

Optimization of the Sequence of Antigen Loading and CD40-Ligand-induced Maturation of Dendritic Cells¹

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Abstract

Dendritic cells (DCs), matured by CD40-ligand (CD40L), undergo marked changes in their ability to process and present antigen, resulting in augmented lymphocyte stimulatory activity. We demonstrate that the form of the tumor antigen (peptide or genetic material) used to load the DCs dictates the required sequence of antigen loading and maturation for antitumor immunotherapy. Optimal stimulation of carcinoembryonic antigen (CEA)-specific CTLs by peptide-loaded DCs occurs when DCs from cancer patients are matured with CD40L before exposure to CEA peptide, whereas optimal stimulation by RNA-transfected DCs occurs when the DCs are loaded with CEA RNA before maturation with CD40L.

Introduction

Immunotherapy strategies using DCs,³ professional antigen-presenting cells capable of initiating potent primary antitumor immune responses *in vivo* (1–4), have become feasible following the development of methods for generating DCs from adherent PBMCs in media containing the cytokine combination of GM-CSF and IL-4 (5–8). These DCs are phenotypically and functionally immature (6), expressing high levels of HLA-DR but minimal CD83 and CD80 and retaining the ability to phagocytose antigen. Although these immature DCs are capable of presenting antigen to T cells, mature DCs are more effective stimulators of antigen-specific T-cell responses. Cross-linking CD40 on DCs by cell-bound CD40L results in DC maturation and activation (9), prolongation of survival (10), expression of accessory molecules, and IL-12 secretion (11, 12). It would be desirable, therefore, to determine how to apply trimeric soluble CD40L to the maturation of dendritic cells for immunotherapy studies. Ongoing Phase I studies are using DCs generated in serum-free media supplemented with GM-CSF and IL-4 and loaded with either an HLA-A2-restricted peptide of CEA or the mRNA encoding CEA. Although CEA peptide likely binds directly to surface-expressed HLA molecules, CEA RNA must be taken up by the DCs (a function of immature DCs) and transcribed to CEA protein that is then degraded into peptides capable of forming complexes with nascent HLA molecules for presentation on the cell surface. We hypothesized that the type of antigen would affect the optimal sequence of antigen loading and maturation. We demonstrate that CD40L-matured DCs from patients with advanced cancers effectively stimulate a CEA-specific T-cell response *in vitro* when CD40L is applied before CEA peptide, but after CEA RNA, loading.

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³ The abbreviations used are: DC, dendritic cell; PBMC, peripheral blood mononuclear cell; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL, interleukin; CD40L, CD40-ligand; CEA, carcinoembryonic antigen; HCV, hepatitis C virus; GFP, green fluorescent protein; TNF, tumor necrosis factor; MLR, mixed lymphocyte reaction.

Materials and Methods

Media and Reagents. The culture medium for generation of DCs was AIM V (Life Technologies, Inc., Grand Island, NY). The following cytokines were used: human recombinant GM-CSF and recombinant IL-4 (gifts of Dr. Mary-Ellen Ryback, Schering-Plow, Kenilworth, NJ) and recombinant IL-2 and recombinant IL-7 (Genzyme, Cambridge, MA). Human CD40L trimer (hCD40LT) was provided by Dr. Elaine Thomas (Immunex Corporation, Seattle, WA). Mouse anti-human CD40-ligand was purchased from PharMingen, San Diego, CA. The following HLA-A2-restricted peptides were used [and have been described previously (13)]: influenza virus peptide (M1; amino acids 58–66, GILGFVFTL); CEA peptide (CAP-1; amino acids 571–579, YLSGANLNL; a gift of Jeffrey Schlom, National Cancer Institute, Bethesda, MD); HCV peptide (NS3 protein; amino acids 1077–1085, CINGVCWTV). CEA RNA and GFP RNA were produced and polyadenylated as described previously (14). The following monoclonal antibodies were used for immunofluorescence staining: anti-CD14; anti-CD80; anti-CD86; anti-HLA A, -B, and -C; anti-HLA-DR (Becton Dickinson, San Jose, CA); and anti-CD83 (Immunotech, Westbrook, ME).

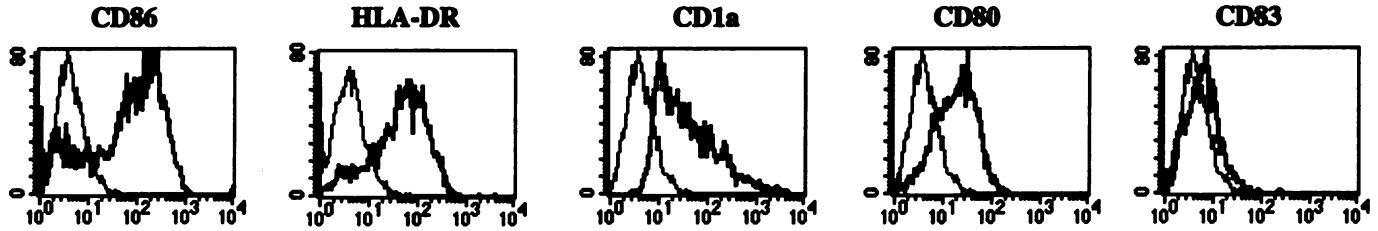
DC Generation, Antigen Loading, and Maturation with CD40L. PBMCs were obtained by leukapheresis from healthy volunteers and patients with advanced cancers after they gave informed consent. The protocols for the leukaphereses were approved by the Duke University Medical Center Institutional Review Board and were performed in accordance with an assurance filed with and approved by the Department of Health and Human Services. DCs were generated over 7 days from adherent PBMCs in AIM V media containing GM-CSF (800 units/ml) and IL-4 (500 units/ml) as described by Romani *et al.* (7) with minor modifications (8). DCs and autologous PBMCs to be used as responders were cryopreserved in 90% FCS (or autologous plasma)/10% DMSO. For studies using the HLA-A2-restricted CAP-1 peptide, only DCs from HLA-A2 donors were tested, whereas there was no HLA type required for the CEA RNA studies. For the peptide pulsing protocol, DCs ($2\text{--}5 \times 10^6$ /ml), prepared as described above, were incubated in AIM V with GM-CSF, IL-4, and CD40L (1 $\mu\text{g}/\text{ml}$) for 18–20 h, washed, and resuspended in AIM V; then CEA peptide and β_2 -microglobulin were added for 4 h. Alternatively, DCs, prepared as described above, were incubated with CEA peptide and β_2 -microglobulin for 4 h and washed; then GM-CSF, IL-4, and CD40L were added for 18–20 h. As a control, DCs were incubated in AIM V with CEA peptide (20 $\mu\text{g}/\text{ml}$) and β_2 -microglobulin (3 $\mu\text{g}/\text{ml}$) for 4 h at 37°C. In some experiments, neutralizing monoclonal antibody to CD40L (5 $\mu\text{g}/\text{ml}$) was added with CD40L to demonstrate the specificity of CD40L-mediated effects. In experiments using DCs transfected with RNA, CEA RNA (10 $\mu\text{g}/\text{ml}$) was substituted for CEA peptide in each of the above groups, and no β_2 -microglobulin was used. DCs were washed twice to remove any cytokines, peptides, or RNA prior to their use as stimulators.

Induction of Primary CTL Responses *in Vitro*. Stimulation of PBMCs and expansion of CTLs were performed as described previously (13). Targets for experiments with CEA peptide were autologous DCs loaded with CAP-1 peptide or HCV peptide as a control, whereas targets for experiments with CEA RNA were autologous DCs loaded with CEA RNA or GFP RNA as a control. A standard europium release CTL assay was performed. Spontaneous release was <25%. SEs of triplicate cultures were <5%.

Results and Discussion

Upon maturation, DCs generated from PBMCs of normal donors with GM-CSF and IL-4 in a clinically applicable serum-free media

No CD40L



+ CD40L

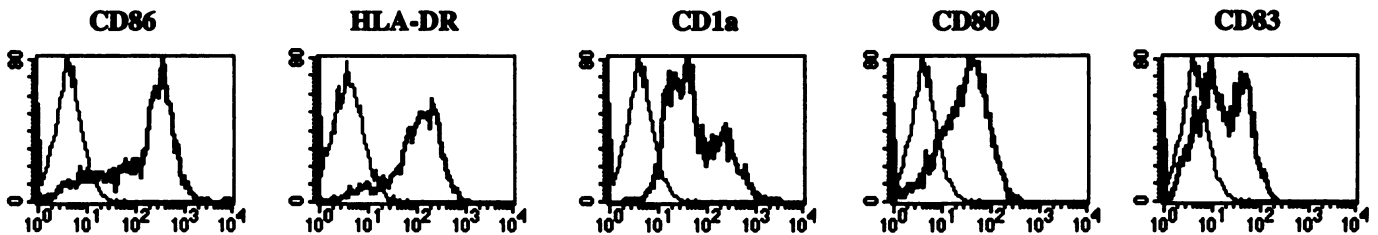


Fig. 1. Fluorescence-activated cell sorter analysis of DCs before and after CD40L treatment. DCs generated in GM-CSF and IL-4 for 7 days were harvested, and a portion were cultured for an additional 18 h with 1 μ g/ml CD40L. The DCs were then stained with monoclonal antibody to CD80, CD83, CD86, and HLA-DR. CD83 was up-regulated after CD40L exposure. Solid lines represent the isotopic control. Gray lines represent the sample stained with the antibody for the dendritic cell marker listed above the histogram.

up-regulate CD80 and CD83 (5, 6). Using DCs generated in serum-free media from the PBMC of patients with metastatic malignancies, we observed that CD40L caused up-regulation of CD83 on at least a portion of the DCs (Fig. 1). There was considerable variability in the levels of CD83 expression pre- and post-CD40L treatment, with CD83 expression on as many as 11% of the cells preexposed to CD40L and on as many as 61% of the cells post-CD40L exposure. As we have observed previously with exposure of DCs to TNF- α (15), CD83 expression is not as complete as if the cells had been cultured in a serum-containing media. The up-regulation did not differ when CD40L was added before or after loading the DCs with antigen or when the antigen was in the form of peptide or RNA (data not shown). Although CD80 is often low in DCs cultured with GM-CSF and IL-4, we observed variable CD80 expression before exposure of DC to CD40L (37–53% positive), but in all cases the percentage of DCs expressing CD80 increased after CD40L exposure. The expression of HLA-DR, CD86, and CD1a were not altered by CD40L treatment. We also observed an increase in the allostimulatory activity of DCs after exposure to CD40L, as determined in a MLR (Fig. 2).

We have shown previously that DCs loaded with the CEA peptide CAP-1 are highly effective in stimulating a primary CAP-1-specific CTL response *in vitro* (13). To test whether the sequence of loading and maturation affects the potency of the T-cell response, we evaluated the ability of DCs loaded with CAP-1 before and after the application of CD40L to stimulate a CAP-1-specific CTL response (Fig. 3A). CD40L-matured DCs induced T cells with greater CAP-1 specific lytic activity (as judged by the lysis of CEA peptide, but not HCV peptide, pulsed target cells) if peptide pulsing was performed after maturation. Addition of the CD40L after loading with CAP-1 resulted in no improvement in CTL stimulation over that of DCs, which were not exposed to CD40L.

DCs transfected with CEA RNA induced CEA-specific T-cell responses comparable with CEA peptide-pulsed DCs (15). Because CEA RNA must first be taken up by the DCs, followed by translation and processing of the CEA protein, we hypothesized that the sequence

of RNA transfection and maturation would have a significant effect on the potency of the T-cell response induced. We have demonstrated previously that “immature” DCs transfected with CEA RNA and then treated with TNF- α were significantly more potent stimulators of CEA-specific CTLs than DCs treated with TNF- α before transfection with RNA (15). Therefore, we evaluated the ability of DCs transfected with CEA RNA before and after exposure to CD40L to stimulate a CEA-specific CTL response (Fig. 3B). CD40L-matured DCs induced T cells with greater CEA-specific lytic activity (as judged by the lysis of CEA RNA, but not GFP RNA, transfected target cells) if the CEA RNA transfection was performed before maturation. Addition of the CD40L before CEA RNA transfection resulted in similar CTL stimulation as that of DCs that were not exposed to CD40L.

Clinical trials using CAP-1 peptide and CEA RNA-loaded DCs to

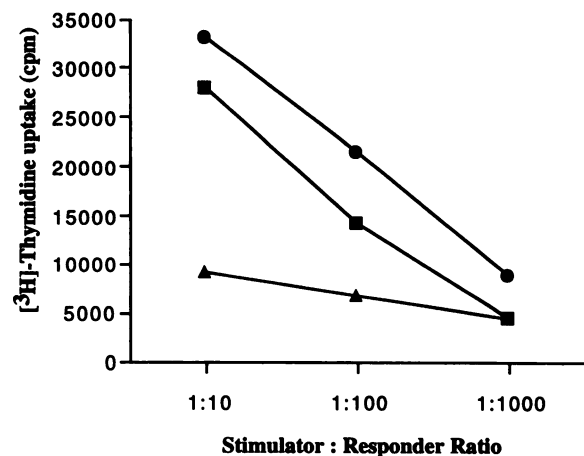
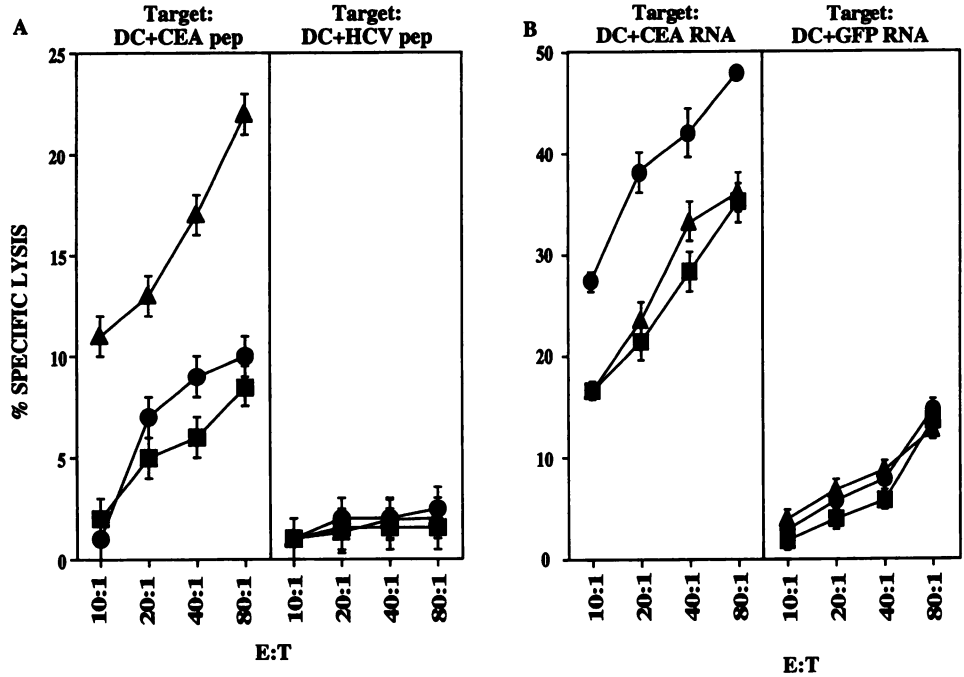


Fig. 2. Allogeneic MLR of DC before and after exposure to CD40L. Allogeneic PBMCs were cocultured with DCs, and the proliferation of the allogeneic T cells was measured by [3 H]thymidine incorporation. DCs matured with CD40L (●) were more potent allostimulators than immature DCs (■) and PBMCs (▲).

Fig. 3. Stimulatory activity of DCs pulsed with CEA peptide or transfected with CEA RNA before and after CD40L exposure. In A, DCs loaded with CEA peptide without exposure to CD40L (■) and before (●) and after (▲) 18 h of incubation with CD40L were used as stimulators of autologous PBMC. Autologous DCs loaded with CAP-1 or HCV peptide were used as targets. Data are representative of three experiments; bars, SE. In B, DCs transfected with 10 μ g of IVT CEA RNA alone (■) and before (●) and after (▲) 18 h incubation with CD40L were used as stimulators of autologous PBMCs. Autologous DCs loaded with CEA or GFP RNA were used as targets. Data are representative of three experiments; bars, SE.



stimulate antitumor immunity are ongoing for patients with metastatic malignancies expressing CEA, but the most potent active immunotherapy strategy remains to be determined. To apply the observations regarding the increased potency of T-cell responses induced by CD40L-matured DCs to clinical settings, it is necessary to evaluate *in vitro* the function of DCs derived from cancer patients and matured with CD40L. Because tumor-bearing hosts often have increased levels of cytokines and other factors that may affect DC generation such as vascular endothelial growth factor (16), it is also necessary to demonstrate that the observed maturational effects are specific to CD40L by evaluating the induction of antigen-specific CTLs by DCs exposed to CD40L in the presence and absence of neutralizing anti-CD40L monoclonal antibody. DCs loaded with CAP-1 peptide (Fig. 4A) or CEA RNA (Fig. 4B) before and after CD40L administration were incubated with and without anti-CD40L (simultaneously with the

CD40L) and were then cultured with autologous PBMCs to stimulate antigen-specific T cells. The results for one representative patient are presented in Fig. 4. For all three patients, who had received prior therapy for their metastatic malignancies (colon and breast cancer), the results are similar to those of normal individuals. Thus, CD40L treatment can potentially enhance the stimulatory activity of PBMC-derived DCs pulsed with peptide or transfected with RNA in patients with metastatic cancer. Importantly, Fig. 4 demonstrates that anti-CD40L abrogated the increased potency of the cytotoxic T cells induced by the DCs, regardless of the timing of the CD40L addition or the antigen used.

In this study, we demonstrate that the optimal sequence of antigen loading and maturation of DCs generated under clinically relevant conditions depends on the form of the antigen used. We hypothesized that CAP-1 peptide is not necessarily taken up or processed by the

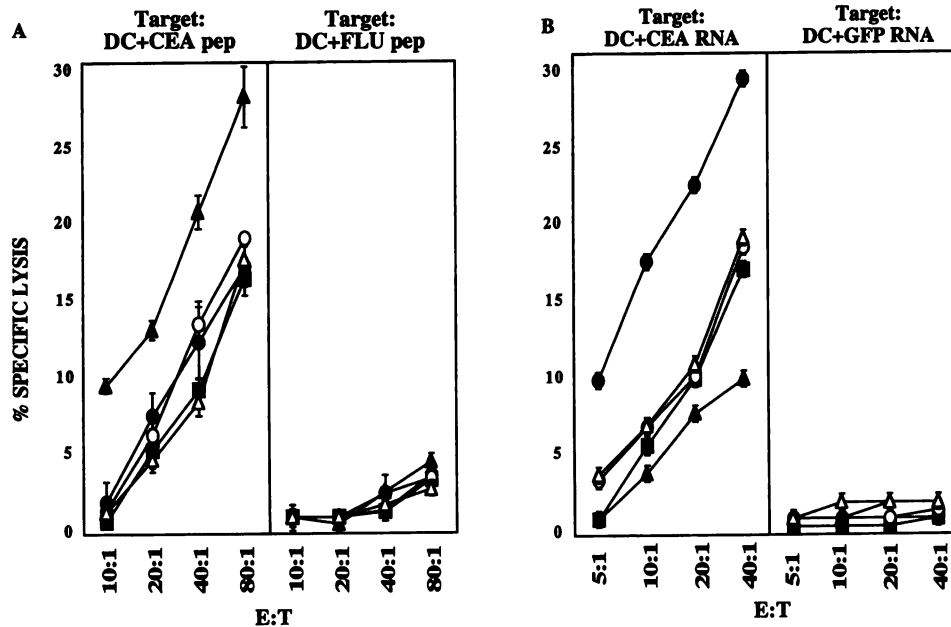


Fig. 4. DCs generated from a cancer patient and matured with CD40L stimulate CEA-specific CTL responses *in vitro*. In A, DCs from an HLA-A2⁺ patient with metastatic colon cancer were generated in serum-free AIM V in the presence of GM-CSF and IL-4 and then incubated with CEA peptide alone (■) and before (●) and after (▲) exposure to CD40L. The DCs were used to stimulate autologous PBMCs, and after two rounds of stimulation, the CD8⁺ T cells were tested for CTL activity against a CEA-expressing target. In B, DCs from an HLA-A2-negative individual with metastatic, CEA-expressing breast cancer were generated as described above and loaded with CEA RNA alone (■) and before (●) and after (▲) exposure to CD40L. Autologous DCs transfected with CEA RNA or GFP RNA were used to measure CEA-specific lysis. DCs coincubated with 1 mg/ml CD40L and 5 mg/ml anti-CD40L before (Δ) and after (○) loading with CAP-1 peptide or CEA RNA were used as stimulators of autologous PBMCs.

DCs but rather binds to and stabilizes HLA molecules expressed at the surface of the DC. Consistent with this hypothesis is the observation that maturation of DCs before peptide loading induces a more potent CTL response (Figs. 3A and 4A) than maturation after peptide loading or when maturation was not induced at all. Importantly, we hypothesized that the efficient uptake of RNA by DCs is a reflection of their degree of immaturity because immature DCs are the most active in uptake of macromolecules via fluid phase macropinocytosis or a variety of receptors (17–19). In support of this hypothesis is our demonstration that maturation of DCs by incubation with CD40L increased the CTL potency only after RNA exposure. Surprisingly, application of CD40L prior to addition of the CEA RNA did not lead to a reduction of CTL potency compared with DCs that had never been exposed to CD40L. This suggests that CD40L-matured DCs are still able to take up RNA, or that the maturation effected in the clinically relevant condition of serum-free media is incomplete. The latter is supported by the demonstration of incomplete up-regulation of CD80 and CD83. This maturation effect was specific to CD40L binding to the DCs and could be abolished by the addition of a neutralizing antibody for CD40L (Fig. 4). The ability to recapitulate our findings in patients with advanced malignancies (Fig. 4) under clinically relevant conditions suggests a strategy for improving the induction of antitumor immunity. The results of the MLR are consistent with the observation that CD40L up-regulates costimulatory molecules but is in contrast to a study (20) that found that CD40L leads to a decrease in T-lymphocyte proliferation. In our study, DCs were exposed to CD40L for 18 h and washed prior to use as stimulators, whereas in the study of McLellan *et al.* (20), it was present in the media during the entire MLR.

In summary, strategies that rely on maturation of DCs with CD40L will need to consider the temporal relationships of antigen loading and maturation signals to achieve optimal induction of tumor-specific T-cell responses in cancer patients.

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