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Primming of a Novel Subset of CD28+ Rapidly Expanding High-Avidity Effector Memory CTL by Post Maturation Electroporation-CD40L Dendritic Cells Is IL-12 Dependent

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Dendritic cell (DC)-based immunotherapeutics must induce robust CTL capable of killing tumor or virally infected cells in vivo. In this study, we show that RNA electroporated post maturation and coelectroporated with CD40L mRNA (post maturation electroporation (PME)-CD40L DC) generate high-avidity CTL in vitro that lyse naturally processed and presented tumor Ag. Unlike cytokine mixture-matured DC which induce predominantly nonproliferative effector memory CD45RA+ CTL, PME-CD40L DC prime a novel subset of Ag-specific CTL that can be expanded to large numbers upon sequential DC stimulation in vitro. We have defined these cells as rapidly expanding high-avidity (REHA) CTL based on: 1) the maintenance of CD28 expression, 2) production of high levels of IFN-γ and IL-2 in response to Ag, and 3) the demonstration of high-avidity TCR that exhibit strong cytolytic activity toward limiting amounts of native Ag. We demonstrate that induction of REHA CTL is dependent at least in part on the production of IL-12. Interestingly, neutralization of IL-12 did not effect cytolytic activity of REHA CTL when Ag is not limiting, but did result in lower TCR avidity of Ag-reactive CTL. These results suggest that PME-CD40L DC are uniquely capable of delivering the complex array of signals needed to generate stable CD28+ REHA CTL, which if generated in vivo may have significant clinical benefit for the treatment of infectious disease and cancer. The Journal of Immunology, 2008, 181: 5296–5305.

The generation of long-lasting immunity after antigenic stimulation is dependent on the establishment of a residual pool of memory cells retaining the capacity for rapidly renewed effector responses upon Ag reencounter (1–3). Under conditions of chronic viral exposure, there is a progressive deterioration of the Ag-reactive CTL pool, characterized by a loss of the costimulatory receptors CD28 and CD27 with a concurrent gain of cytokine production and cytolytic function (4–7). The loss of costimulatory molecules such as CD28 on effector CTL serve as a natural negative regulatory control mechanism when Ag is no longer present in sufficient quantities to induce T cell activation (6). Deregulation of T cell differentiation has been reported in certain cases of chronic viral exposure such as HIV, where the lack of CD28 expression on HIV-specific CTL was associated with disease progression (7). Furthermore, HIV-specific CTL that maintain expression of CD28 have been associated with control of viral replication in HIV-infected long-term nonprogressors (8). These data suggest that reexpression of costimulatory molecules could be vital to generating effector memory CTL that control viremia. A similar situation may arise during the differentiation of tumor-reactive CD8+ T cells. Recent clinical trials using adoptively transferred anti-melanoma effector memory CTL expressing CD27 and CD28 show that subjects receiving these cells were more likely to experience tumor regression than subjects receiving CD28- T cells (9, 10). These data suggest that the most clinically relevant and most robust Ag-reactive CTL reside in the effector memory transition stage without commitment to late terminal differentiation. Thus, the therapeutic efficacy of Ag-experienced CTL may hinge on their resistance to CD28 down-regulation while maintaining CD28 expression under conditions of chronic viral (7, 11, 12) or tumor (9) Ag stimulation in vivo.

Even though evidence from murine models suggest that central memory T cells are more efficient at controlling tumor growth after adoptive transfer than effector/memory T cells (13), human trials in melanoma patients suggest that the persistence of human CD28+ effector/memory T cells correlate with improved clinical outcome (14). The ability to generate a stable population of long-lived CD28+ effector/memory T cells may greatly improve the clinical activity of adoptively transferred T cells (15). Methods using artificial APCs to expand T cells show promise (16); however, they are limited by the HLA haplotypes they express, as they represent nonautologous APC products requiring Ag loading, typically defined as Ags or peptides. An alternative strategy to obtain CD28+ T cells from bulk T cell populations include T cell transduction with lentiviral vectors encoding CD28 (11). Although this method can generate CD28+ T cells with reconstituted function, for instance the release of IL-2, the inherent risks of using lentiviral constructs renders this strategy less suitable for clinical use.

Dendritic cells (DC) are highly efficient at priming naive T cell responses (17–19). However, the methodology used to mature DC define the type and breadth of the T cell responses induced (20, 21).

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2 Address correspondence and reprint requests to Dr. Mark A. DeBenedette, Argos Therapeutics, Inc., 4233 Technology Drive, Durham, NC 27704, E-mail address: mdebenedette@argostherapeutics.com
3 Abbreviations used in this paper: DC, dendritic cell; REHA, rapidly expanding high avidity; PME, post maturation electroporation; MART-1, melanoma Ag recognition by T cell 1; PSA, prostate-specific Ag; APL, altered peptide ligand; ECD, PE-Texas Red-X.
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and their phenotypic characteristics and effector functions may influence the persistence of immunological memory (22, 23). Mono
cytodervived DC loaded with Ag have been tested as an immunotherapeutic approach to treating infectious and malignant disease via the induction of Ag-reactive CTL in vivo (24–27). One such approach to produce clinical DC therapeutics employs electroporation with tumor mRNA before maturation with a cytokine mixture consisting of TNF-α, IL-1β, and IL-6 with PGE2 (28). These inflammatory DC (referred to as cytokine mixture DC (CC-DC)) can induce IFN-γ-producing CTL (29, 30); however, only limited cytolytic effector activity was observed in patients treated with this type of DC (31–33). It has been reported that the sequential delivery of maturation signals and Ag loading is critical to generating DC with improved immunopotenency (34, 35). Previously, studies in our laboratory were undertaken to provide a direct head-to-head comparison of four DC preparations to determine the value of the CD40L signal to the immunopotenency of the DC matured with cytokines and electroporated with Ag-encoding RNA (36). Results from these studies revealed that only the two processes receiving CD40L RNA could induce high levels of IFN-γ by MART-1-specific CTL due to the induction of CD8⁺ T cells with a CD28⁺ CD45RA⁻ phenotype. In an effort to mimic the maturation of DC in vivo, the post maturation electroporation with CD40L (PME-CD40L) process was developed. This consists of adding TNF-α, IFN-γ, and PGE2 to provide proinflammatory signals to immature DC. At this stage, the post matured DC express CD80, CD86, and CD83 costimulatory molecules but are inefficient at priming strong CTL responses to electroporated RNA-encoded Ag (36). A second maturation signal is delivered by coelectroporation with RNA encoding CD40L along with the RNA Ag payload. This process leads to the integration of three critical signals: 1) expression of Ag-MHC complexes (signal 1), 2) costimulatory molecules (signal 2), and, of equal importance, 3) the secretion of IL-12 (signal 3) to achieve priming of high-avidity, polyfunctional CTL. Data presented here show that PME-CD40L DC prime naive T cells in vitro characterized by the expression of high-avidity T cell receptors, the ability to secrete IL-2 in response to Ag stimulation, and rapid proliferation kinetics, all attributes typically associated with memory cells. In addition, they also display the qualities of terminally differentiated effector cells by expressing IFN-γ and granzyme B and possessing cytolytic activity. Phenotypically, these effector/memory CTL maintain the expression of both CD27 and CD28 but are CD45RA and CCR7 negative, unlike CTL induced with DC electroporated with IL-12 encoding RNA (37). We have termed this novel subset of T cells: CD28⁺ rapidly expanding high-avidity (REHA) CTL.

To our knowledge, this is the first description of an ex vivo-generated fully autologous DC therapeutic capable of inducing CD28⁺ REHA CTL from a naive T cell population. The model system described, based on raising MART-1-specific CTL in vitro, allows the definition of the characteristics of this novel subset of CTL. PME-CD40L DC could be used within a fully autologous system to generate CD28⁺ REHA CTL for adoptive T cell transfer. Alternatively, a PME-CD40L DC immunotherapeutic could be administered in vivo to achieve priming of CD28⁺ REHA CTL to treat infectious or malignant disease.

Materials and Methods

Generation of mRNA for DC electroporation

MART-1 RNA. The MART-1 wild-type sequence was cloned from SK-Mel-28 total RNA. First, total cDNA was produced by RT-PCR using Powerscript reverse transcriptase (BD Clontech). To generate cDNA encoding MART-1, the following primers were used in the presence of PFU of enzyme (Stratagene). MART-1 forward: 5’-CCACCATTGCCAA GAGAAC-3’ and MART-1 reverse: 5’-TTAAGGTAATAAGGTTG-3’. The PCR products were cloned into a TA vector (Invitrogen). The MART-1 insert was then subcloned into a pGem 4Z 64T vector using KpnI and Xhol restriction enzymes. To obtain a mutation in the MART-1 open reading frame at position 27, alanine was converted to leucine by changing codon GCC to CTC. The site-directed mutagenesis was performed using QuickChange methodology (Stratagene) according to the manufacturer’s guidelines for reaction components and oligonucleotide design. Forward primer GCCCTGAAAGACGTGCGC-ATC-3’ and reverse primer 5’-GATGGCGATCCGAGGACCTCAGCCG-3’ carrying mutations (indicated in bold) were used in the mutagenesis. Clones carrying the mutated codon served to generate the plasmid template for in vitro transcription. Both MART-1 native and MART-1 altered peptide ligand (APL) RNA were generated by in vitro transcription using an mMessage Machine T7 kit (Ambion).

CD40L RNA. An optimized CD40L cDNA template (pcRC2.1 CD40L AXE-MET-1) was used which employed a modified 5’ CD40L untranslated region for optimal transcription initiation, and the first ATG in the open reading frame was deleted to generate a single-species CD40L RNA product for optimal CD40L protein expression. Details describing the generation of the cDNA are described elsewhere (38). Post transcriptional capping of CD40L RNA was performed to generate a type I cap using a ScrtisCap Kit (Epiei centre Biotechnologies). Polyadenylation was performed on purified capped RNA using A-plus Poly(A) Tailing Kits (Epici centre Biotechnologies). Final RNAs were purified as described above and the length of the poly(A) tail was determined by comparing sizes of pre-polyadenylated and polyadenylated RNAs on denaturing gel electrophoresis. A typical preparation of posttranscriptionally polyadenylated RNA contained a poly(A) tail of >150 nt.

Generation of monocyte-derived DC using the PME-CD40L process

Leukapheresis from HLA-A2-positive healthy volunteers was provided by Lifeblood (Memphis, TN). PBMCs (200 × 10⁶) were cultured in 30 ml of AIM-V medium (Invitrogen) in T150 flasks (Corning) for 2 h. Nonadherent cells were removed by washing with modified PBS (Cambrex) and the remaining monocytes were cultured in X-VIVO 15 (Cambrex) medium for 5 days, supplemented with 1000 U/ml each of GM-CSF (Leukine liquid; Berlex) and IL-4 (R&D Systems). To prepare mature PME-CD40L DC, on day 5, 10 ng/ml TNF-α (R&D Systems), 1000 U/ml IFN-γ (Ac- tummine), and 1 µg/ml PGE2 (Sigma-Aldrich) were added to immature DC cultures. On day 6, mature DC were coelectroporated with RNA encoding CD40L (Argos Therapeutics) at a concentration of 3 µg/ml DC plus either human MART-1 APL (Argos Therapeutics) or MART-1 native (Argos Therapeutics) encoding RNA at 2 µg/ml DC. MART-1 APL RNA encodes the analog variant of the immunodominant HLA-A*0201-binding peptide (AAGIGILTV) with a leucine substitution at position 27 of the amino acid sequence of the native MART-1 sequence (LLAGIGLTV). Post electroporated DC were cultured for an additional 4 h in X-VIVO 15 medium supplemented with 800 U/ml GM-CSF and 500 U/ml IL-4 at 1 × 10⁶ DC/ml in low adherence 6-well plates (Costar). Cytokine mixture DC (CC-DC) were prepared by electroporation using the indicated MART-1 RNA resuspended at 1 × 10⁶ cells/ml post electroporation and treated with a cytokine mixture comprised of TNF-α (10 ng/ml), IL-1β (10 ng/ml), IL-6 (100 ng/ml), and PGE2 (1 µg/ml). The cells were cultured for 18–22 h in low adherence 6-well plates. Post electroporated DC were formulated for freezing at 10 × 10⁶ DC/ml in 10% DMSO, 10% dextrose, and 80% autologous plasma.

Generation of MART-1-specific CTL

Either PME-CD40L DC or CC-DC electroporated with either MART-1 APL or MART-1 native RNA were cocultured with CD8⁺ T cells purified using a CD8⁺ T Cell Isolation kit II (Miltenyi Biotec). Purified CD8⁺ T cells were mixed with DC at a 10:1 CD8⁺ T cell:DC ratio in 6-well tissue culture dishes (Corning) at 5 ml (5 × 10⁶ CD8⁺ T cells/well) in R-10 medium (10% FBS (Atlanta Biologicals), RPMI 1640 (supplemented with 10 mM HEPES (pH 7.4), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM sodium glutamate, and 55 µM 2-ME (Invitrogen)). Cocultures were maintained in medium supplemented with 0.2 U/ml IL-2 and 10 ng/ml IL-7 (R&D Systems) for the first 3 days. On day 3, the medium was supplemented with 20 U/ml IL-2. CD8⁺ T cells were stimulated on a 7-day cycle with fresh DC stimulators at a 10:1 ratio in R10 medium supplemented with 20 U/ml IL-2 and 10 ng/ml IL-7. Detection of MART-1-specific CTL present in the cocultures as well as all functional assays were performed 3 days after the last cycle of stimulation. In some cases, MART-1-specific CTL were purified from bulk cultures after six
cycles of PME-CD40L DC stimulation by selection with PE-labeled HLA-A*201/ELAGIGLTV pentamer (MART-1 PE-pentamer), (Proimmune) and anti-PE microbeads (Milteny Biotec). Purified MART-1-specific CTL were cultured for 7 days in IL-2/IL-7 containing R10 medium before further cycles of DC stimulation.

Measurement of cytotoxic T cell activity

T2 cells (American Type Culture Collection) were used as CTL targets and pulsed with the indicated concentrations of HLA-A*201-restricted peptides, MART-1-APL (LAGILGTV), MART-1 native (AAAGGILTV) or prostate-specific Ag (PSA) 1 (FLTPKKLQCV) (Sigma Genosys). Peptide-pulsed T2 cells or melanoma tumor cell lines MEL-687 (HLA-A*2, MART-1) or SK-MEL-28 (HLA-A*2, MART-1) (American Type Culture Collection) were incubated with 100 μCi of Na125I (PerkinElmer) for 90 min at 37°C. Excess 125I was washed away and 5,000 labeled targets were incubated with various E:T ratios of CD8+ T cells for 4 h. Nonspecific lysis was reduced by the addition of unlabeled, unpulsed T2 cells at 25,000 cell/well. Released 125I was measured in the supernatant by scintillation counting. Specific lysis was calculated by subtracting out background lysis seen using T2 cells pulsed with the control peptide PSA from experimental lysis using T2 cells pulsed with MART-1 peptide.

Flow cytometry and intracellular cytokine labeling

CTL were labeled with PE-labeled HLA-A*201/ELAGIGLTV pentamer (MART-1 PE-pentamer) (Proimmune) for 10 min at room temperature. MART-1-PE-labeled cells were washed with FACS buffer (PBS (Cambrex), 2% FBS, 0.01% sodium azide (Sigma-Aldrich), and 2 mM EDTA (Sigma-Aldrich), then labeled with the following human specific Abs, anti-CD8-PerCP-Cy5.5, anti-CD28-allophycocyanin, anti-CD45RA-FITC, anti-CD62L-FITC (BD Biosciences), anti-PD-1-FITC, anti-CD27-FITC (eBioscience), anti-CXCR3-FITC, or anti-CCR5-FITC (R&D Systems) for 20 min at 4°C. To determine CTL intracellular cytokine labeling, in combination with cell surface labeling, responder CTL harvested at the indicated time points were incubated with peptide-pulsed T2 target cells at a ratio of 10:1 for 4 h, with the addition of brefeldin A (BD Biosciences) for the last 3 h of culture. For the determination of degranulation, 5 μl of each Ab anti-CD107a-FITC, anti-CD107b-FITC (BD Biosciences) was added at the time CTL were stimulated. Monensin (BD Biosciences) was added for the last 3 h of a 4-h incubation. MART-1-specific CTL were identified by labeling with MART-1 PE-pentamer and the indicated cell surface markers. Labeled cells were then fixed in 1% parafomaldehyde (BD Biosciences) and permeabilized with 0.1% saponin in FACS buffer. In some instances, permeabilized cells were then labeled with anti-IL-2-FITC and anti-IFN-γ-allophycocyanin or anti-TNF-α-AF700 (BD Biosciences) in the presence of 0.1% saponin buffer to detect intracellular cytokine expression. Cells were washed in FACS buffer and fluorescence was measured on a FACSCalibur or LSRII (BD Biosciences). A total of 300,000–400,000 lymphocyte gated events (based on forward vs side scatter) were collected for each sample tested. Further subgating was performed on the MART-1+CD8+ T cells to define the cytokine- or lytic-positive cells. Data were analyzed with FlowJo software (Tree Star).

CSFE labeling of MART-1-specific CTL

Before DC stimulation 1 × 10^6/ml CTL were labeled with 0.5 μM CSFE (Invitrogen) in X-VIVO-15 medium for 10 min at 37°C washed twice with PBS and resuspended at 1 × 10^6/ml in R10 medium. Labeled CTL were then stimulated with the indicated DC preparations and 3 days later colabeled with MART-1 PE-pentamer, anti-CD8-PerCP-Cy5.5, and anti-CD45RA-allophycocyanin Ab to identify proliferating MART-1-specific CTL. Proliferation was measured by CSFE dilution.

MART-1 pentamer stimulation to detect high-avidity CTL. MART-1 PE-pentamer was used as a stimulating reagent to induce MART-1-specific CTL effector function from CTL harvested from DC cocultures. Indicated concentrations of MART-1 PE-pentamer were added directly to 1 × 10^6 CTL in 200 μl of R10 medium. Five microliter of CD107a-FITC Ab along with brefeldin A and monensin were added at the start of the stimulation. Four hours poststimulation, CD107a-positive MART-1-specific CTL were identified by subsequent labeling with anti-CD8 PE-Texas-Red-X (ECD) (Beckman Coulter) and MART-1 allophycocyanin-labeled HLA-A*201/ELAGIGLTV pentamer (MART-1 allophycocyanin-pentamer; Proimmune) as a means to detect the overall frequency of MART-1-positive CTL at each dilution of stimulating pentamer tested. By using the MART-1 pentamer conjugated with a different fluorochrome to label stimulated CTL, the total number of MART-1-specific CTL can be tracked, as the stimulating pentamer does not saturate all of the available TCR binding sites. Degranulation is expressed as the percentage of CD107a+ cells

![FIGURE 1. Expansion of MART-1-specific CTL. A, CD8+ T cells were cocultured with either PME-CD40L DC (■ and □) or CC-DC (▲ and ○). MART-1-specific CTL were identified at the indicated time points by labeling cocultures with MART-1 PE-pentamer and anti-CD8-PerCP-Cy5.5 Ab to identify the percentage of MART-1-specific CD8+ T cells present at each time point. Arrows indicate the day of a cycle of stimulation with fresh DC. Closed symbols represent CTL expansion using DC electroporated with MART-1 APL RNA and open symbols represent CTL expansion using DC electroporated with MART-1 native RNA. Data are representative of three separate donors. B, The absolute number of MART-1-specific CD8+ T cells present after three cycles of DC stimulation was calculated as follows: (number of viable CTL in bulk cocultures × percent MART-1+CD8+ T cells identified by labeling with MART-1 pentamer). Data shown are the absolute number of MART-1+CD8+ T cells stimulated with CC-DC encoding either MART-1 native Ag (■) or MART-1 APL Ag (△) or PME-CD40L encoding with either MART-1 native Ag (▽) or MART-1 APL Ag (○). C, The fold increase was defined as the absolute number of MART-1+CD8+ T cells generated using PME-CD40L DC encoding either MART-1 native Ag (▽) or MART-1 APL Ag (○) for each data point divided by the average number of MART-1+CD8+ T cells generated using CC-DC encoding either MART-1 native Ag or MART-1 APL Ag, respectively. The average number of MART-1+CD8+ T cells generated using CC-DC (native) was 129,300 for the three data sets shown in B and the average number of MART-1+CD8+ T cells generated using CC-DC (APL) was 292,600 for the four data sets shown in B.](http://www.jimmunol.org/)}
within the MART-1 CD8+ T cell population. A total of 300,000–400,000 lymphocyte gated events (based on forward vs side scatter) were collected for each sample tested. Further subgating was performed on the MART-1 CD8+ T cells to the lytic-positive cells. Samples were analyzed on an LSRII (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

**Results**

PME-CD40L DC loaded with MART-1 Ag prime rapidly expanding CD28+CD45RA-CD62L+ REHA CTL

DC were generated from HLA-A2 healthy donors using two alternate maturation methods and used to prime CTL in vitro to the melanoma-associated Ag MART-1. The MART-1 Ag was chosen as a model system based on the relatively high frequency of MART-1-specific CD8+ T cells present in the HLA-A2 healthy donors compared with other tumor Ags (36). RNA-electroporated DC were generated from HLA-A2 healthy donors using two alternatives: two al-mixture (TNF-α, IL-1β, PGE2, and IL-6, CC-DC) or by using TNF-α, IFN-γ, and PGE2 before coelectroporation with MART-1-encoding and CD40L RNA (PME-CD40L DC). In one set of experiments, CD8+ T cells were primed with PME-CD40L DC or CC-DC electroporated with a full-length MART-1 RNA modified to encode an APL with enhanced HLA-A*0201 affinity and subsequently restimulated weekly with the indicated DC for a total of four cycles. Priming with PME-CD40L DC induced greater expansion of MART-1-specific CTL in contrast to CD8+ T cells primed with CC-DC as detected by binding of MART-1 pentamer (Fig. 1A). Moreover, PE-MCD40L DC could continually drive increasing numbers of MART-1-specific CTL with each DC restimulation. To confirm this observation under conditions using the unmolded native MART-1 Ag, a second experiment was performed, but with the CD8+ T cells primed with either PE-MCD40L DC or CC-DC electroporated with MART-1 RNA encoding the wild-type sequence. CTL expansion was observed when priming with PE-MCD40L DC but not when using repetitive stimulation with CC-DC (Fig. 1A). To gauge the overall robustness of PE-MCD40L DC to expand MART-1-specific CTL compared with using DC matured just with a cytokine mixture (CC-DC), data were compiled from a total of 13 independent experiments using native MART-1 Ag-encoding RNA and a total of 8 experiments using MART-1 APL encoding RNA using six different HLA-A2 donors. The absolute numbers of MART-1 pentamer-positive CTL generated from a CD8 T cell starting population of 5 million T cells were calculated for each of the given data points from the bulk cocultures after three cycles of DC stimulation (Fig. 1B). The data set derived from CC-DC-stimulated cocultures is limited due to the low frequency of MART-reactive T cells induced by CC-DC, but nonetheless served as a baseline measurement to calculate the fold increase seen when PE-MCD40L DC are used to stimulate MART-1-specific T cells (Fig. 1C). Overall, the fold increase of PME-CD40L DC over CC-DC was 3.4- to 107.3-fold when using the native MART-1 Ag and 5.2- to 46.9-fold when using MART-1 APL Ag. Given the differences in the expansion capacity of MART-1-specific CTL as a consequence of the type of DC used for priming, we next set out to characterize the state of differentiation of MART-1-specific CTL generated with PME-CD40L DC vs CC-DC. The phenotypes of functionally distinct T cell populations can be subdivided by measuring expression of CD28, CD45RA, CD45RO, and CCR7 (39, 40). Using these cell surface markers, multiparametric flow cytometry was performed on CTL generated with PME-CD40L DC or CC-DC (Fig. 2). The majority of PE-MCD40L DC-primed MART-1-specific CTL are characterized by the expression of CD28 (79.6%), with a loss of CD45RA (Fig. 2A). In contrast, CC-DC primed MART-1-specific CTL lack CD28 expression and comprise two subsets, either CD45RA+ (69%) or CD45RA− (19.2%) (Fig. 2A). The expression intensity of CD62L was 10-fold higher on PME-CD40L DC-induced CTL (mean fluorescence intensity, 191) compared with CC-DC-induced CTL (mean fluorescence intensity, 18) (Fig. 2A). Neither PE-MCD40L DC nor CC-DC induced MART-1-specific CTL expressing CCR7, a marker associated with central memory T cells (data not shown). Given the lack of CCR7 expression on the activated cells, it is unlikely that CC-DC-MART-1-specific CTL represent a pool of long-term memory CTL. The bulk of CC-DC-induced MART-1-specific CTL do not express CD28, but due to the expression of CD45RA, these CTL are classified as either effector CTL (CD45RA+), or effector memory CD45RA+ (EMRA) CTL (41, 42). MART-1-specific CTL were analyzed for CD28 expression along with the following markers: CD27, PD-1, CXCR3, and CCR5 (Fig. 2, C–F, PE-MCD40L, and I–L, CC-DC). PME-CD40L DC induced CD28+ CTL, whereas CC-DC induced CD28- CTL. However, both populations of CTL displayed a CD27+PD-1−/CXCR3−CCR5− profile.
A separate set of experiments was designed to characterize the expansion potential of MART-1-specific CTL by either PME-CD40L DC or CC-DC. CTL were labeled with CFSE before the fifth stimulation cycle using DC electroporated with either MART-1 APL RNA or MART-1 native RNA. When PME-CD40L DC-presenting MART-1 APL Ag (Fig. 3A) were used to stimulate MART-1 APL-specific CTL, the majority of proliferating (CFSElow) CTL were CD28+/H11001 (78.5%). In contrast, when CC-DC-presenting MART-1 APL Ag (Fig. 3B) were used, the profile was reversed with the majority of proliferating CTL induced by CC-DC residing within the CD28−/H11002 subset (54.2%). The ability of PME-CD40L DC to induce a rapidly expanding population of MART-1-specific CD28+/H11001 CTL was most pronounced in the context of native Ag where nearly one-half of the proliferating CTL expressed CD28 (Fig. 3C) compared with a striking lack of proliferation induced by CC-DC (Fig. 3D). We conclude that PME-CD40L DC can prime CTL with greater expansion capacity upon Ag re-encounter, which maintain expression of CD28 in response to repeated Ag presentation of both heteroclitic and wild-type self-Ag.

The lytic activity of CD28+/H11001 CD45RA− effector memory CTL is not dependent on IL-12 secretion from PME-CD40L DC when using targets displaying high Ag density

Previously, we have shown that PME-CD40L DC produce IL-12 which may serve as a necessary third signal for CTL expansion (36). Therefore, experiments were set up to examine the role played by IL-12 in the induction of lytic activity in MART-1-specific CD28+/H11001 CD45RA− CTL expanded by PME-CD40L DC. In

#### FIGURE 3

Proliferation of CD28+/H11001 effector memory MART-1-specific CTL. On day 28 before the fourth cycle of stimulation, CTL were labeled with CFSE and stimulated with either PME-CD40L DC (A and C) or CC-DC (B and D) and 3 days later cocultures were labeled with MART-1 PE-pentamer, anti-CD8-PerCP-Cy5.5, and anti-CD28-allophycocyanin Ab to detect proliferating MART-1-specific CD28+ CTL. Proliferation measured by CFSE dilution to DC loaded with either MART-1 APL (A and B) Ag RNA or MART-1 native Ag RNA (C and D) is shown. Numbers indicate percentage of cells in each quadrant defined by CD28 labeling vs CFSE dilution. CTL were first gated on MART-1 pentamer+/H11001 CD8+ CTL. Data are representative of two separate experiments.

#### FIGURE 4

IL-12 produced by PME-CD40L DC induces the generation and expansion of CD28+/H11001 CD45RA− effector memory CTL with enhanced cytolytic activity. Isotype control Ab (A and B), anti-p40-neutralizing Ab (C and D), or anti-p19-neutralizing anti-IL-23 Ab (E and F) was added at the initiation and at each cycle of CTL-DC stimulation. At days 10 and 17, CTL were labeled with MART-1 PE-pentamer, CD8-PerCP-Cy5.5, CD28-allophycocyanin, and CD45RA-FITC Abs to identify MART-1-specific CTL subsets. Numbers indicate percentage of cells in each quadrant defined by CD28 labeling vs CD45RA labeling. CTL were first gated on MART-1 pentamer+/H11001 CD8+ CTL. At day 10 (G) and day 17 (H), 3 days after stimulation cycles 2 and 3, respectively, killing activity was measured in a chromium release assay using MART-1 peptide-pulsed T2 targets. Neutralizing anti-p40 Ab was added on days 0, 7, and 14 at the time point when CTL cultures were stimulated with PME-CD40L DC. Percent specific lysis is shown. Data using anti-p40 Ab is representative of two separate experiments.
an independent experiment, CD8 T cell cocultures were primed with PME-CD40L DC electroporated with native MART-1-encoding RNA. Neutralizing Ab to the p40 subunit of IL-12 and IL-23 or to the p19 subunit of IL-23 was added each time the cocultures were restimulated with DC (Fig. 4). When p40 was neutralized, the percentage of MART-1-specific CD28+/H11001CD45RA/H11002CTL present in the cocultures was decreased on day 10 from 84.7% (Fig. 4A) to 38.5% (Fig. 4C). However, p19 neutralization had no effect (Fig. 4E), indicating that IL-12, as opposed to IL-23, was associated with the maintenance of a population of Ag-specific CTL that are CD28 positive as well as CD45RA negative. The change in phenotype was not a consequence of activation-induced T cell death since the percentage of MART-1-specific CTL were equivalent between cocultures treated with control Ab (0.6%) and anti-p40 Ab (0.7%). In the absence of IL-12, the CD28− subset was subdivided, such that 22% of the MART-1-specific CTL lack CD45RA and 23.3% express CD45RA (Fig. 4C). The requirement for PME-CD40L DC to produce IL-12 as a third stimulatory signal is more pronounced at later stimulation cycles. Three days after the third stimulation in the presence of p40 blockade (day 17, Fig. 4D), but not p19 blockade (day 17, Fig. 4F), there is not only a predominant shift to a CD45RA− phenotype (56.1%), but there is a 1.5-fold decrease in the overall expansion of MART-1-specific CTL from 2% of the CD8 T cell population to only 0.78% in the presence of p40 neutralization (data not shown).

On day 10, when the number of MART-1-specific CTL were similar in both control and p40-neutralized cultures (0.6 and 0.7%, respectively), there was no difference in the ability of MART-1-specific CD28+ and CD28− CTL to lyse peptide-pulsed targets, (Fig. 4G), suggesting that lytic activity is not IL-12 dependent. After a third cycle of DC stimulation, day 17 cocultures exhibited a 1.5-fold decrease in MART-1-specific lysis in the presence of neutralizing p40 (Fig. 4H). However, this loss of CTL activity is most likely a reflection of the decrease in the percentage of MART-1-specific CTL in the cocultures from 2 to 0.78% (in the presence of p40-neutralizing Ab) as defined by MART-1 pentamer binding, as opposed to an absolute requirement for IL-12 to license CTL for cytotoxicity. Thus, based on recognition of Ag presented in saturating concentrations on peptide-pulsed targets, both the CD28+ and CD28− MART-1-specific T cells are cytolytic. Only PME-CD40L DC-secreting IL-12 can prime MART-1-specific CTL which retain a CD28+ phenotype and display an increased proliferative potential with immediate lytic activity.
Effector memory cytokine production is dependent on IL-12

To understand the importance of IL-12 in supporting the development of T cells with the capacity to secrete cytokines, MART-1 reactive T cells were generated in the presence or absence of neutralizing anti-p40 Ab using PME-CD40L DC. CTL generated in the presence of anti-p40-neutralizing Ab required target cells pulsed with 100 ng/ml MART-1 native peptide to induce 9.26% MART-1-specific IFN-γ and IL-2 CTL (Fig. 5A, lower panels), whereas CTL generated in the absence of p40-neutralizing Ab required target cells pulsed with only 10 ng/ml native peptide to induce a similar percentage (7.75%) of MART-1-specific IFN-γ and IL-2 CTL (Fig. 5A, upper panels).

In two separate experiments, assaying cytokine production to either MART-1 native Ag (Fig. 5B, left panel) or MART-1 APL Ag (Fig 5B, right panel), the frequency of MART-1-specific CTL secreting TNF-α, IFN-γ, and IL-2 was measured and the impact of neutralizing IL-12 on each parameter was determined (Fig. 5B). In the presence of anti-p40-neutralizing Ab, the simultaneous production by CTL of either two or three cytokines was decreased (Fig. 5B). This suggests that IL-12 acting as signal 3 may not only influence the size of the Ag-reactive T cell pool but may also alter the subsequent polyfunctional cytokine secretion profile and thereby the overall breadth of the effector/memory CTL response (43).

PME-CD40L DC induce high-avidity CTL capable of degranulation-dependent lysis on recognition of low Ag-bearing target cells

The measurement of CD107 surface expression was used to determine the functional avidity of MART-1-specific CTL. In the first experiment using an additional healthy donor, MART-1-specific CTL were primed with PME-CD40L DC electroporated with native MART-1 encoding RNA for five cycles of stimulation. To minimize the contribution of non-MART-1-specific CD8+ T cells in the assay, MART-1-specific CTL present in the bulk CD8+ T cell coculture were purified using MART-1 PE-pentamer and anti-PE Ab-coated microbeads to isolate a homogenous source of MART-1-specific CTL for avidity analysis. When purified MART-1-specific CTL were stimulated with titrated concentrations of MART-1 peptide pulsed onto T2 cells, a linear relationship between the concentration of Ag and the ability of MART-1-specific CTL to degranulate was observed. More than 80% MART-1-specific CTL degranulation was achieved at the highest concentration of peptide tested (10 ng/ml). Moreover, just 1 pg/ml peptide pulsed onto T2 cells still resulted in >20% of the MART-1-specific CTL undergoing degranulation (Fig. 6A). No degranulation was observed using T2 cells pulsed with irrelevant peptide (PSA).
CTL raised against the native Ag by cycles of stimulation reduced the expansion of MART-1-specific IL-12p70 Ab to the CTL/DC cocultures during each of the three. The addition of neutralizing anti-Ab MART-1 native Ag (Fig. 6) and MART-1 native-specific CTL compared with using PME-CD40L DC-presenting fold (donor 1) and 5.5-fold (donor 2) greater number of MART-1 Ag presented by melanoma tumor lines. Three days after the fifth cycle of DC stimulation, killing activity was measured in a chromium release assay using either MEL-687 cells (HLA-A2) or SK-MEL 28 (HLA-A2) cells as targets. The percentage of MART-1 APL-specific CTL tumor lysis (A) and MART-1 native-specific CTL tumor lysis (B) in a 4-h chromium release assay is shown.

CTL from two different donors were stimulated with their respective PME-CD40L DC electrooporated with RNA encoding native MART-1 or the heteroclitic variant MART-1 APL for three cycles. On day 17, 3 days after the third stimulation cycle PME-CD40L DC-presenting MART-1-APL were able to generate a 14-fold (donor 1) and 5.5-fold (donor 2) greater number of MART-1-specific CTL compared with using PME-CD40L DC-presenting MART-1 native Ag (Fig. 6B). The addition of neutralizing anti-IL-12p70 Ab to the CTL/DC cocultures during each of the three cycles of stimulation reduced the expansion of MART-1-specific CTL raised against the native Ag by ~65% (donor 1) and 31% (donor 2) vs 10% (donor 1) and 35% (donor 2) for CTL raised against MART-1-APL (Fig. 6B). To further examine functional avidity, an assay was developed using soluble MART-1 PE-pentamer to cross-link the TCR without a requirement for secondary and tertiary signals (e.g., CD70 expression by T2 cells facilitating signaling via CD27). MART-1 pentamers are multimeric molecules that bind to the TCR potentially resulting in cross-linking of the TCR and allowing delivery of an activation signal. Therefore, only those CTL of the highest avidity would exhibit effector function in this assay. CTL effector function was measured by the percentage of MART-1-specific CTL coexpressing CD107a after TCR cross-linking with MART-1-PE-pentamer. Concurrently, on day 17, the functional avidity of CTL generated from the same two donors (depicted in Fig. 6B) with either MART-1 native or MART-1 APL Ag-expressing PME-CD40L DC in the presence of IL-12-neutralizing Ab was assessed by incubating the T cells with a titration of MART-1 pentamer as a stimulating agent, followed by colabeling with anti-CD107a Ab to measure degranulation. The overall frequency of MART-1-specific CTL detected did not change regardless of the amount of pentamer used to stimulate the cells. CTL raised on PME-CD40L DC-presenting native MART-1 Ag displayed a higher level of CD107a expression compared with CTL raised on PME-CD40L DC-presenting MART-1-APL Ag (Fig. 6C, donor 1, left panel; donor 2, right panel). By neutralizing IL-12p70 during DC priming and subsequent cycles of stimulation, there was a decrease in the overall avidity of the TCR on the MART-1-specific CTL. This was evident by the need for higher concentrations of MART-1 pentamer to stimulate the same level of degranulation of MART-1-specific CTL primed in the absence of IL-12-neutralizing Ab (Fig. 6C). Finally, the lytic activity of CTL primed on PME-CD40L DC electrooporated with RNA-encoding MART-1 APL (Fig. 7A) or MART-1 native (Fig. 7B) Ag was determined using melanoma cell lines as a source of naturally processed and presented Ag. Both MART-1-specific CTL primed against the native Ag or the heteroclitic Ag (APL) could lyse MART-1+ melanoma cells in a MHC-restricted manner. Interestingly, CTL primed with MART-1 native Ag exhibited a higher overall level of lysis. However, the frequency of MART-1-specific CTL, as defined by MART-1 pentamer labeling, was significantly lower when compared with CTL raised against the MART-1-APL (Fig. 7B). MART-1-specific CTL primed with native Ag contained 9.6% MART-1 PE-pentamer-positive cells, compared with 23.8% for CTL stimulated with MART-1 APL (Fig. 7, A and B).

**Discussion**

We have previously published studies showing the relative immunopotency of different DC preparations and the induction of CD8\(^+\) CTL responses (36). Additional data are provided herein that support those studies by comparing T cell immunity induced using cytokine mixture-matured DC, considered current “best practice,” particularly for use in the clinic, to T cell responses induced by PME-CD40L DC. The primary goal of the studies present in this manuscript was not to exhaustively compare DC preparations, but to study in detail the phenotype and function of a novel subset of CD8\(^+\) CTL induced by PME-CD40L DC. The results presented here show that PME-CD40L DC possess the appropriate complement of stimulatory signals required to prime a unique population of Ag-reactive CTL. The T cells display a previously unreported combination of phenotypic and functional properties that do not allow definitive classification under current nomenclature describing naive, effector, effector memory, or central memory CTL, since models of CTL differentiation propose down-regulation of CD28 CD27 and CCR7 with a concurrent up-regulation of cytolytic effector molecules (3–5, 44, 45). Others have recently shown that effector memory CTL isolated from the peripheral blood of normal donors can be defined functionally by four distinct subsets based on the expression patterns of CD28 and CD27 along with effector function. One such subset (EM1) displays a CD28\(^-\)CD27\(^+\) phenotype but they do not display high levels of effector activity and have poor lytic capability when compared with their CD28\(^+\)CD27\(^-\) counterparts (4). This is in contrast to T cells induced with PME-CD40L DC.

The phenotype of PME-CD40L DC-primed CTL described here is defined as CD28\(^-\)CD27\(^+\)CXC\(_{CR3}\)\(\Delta\)CD62L\(^-\) with a loss of CD45RA and CCR7. These CTL are not canonical central memory CTL due to lack of CCR7, nor true effector CTL due to the lack of CD45RA reexpression after subsequent Ag encounter (41). Since PME-CD40L DC-primed CTL do not fit into the current nomenclature, they are defined as CD28\(^-\)REHA CTL. Other approaches using artificial APC in conjunction with IL-15 have been used to generate large numbers of Ag-specific CTL in vitro for use in adoptive T cell therapy (16). Although these CTL express CD28 and CD27 and
mixed expression for CD62L, Ag-specific production of IFN-γ declined after multiple rounds of stimulation (16). In contrast, REHA CTL primed with PME-CD40L DC, producing both IFN-γ and IL-2, could be detected for at least six weekly cycles of stimulation (42 days). One important distinction between PME-CD40L DC vs artificial APC is that PME-CD40L DC do not produce detectable amounts of IL-15 (data not shown). The maintained expression of both CD28 and CD27 on CTL with strong effector function has been used to identify CTL in vivo that are linked to positive clinical outcome in adoptive T cell immunotherapy (46). However, this is the first report showing the generation of CD28+ REHA CTL using DC in vitro, allowing the possibility of using these DC in in vivo immunotherapy as an alternative to adoptive T cell therapy. In addition to the expression of CD28 and CD27, REHA CTL induced by PME-CD40L DC also express CD62L, which would impart a selective advantage on CTL, allowing trafficking back through lymph nodes to receive additional rounds of DC stimulation, perhaps perpetuating the expansion of long-lasting Ag-reactive cells.

One of the critical factors responsible for the enhanced efficiency of priming Ag-reactive CTL is the ability of PME-CD40L DC to secrete IL-12 to act as a critical third signal. This is in contrast to other forms of DC maturation, including the use of standard cytokine mixtures, which do not lead to DC IL-12 secretion (47), and thus do not prime high numbers of CD28+ REHA CTL. Additionally, blockade of IL-12 in PME-CD40L DC in T cell cocultures also prevents the generation of the CD28+ REHA CTL phenotype. IL-12-secreting DC populations have been described by others which could induce CTL, but which do not meet the criteria defined here for REHA CTL. For instance, in cases where monocyte-conditioned medium or TNF-α-matured DC were used with exogenous IL-12, high-avidity MART-1-specific CTL could be induced but these cells were CD27 and CD28 negative, having an effector CTL phenotype (48). Similar results were seen using monocyte-conditioned medium or TNF-α-matured DC electroporated with IL-12 mRNA, which increased MART-1-specific CTL proliferation and IFN-γ secretion, but once again only a CD27−CD28− effector CTL population was generated (37), in stark contrast to the REHA CTL reported in this study using PME-CD40L DC. Therefore, PME-CD40L DC provide additional costimulation signal(s) in addition to, but dependent on, IL-12 secretion.

A link between CD28 expression and restored IL-2 production in situations of chronic viral infections has been reported (11). In these studies, IL-2 production of both CMV- and HIV-specific CTL clones could only be restored in vitro by retroviral transfection of the CD28 receptor into CD28-negative clones (11). Restoration of both IFN-γ and IL-2 secretion may be critical to generate CTL capable of controlling viral infections and tumor persistence subsequent to immunotherapy. The positive advantage of REHA CTL to secrete both IFN-γ and IL-2 is linked to the ability of PME-CD40L DC to secrete IL-12 during CTL priming.

There is little doubt that numerous immunotherapeutic interventions used to treat cancer or infectious disease can induce tumor- or pathogen-reactive T cells, both in vitro and in vivo. A difficulty with many of these approaches is their inability to prime CTL that are of sufficient avidity to demonstrate clinically relevant activity. Data in this present study show that DC-derived IL-12 can greatly impact the recognition of Ag, decreasing the amount of peptide-MHC complexes needed to induce cytokine or cytolytic activity. This was most evident using native MART-1. Although it has been shown that using the MART-1 APL can generate CTL with higher avidity TCR (49), there are other reports showing that the use of APLs can induce CTL with lower avidity than the native epitope and that these cells do not recognize naturally processed and presented Ag expressed on targeted tumor cells (50, 51). Furthermore, recent clinical trials comparing the modified MART-1 peptide analog to the unmodified MART-1 self-peptide mixed in conjunction with CpGs showed increased functional avidity and enhanced effector function using the natural Ag (52). This is in agreement with data presented here. When MART-1 native Ag and MART-1 APL Ag were compared head-to-head using PME-CD40L DC as a means to prime REHA CTL, the native Ag generated CTL of the highest avidity. These data suggest that avidity is dependent not only on the MHC/peptide/TCR synapse, but also on other contributing factors derived from the APC. The ability to signal CD28+ REHA CTL with low doses of Ag may be particularly important when the availability of Ag in vivo may be a limiting factor in the process, for instance, due to tumor- or pathogen-mediated down-regulation of Ag presentation. In the context of immunotherapy of malignant and infectious disease, generating a large pool of terminally differentiated effectors with limited expansion ability and effector function may be a major factor in inducing only transient immune responses, particularly where Ag-reactive CTL have lost the capacity for self-renewal during chronic stimulation by persistent tumors or chronic infections. Therefore, cell-based immunotherapeutic strategies relying on Ag-loaded DC must take into account the ability to deliver a full complement of signals during priming. Herein, we show an optimal strategy using PME-CD40L DC that, due to the unique delivery of the Ag and CD40L signal as electroporated RNA, allows the optimally timed release of IL-12 during priming of the CD8 immune response. PME-CD40L DC are capable of expanding a unique population of CD28+ REHA CTL that maintain reactivity upon repeated antigenic challenge, a functional trait that would favor the use of PME-CD40L DC as a DC immunotherapeutic platform for cancer and infectious disease. Current best practices rely on using the cytokine mixture DC platform which may not generate a large number of Ag-specific T cells. In this study, we show that PME-CD40L DC are able to drive a more robust, polyfunctional CTL response when compared with the CC-DC platform, ranging from 3- to a 100-fold increase in the number of Ag-specific cells.

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Disclosures

All authors are full-time employees of Argos Therapeutics and have stock options in the company.

References


