experiments performed. (C) NLV-specific T cells (96% NLV/A*0201 tetramer+) were restimulated with SCT- and AT-SCT–expressing cells, and apoptotic T cells were identified on the indicated days by staining with 7AAD and AnnexinFITC. Camptothecin-treated (10 μM) T cells were used as positive controls to set up dot plot quadrants (data not shown). Percentages represent annexin+/annexin−/7AAD+ T cell populations. Media indicates T cell cultures in Stemline-5 in the absence of stimulation. Equal numbers of events were collected on the FSC/SSC-gated populations. Results are representative of three experiments performed.
(NIMD and TIMD decamer)-expressing K562 cell lines. Similarly to our observations in the CMV Ag system, the G209-2M TCR multimer reagent failed to stain either AT-SCT, whereas it demonstrated positive staining on SCT (Fig. 7A). The functional consequences of AT-SCT stimulation were examined using CD8+ T cells obtained from HLA-A*0201+ melanoma patients after autologous mDC vaccination. CD8+ T cells purified from peripheral blood were stimulated with G209-2M peptide-pulsed autologous mDC for 14 d and restimulated with SCT- or AT-SCT–expressing K562 cells. Live cell growth kinetics were determined by manual counting using trypan blue exclusion at initiation and termination of the culture; the surviving cells were stained with anti-CD8 and G209-2M/A*0201 tetramer to assess the frequency of peptide-specific CD8+ T cells by flow cytometry (Fig. 7B). Memory (G209-2M Ag-specific) T cells restimulated with AT-SCT showed significant growth over 10 d, whereas the SCT-stimulated memory T cells displayed minimal growth and dramatically decreased in frequency (Fig. 7B, 7C). As shown for a representative patient, cultures started with $5 \times 10^6$ cells (0.38 $\times 10^8$ G209+) yielded $1.4 \times 10^8$ (6.1 $\times 10^7$ G209+) and $4.9 \times 10^8$ (1.8 $\times 10^8$ G209+) total T cells after stimulation with AT-SCT NIMD and TIMD, respectively (Fig. 7C). In contrast, cells stimulated with SCT decreased in frequency, suggesting clonal deletion. AT-SCT yields represent Ag-specific T cell increases of 10- and 30-fold over cultures stimulated with SCT (Fig. 7D).

**FIGURE 6.** Functional and phenotypic characteristics of AT-SCT–stimulated NLV-specific T cells. (A) NLV-specific T cells obtained from five individual donors (D1–D5) after AT-SCT restimulation were functionally and phenotypically characterized. Ag-specific frequencies, as determined by NLV/A*0201 tetramer staining, range from 75 to 96% of CD8+ T cells. To evaluate polyfunctionality, T cells were restimulated with SCT for 16 h in the presence of brefeldin A, permeabilized, and stained for cytokine expression. Total frequency of Ag-specific T cells represents the summation of T cell frequencies within each unique cytokine combination (data not shown); the responses are grouped according to the number of cytokines produced. Pie charts depict polyfunctional profiles, with responses grouped according to the number of functions (IFN-γ, IL-2, TNF-α, and MIP-1β). (B) Cells characterized in (A) were stained with NLV/A*0201 tetramer and Abs directed at the indicated cell surface markers. A total of 25,000 events was collected on NLV/A*0201 tetramer+ gated cells; the percentages of T cells for the indicated cell surface molecules within the tetramer+ population are reported. (C) Avidity of NLV-specific T cells was determined in a standard 4-h [3H]-release assay using peptide titrations and T2 cells as targets. The percentage of specific lysis (mean of triplicates) is shown for each peptide concentration. SD was <20% and is omitted for clarity; spontaneous lysis was <5%. Results are representative of two experiments performed. (D) To demonstrate recognition of native CMV Ag by AT-SCT–expanded T cells, T98G glioma cells were infected with CMV (27) for 72 h or pulsed with peptide, labeled with [3H]Cr, and used as targets in a standard assay. The percentage of specific lysis (mean of triplicates) ± 1 SD is shown for each target. Spontaneous lysis was <10%. Results are representative of three experiments performed. (E and F) After DC stimulation, NLV-specific T cells were purified by cell sorting using NLV/A*0201 tetramers. The sorted populations were stimulated with AT-SCT in the presence of IL-2, as described in Materials and Methods, and the frequency of NLV/A*0201 tetramer CD8+ T cells on day 14 after restimulation is shown as dot plots. RNA was isolated from T cells, and TCR Vβ usage was determined as described in Materials and Methods. A similar analysis was performed with CD8+ T cells isolated from fresh PBMC prior to Ag stimulation. Top panel, TCR Vβ usage in CD8+ T cell population prior to Ag stimulation. Bottom panel, TCR Vβ usage in NLV-specific T cells after AT-SCT stimulation. The results obtained with two representative donors are shown.
specific T cells exhibited slower TCR downregulation after AT-SCT activation compared with SCT activation, and the expanded T cells killed human HLA-A*0201+gp100+ melanoma cells in vitro (Fig. 7E).

Discussion

Using HLA-A*0201–restricted viral and self-Ags (CMV pp65 NLV peptide and melanoma gp100 20-2M peptide, respectively), we provide evidence that N-terminal extended peptides (decamers), when presented as single-chain MHC class I trimers (AT-SCT), are highly stimulatory for memory human CD8+ T cells, resulting in the growth of a clonally diverse population of Ag-specific T cells with polyfunctional traits. The expanded T cell population displays phenotypic traits of early effector CD8+ T cells, CD45RA2/CD28+/CD27high/low/CCR5+, and retains high avidity for Ag (29). Our results demonstrate that activation with AT-SCT leads to increases in the frequency and numbers of Ag-specific CD8+ T cells. In both viral and self-Ag systems, we routinely observe >400-fold increases in Ag-specific T cell numbers after AT-SCT restimulation. Thus, AT-SCT defines a novel p-MHC entity that can act as a potent agonist for the expansion of memory CD8+ T cells elicited by natural infection (CMV), as well as therapeutic vaccination (melanoma gp100 Ag).

The SCT scaffold for peptide presentation was used for two reasons. First, SCT ensures structural integrity and assumes that each p-MHC is equivalent. Second, SCT constructs can be exclusively presented by mutant cell lines (such as K562), which lack endogenous MHC molecules, minimizing peptide exchange and allowing a uniform expression of a single p-MHC species. Previous studies demonstrated that single-chain p-MHC can induce proliferation, inhibit CTL-mediated cytotoxicity, or induce cell death (21, 37, 38). Structural features of single-chain p-MHC, such as short versus long linkers or soluble versus cell bound, as well as the differentiation status of T cells, play a role in determining the outcome of single-chain p-MHC/T cell interaction (21, 37, 38). In this study, we find that insertion of an amino acid residue at the N terminus of antigenic peptide of a single-chain trimer (AT-SCT) creates a novel structure that promotes ex vivo numerical expansion of memory CD8+ T cell with maintenance of functional capacity (Fig. 6). We report these results in two clinically relevant antigenic systems: CMV pp65 and melanoma gp100 (39, 40). However, in our experience, random insertion of any amino acid...
residue at the N-terminal position may not always be sufficient to create a stimulatory AT-SCT. For example, addition of K (naturally occurring amino acid residue) or N to the N terminus of the influenza A matrix (MP 58-66, HLA-A*0201–restricted) nonamer peptide failed to confer improved agonist activity to the flu M1 AT-SCT relative to the SCT (B.M. Carreno and M. Becker-Hapak, unpublished observations). Structural data on the AT-SCT entities described in this article should be instructive in modeling predictions for additional peptide Ags in the context of HLA-A2, as well as other HLA class I alleles.

AT-SCT stimulation of memory CD8+ T cells leads to slower cell surface TCR (CD3) downregulation, decreased cytokine synthesis, and reduced RICD. These features are discernible quantitative differences from canonical nonamer SCT stimulation and may define a novel mechanism of T cell activation. In contrast, other early signaling events, such as ERK phosphorylation and upregulation of activation markers, such as CD25 and CD69, are identical in AT-SCT– and SCT-stimulated T cells. These findings suggest that AT-SCT represent p-MHC structures distinct from most APL, which induce a state of partial activation (9); indeed, AT-SCT induces full activation based on cell cycle entry kinetics with avoidance of apoptotic cell death. This is particularly striking in the self-peptide G209-2M system (Fig. 7B), in which restimulation with AT-SCT leads to increases in the frequency and number of Ag-specific T cells, whereas SCT leads to clonal deletion, because only ~1% tetramer+ T cells remain after 14 d of culture. In the CMV pp65 system, activation with SCT promotes limited growth, generally without enrichment of tetramer frequencies, whereas AT-SCT promotes marked growth and enrichment. Thus, native Ag leads to RICD of memory T cells, whereas AT-SCT promotes T cell expansion to increase the memory pool. These findings raise the interesting possibility that native Ag may not constitute the optimal immunogen for booster vaccine for-

References

Figure S1 – Phenotype of DC-activated T cells and K562 cells. (A) Purified CD8+ T cells were stimulated with NLV-peptide pulsed autologous DC as described in Materials and Methods. To define the differentiation stage of the DC-stimulated NLV-specific T cells, cells were harvested on day 14 and stained with NLV/A*0201 tetramer and antibodies to the indicated cell surface markers. (B) Single color histograms of K562 cells stained with mAbs confirms the lack of HLA class I/class II expression. Fluorochrome-conjugated monoclonal antibodies to CD54, CD58 were obtained from BD (San Diego, CA); all others were obtained from Invitrogen (Carlstad, CA). Surface staining is shown for corresponding antibodies (solid lines) and isotype controls (dotted lines).
Figure S2 – (A) Stimulation of NLV-specific CD8+ T cells with soluble (fluid phase) 9-mer and 10-mer peptides. DC-activated T cells (10⁶ T cells) were re-stimulated with AT-SCT or peptide pulsed-HLA-A2/b2m (SCD) expressing cells at a 1:1 ratio and cultures maintained as described in Materials and Methods. Antigen-specific T cell yields and folds are shown for a representative donor. Yields and folds were calculated as described in Figure 1. (B) In the AT-SCT format, multiple amino acid residues at the peptide amino-terminal end can stimulate memory T cells proliferation (see Materials and Methods). DC-activated T cells were counted and adjusted to 10⁵ cells/mL and plated 100ul/ well in flat bottom 96 well tissue culture plates. Parental K562, SCT (NLV) or AT-SCT (NNLV, GNLV or YNLV)-expressing K562 were irradiated, adjusted to 10⁵/mL and 100ul added to T cells or media. Cultures were incubated for 5 days in 5%CO₂ 37°C and pulsed with 0.25 uCi/well for the last 6h of culture. Results obtained with 2 representative donors are shown.
Edman degradation analysis confirms the presentation of an amino terminal extended (decamer) peptide by AT-SCT. HLA-A2 molecules were immunoprecipitated from SCT and AT-SCT expressing K562 cells using the HLA-A2 specific mAb BB7.2, resolved by electrophoresis and transferred to PVDF membrane for protein isolation prior to Edman degradation analysis. (A) Coomasie blue staining of BB7.2 immunoprecipitated material is shown. Indicated in brackets is the protein band subjected to Edman analysis and amino acid sequence obtained is shown in box below gel. (B) Identity of the immunoprecipitated molecules was confirmed by western blot analysis using anti-b2microglobulin mAb. Immunoprecipitated molecules were resistant to Endo H digestion and thus, represent peptide/MHC complexes that have egressed the endoplasmic reticulum.
Figure S4 – Phenotype of SCT and AT-SCT expanded T cells. DC-activated T cells were re-stimulated with (A) SCT or (B) AT-SCT as described in Materials and Methods. Fourteen days after stimulation, cells were harvested, stained with NLV/A*0201 tetramer and the indicated antibodies. All dot plots shown are representative of 25,000 events collected on FSC/SSC + NLV/A*0201 gated population. A representative donor is shown.