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Regulation of Melanoma Epitope-specific Cytolytic T Lymphocyte Response by Immature and Activated Dendritic Cells, *in Vitro*[1]

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ABSTRACT

Dendritic cell (DC)-based immunization in cancer has proven to be a promising approach. However, just as DCs are crucial accessory cells in generating immune responses, they also seem to participate in tolerance induction, especially against peripheral "self" antigens. The bulk of the evidence that DCs present peripheral self antigens to induce tolerance has, however, come mostly from studies in transgenic animal models. A tolerogenic function of DCs for peripheral self antigens in a human model has not been critically examined. In this study using the Melan-A/MART-$1_{27–35}$ peptide as a model for self but melanoma-associated antigen–against which human hosts often harbor $CD8^+$ CTL precursors with high frequencies–we confirm that although immature dendritic cells (iDCs) are inefficient antigen presenting cells (APCs), fully activated DCs efficiently activate melanoma epitope-specific $CD8^+$ CTL precursors, *in vitro*. We, however, show that in a direct epitope presentation schema, iDCs neither delete nor anergize epitope-specific $CD8^+$ T cells in primary or secondary stimulation. Interestingly, iDCs and activated DCs can delete a large fraction of the epitope-specific CTLs on tertiary stimulation. The deletion is induced in an epitope-specific manner and through apoptosis. These observations, therefore, have implications on the DC-based cancer vaccine designs and are relevant in the inquiry into the role of DCs on tolerance induction.

INTRODUCTION

DCs[3] play a critical role in the regulation of immune responses (1). For experimental and translational purposes, DCs can be generated, *ex vivo*, from a variety of precursors, and they can be "matured" in a number of DC-maturing protocols (2). As such, *ex vivo* grown DCs have become a useful tool in translational cancer immunotherapy studies, especially for therapeutic immunization with tumor-associated peptide epitopes (3, 4). In the human melanoma model, we had initially demonstrated biological activities with peptide-loaded and tumor lysate-fed monocyte-derived APCs grown in GM-CSF by direct as well as cross-presentation (5, 6). Using *ex vivo* generated DCs, others have also documented both biological as well as clinical responses (7–9). The DC-based approaches to immunotherapy for cancer, therefore, have proven to be promising strategies. DC biology has, however, turned out to be quite complex, *e.g.*, just as DCs play a pivotal role in generating productive effector immune responses, they also induce peripheral "tolerance" (10–14). Furthermore, the great majority of tumor-associated epitopes are essentially "self" epitopes, and DCs have been implicated in presenting peripheral self antigens for tolerance induction (12–14). The evidence that DCs present self antigens to induce tolerance has, however, been mostly demonstrated in transgenic animal models, and the tolerogenic potential of DCs in humans has only been addressed using a foreign antigen, such as influenza (15, 16). A "regulatory" or "tolerogenic" role of DCs in a human model of peripheral self antigen has not been carefully examined.

Because a considerable interest persists on vaccination studies in cancer with DCs and many self epitopes, we examined the context in which DCs might exert regulatory roles in controlling the generation of $CD8^+$ T-cell responses, *in vitro*, to a self and melanoma-associated antigen, Melan-A/MART-1 (17, 18), against which human hosts harbor $CD8^+$ CTL precursors in surprisingly high frequencies (19, 20). Working with monocytes as DC precursors and $CD8^+$ T cells from $HLA\text{-}A2^+$ patients and healthy donors harboring HLA-A2-restricted MART-$1_{27–35}$ epitope-specific CTL precursors, and using IFN-γ response, cytotoxicity, and peptide/MHC tetramer staining as readouts of activation and expansion or contraction, we confirm that although immature DCs are inefficient APCs, fully activated mature DCs efficiently activate MART-$1_{27–35}$ epitope-specific CTL, *in vitro*. We, however, find that iDCs neither delete nor anergize naive or antigen-experienced epitope-specific $CD8^+$ T cells in primary or secondary stimulation, *in vitro*. Interestingly, iDCs and actDCs can delete a large fraction of the epitope-specific CTLs on tertiary stimulation. The deletion is induced in an antigen-specific manner and involves apoptosis. These observations provide a different perspective on the role of DCs in the tolerance induction to self antigens and have practical implications on monocyte-derived, DC-based cancer vaccine designs.

MATERIALS AND METHODS

Study Population. The study population consisted of HLA-A 2-positive melanoma patients or healthy donors. The participants were included in this study with informed consent.

Culture Medium and Reagents. The MART-$1_{27–35}$ peptide (AAGIGILTV) and MAGE-$3_{271–279}$ (FLWGPRALV) was purchased from Multiple peptide systems (San Diego, CA), whereas β2-microglobulin was purchased from Sigma (St. Louis, MO). Culture medium consisted of Iscove's modified Dulbecco's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Inc., Calabasas, CA), 0.55 mM L-arginine, 0.24 mM L-asparagine (both from Life Technologies, Inc., Grand Island, NY), 1.5 mM L-glutamine (Sigma), 50 units/ml penicillin, and 50 μg/ml streptomycin (both from Abbott Laboratories, North Chicago, IL). This will be referred to as complete medium. The TAP-deficient line, T2, with empty surface HLA-A2 molecules, was used as targets to present peptides for the epitope-specific effectors. Recombinant human GM-CSF was purchased from Immunex (Seattle, WA). rhIL-4, rhIL-2, and rhIFN-γ were purchased from R&D Systems, Inc. (Minneapolis, MN). LPS from *Escherichia coli* 055:B5 was purchased from Sigma. Trimeric CD40L was a gift of Immunex. Annexin V kit was purchased from R&D Systems, Inc. Mart-$1_{27–35}$ (ELAGIGILTV) tetramer labeled with phycoerythrin with and without FITC-labeled anti-CD8 was purchased from Beckman Coulter, Inc. (Fullerton, CA). Flurochrome-labeled monoclonal antibodies to CD14, CD40, CD80, CD83, CD86, MHC class I, and MHC class II were purchased from BD Biosciences (San Jose, CA).

Generation of Dendritic Cells from Peripheral Blood. The original procedure of Sallusto *et al.* (21) and our minor modifications were followed to

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[3] The abbreviations used are: DC, dendritic cell; APC, antigen-presenting cell; iDC, immature dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; actDC, activated dendritic cell; FACS, fluorescence-activated cell sorter; IL, interleukin; LPS, lipopolysaccharide; rhIL, recombinant human interleukin.

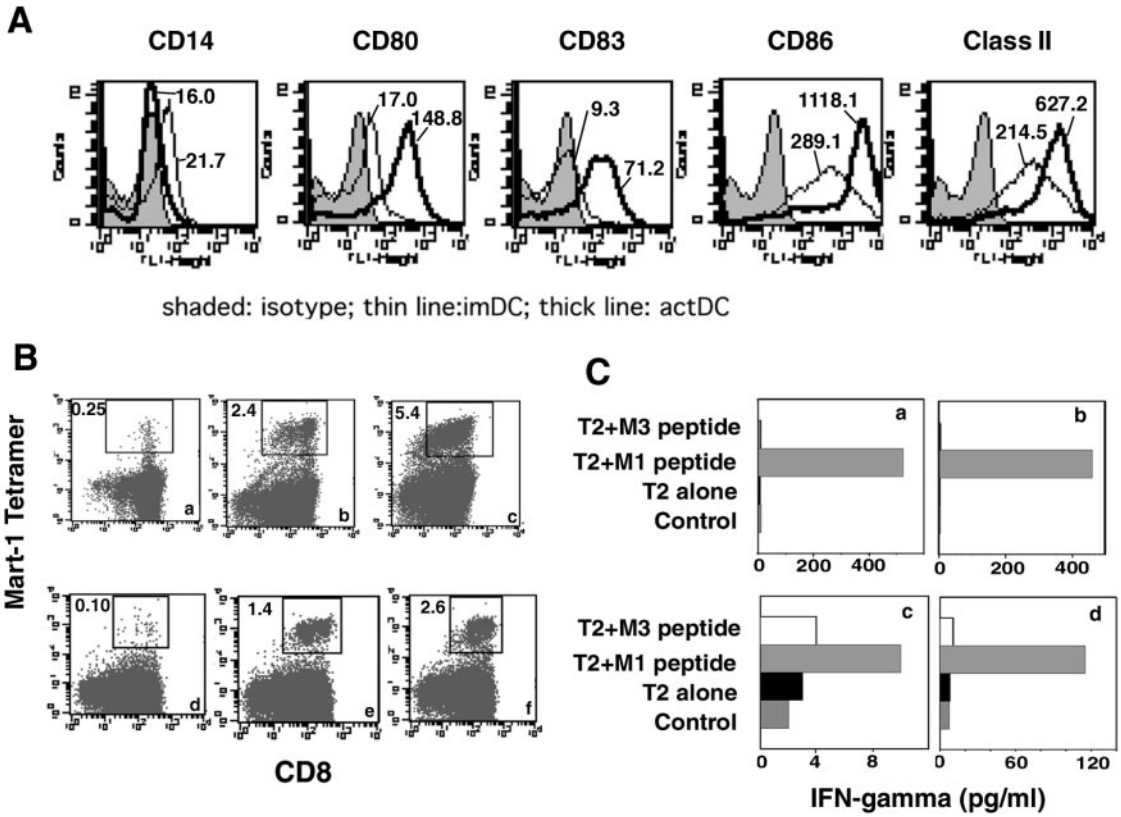

Fig. 1. Activation of MART-$1_{27–35}$ epitope-specific CTLs by immature and activated dendritic cells, *in vitro*. *A*, a comparison of different cell surface markers expressed by iDCs and actDCs is shown. Figures indicate mean fluorescence intensity (*MFI*) of the respective markers expressed by the two DC types. *B*, expansion of MART-$1_{27–35}$ epitope-specific CTLp primed by iDCs and actDCs. $CD8^+$ T cells cocultured with the MART-$1_{27–35}$ epitope (100 μg/ml) loaded DCs in the presence of 50 units/ml IL-2 were analyzed for MART-$1_{27–35}$ peptide complexed HLA-A2 tetramer-positive population on day 10. Numbers in the figure indicate the tetramer-positive gated cells. *Top panel*, healthy donor. Number of tetramer-positive cells in: (*a*) fresh $CD8^+$ population; (*b*) iDC-primed population; (*c*) actDC-primed population. *Bottom panel*, a melanoma patient. Number of tetramer-positive cells in: (*d*) fresh $CD8^+$ population; (*e*) iDC-primed population; (*f*) actDC-primed population. *C*, IFN-γ response by the $CD8^+$ effector cells primed by MART-$1_{27–35}$ epitope-loaded iDCs and actDCs. The $CD8^+$ T cells primed by the MART-$1_{27–35}$ epitope-loaded DCs from a healthy donor (*top panel*) and melanoma patient (*bottom panel*) were tested for IFN-γ response against the peptide-loaded T2 cells. Twenty-four-h supernatants were assayed for IFN-γ in ELISA. *a* and *c*, primed by iDCs; *b* and *d*, primed by actDCs. Both iDCs and actDCs were pulsed with MART-$1_{27–35}$ epitope in all experiments. M1 peptide, MART-$1_{27–35}$; M3 peptide, MAGE-$3_{271–279}$ peptide.

generate myeloid dendritic cells from peripheral blood. Briefly, circulating monocytes were isolated by 2-h adherence of Ficoll-Hypaque density gradient-cut peripheral blood mononuclear cells as described previously (22). The adherent cells were cultured in complete medium with 1000 units/ml GM-CSF and 500 units/ml IL-4 for 5–7 days to obtain a population of iDCs. To obtain fully activated DCs, iDCs were further primed in IFN-γ (1000 units/ml) for 2 h and then matured in 100 ng/ml LPS and trimeric CD40L (5 μg/ml).

IFN-γ Response Assay. IFNγ response by the effector cells was determined by coculturing 1×10^4 effector cells with 1×10^4 peptide-pulsed T2 cells as described previously (22). T2 cells were pulsed with 1 μg/ml the relevant Mart-1 HLA-A2 restricted peptide or an irrelevant HLA-A2 restricted MAGE-3 peptide. After 4–16 h, culture supernatants were harvested, and IFN-γ concentrations were measured by sandwich ELISA as per manufacturer's protocol (Immunotech, Marseilles, France).

FACS Analysis. Dual color FACS analysis was done using phycoerythrin or FITC-labeled antibodies specific to cell surface markers along with an isotype-matched control. Labeled cells were collected using a FACSCalibur cytofluorometer (Becton Dickinson, Mountain View, CA), and analysis was done with CellQuest software (Becton Dickinson).

Tetramer staining and analyses (23) were carried out as per the manufacturer's protocol. Briefly, effector cells were washed twice in PBS and then incubated with 10 μl of MART-$1_{27–35}$ peptide-conjugated HLA-A0201 tetramer at room temperature for 30 min. The effector cells were again washed twice, resuspended in FACS buffer. Thereafter, the number of tetramer-positive cells was determined by flow cytometry using a FACSCalibur, and the acquired cytofluorographic data were analyzed using the CellQuest software.

Microcytoxicity Assay. The chromium release microcytotoxicty assay described previously (24) was performed with the following modification: Effector: ^{51}Cr-labeled target cell interactions in each well were carried out in the presence of 10-fold excess of unlabeled K-562 cells as an irrelevant control target.

Activation of $CD8^+$ T Cells by DC-based Presentation of Epitopes, *in Vitro*. $CD8^+$ T cells were isolated from Ficoll-Hypaque gradient separated blood mononuclear cells by Dynal magnetic bead isolation kits (Dynal, Oslo, Norway). The purity of $CD8^+$ T cells was routinely in excess of 90%. Primary stimulation of MART-1-specific CTL precursors (CTLp) was done by coculturing purified $CD8^+$ T cells with iDCs or actDCs loaded with the relevant peptides [100 μg/ml the HLA-A2 determined MART-$1_{27–35}$ peptide (AAGIGILTV) and 5 μg/ml β2 microglobulin] at a $CD8^+$ T cell:DC ratio of 100 in the presence of rhIL-2 (50 units/ml). Subsequent secondary and tertiary stimulations were done in the similar manner by using primed CTLs according to the experimental design. Before setting up cocultures, the DCs were irradiated to 3000 rad.

RESULTS

Activation of MART-$1_{27–35}$ epitope-specific CTLs from normal donors and melanoma patients by immature as well as matured DCs. Numerous investigators have shown that MART-$1_{27–35}$ epitope or other tumor-associated epitope-specific CTLp can be readily activated to functional CTLs in *in vitro* cocultures with peptide-loaded dendritic cells that are matured in a variety of DC-maturation protocols. It has also been shown that iDCs can also activate CTLs to a certain extent (25–27). iDCs, however, are generally inefficient in priming T cells (1, 2). We have also studied CTL response to the MART-$1_{27–35}$ epitope and other melanoma-associated epitopes, *in vitro*, and have found that although iDCs are indeed inefficient APCs relative to their

Table 1 *Expansion of MART-$1_{27–35}$ epitope-specific circulating precursors after priming with the MART-$1_{27–35}$ peptide loaded autologous iDCs and actDCs, in vitro*[a]

Subjects	No. of MART-$1_{27–35}$/HLA-A2 tetramer-positive cells		
	In the fresh population	After primed by iDCs	After primed by actDCs
UC51	0.25	2.4	5.4
UC52	0.30	3.8	7.8
UC53	0.60	1.1	1.9
UC54	0.24	3.8	7.4
UC55	0.29	2.4	4.3
UC56	0.34	5.0	12.3

[a] Dynal bead-isolated $CD8^+$ T cells were cocultured with the MART-$1_{27–35}$ peptide-loaded autologous DCs ($CD8^+$ T cells: DC = 100) in 48-24-well cluster plates in the presence of 50 units/ml rhIL-2. The cultures were fed every 3^{rd} day. Tetramer assay was carried out between d10 and 14. UC 54 and UC 56, melanoma patients.

matured counterparts, in general, CTLp from normal donors and melanoma patients for the MART-$1_{27–35}$ epitope or other melanoma epitopes can be activated to varied degrees by both immature and matured DCs, *in vitro* (collective data not shown). The MART-$1_{27–35}$ epitope is a self-epitope, yet CTLp for this epitope are often found at high frequencies in normal healthy donors as well as in patients with melanoma (19, 20). Now that DCs appear to have a regulatory role in the generation of immune response to self-epitopes, and as iDCs are increasingly implicated in tolerance induction with peripheral self antigens, we examined the "immunogenic" *versus* "tolerogenic" properties of these two types of DCs in a DC-based CTL activation assay system using the MART-$1_{27–35}$ epitope as a model for peripheral self but tumor-associated antigen. MART-$1_{27–35}$ epitope-specific naïve precursors (*i.e.,* derived from healthy donors; Ref. 20) as well as MART-$1_{27–35}$ epitope experienced precursors (*i.e.,* derived from melanoma patients who have been immunized with the MART-$1_{27–35}$ peptide pulsed autologous GM-CSF cultured monocyte derived APCs; Ref. 5) were primed by both DC types and then restimulated by both DC types in crossover protocol to evaluate the "immunogenic" *versus* "tolerogenic" properties of the respective DCs during priming as well as amplification phase of the responses, *in vitro*.

The representative phenotypic characteristics of the iDCs and actDCs that have been used in these studies and relative capacity of the iDCs and actDCs in activating the MART-$1_{27–35}$ epitope-specific precursors from a normal healthy donor and melanoma patient are shown in Fig. 1. Adherent monocytes cultured in GM-CSF and IL-4 (iDCs) expressed very little CD83 on their surface and only moderate levels of MHC class II molecules and costimulatory molecules. Additional maturation of iDCs in IFN-γ, LPS, and trimeric CD40L (28) led to the expression of CD83 on surface and much higher levels of the relevant antigen presenting and costimulatory molecules (Fig. 1*A*). The relative capacity of the two DC types to activate the MART-$1_{27–35}$ epitope-specific $CD8^+$ T-cell precursors can be seen in Table 1 and Figs. 1, *B* and *C*. Both types of DCs were capable of expanding the epitope-specific, population derived from both healthy donors and melanoma patients to varied degrees (Table 1 and Fig. 1*B*). The populations expanded by iDCs or actDCs did not however exhibit uniform functional activation (Fig. 1*C*). Although the MART-$1_{27–35}$ epitope-specific precursors derived from a healthy donor exhibiting a 10-fold expansion after primary stimulation with the iDCs (Fig. 1*Bb*) were functional in IFN-γ response assay (Fig. 1*Ca*), a similar 10-fold expansion of the precursors from a melanoma patient (Fig. 1*Be*), who has been vaccinated with the MART-$1_{27–35}$ epitope-loaded GM-CSF-activated adherent monocytes (5),[4] was not (Fig, 1*Cc*). These effectors, however, became functional after secondary stimulation (shown later in Fig. 2). Clearly, neither DC type deleted the CTLp after *in vitro* cocultures (Table 1 and Fig. 1*B*). Indeed, the MART-$1_{27–35}$ epitope-specific CTL precursors derived from all donors (four healthy donors and two melanoma patients) harboring CTLp for this epitope at high frequencies were expanded (and not deleted) when they were primed by both DC types, *in vitro* (Table 1).

Effects of Secondary and Tertiary Stimulation by DCs on CTL Expansion and Function. Repetitive stimulation of $CD8^+$ T cells by tumor antigens or peptide-pulsed APCs has been a common practice to generate antigen-specific CTLs, *in vitro*, and repetitive vaccination with antigens, peptides, or peptide-pulsed DCs at various arbitrary intervals, has also been a common practice in the field. Although the assumption has been that such repetitive stimulation would lead to a robust response, the effect of repetitive stimulation on the kinetics of the CTL response to a self peptide has not been carefully examined. Accordingly, we asked how repetitive presentation of the MART-$1_{27–35}$ epitope affected the amplification and contraction of the CTL response. The effect of secondary restimulation by both types of DCs on the epitope-specific CTLp primed in DC-based primary cocultures is shown in Fig. 2. The activated CTLs, whether primed by iDC or actDCs, were neither deleted (Fig. 2*A*) nor did they become functionally

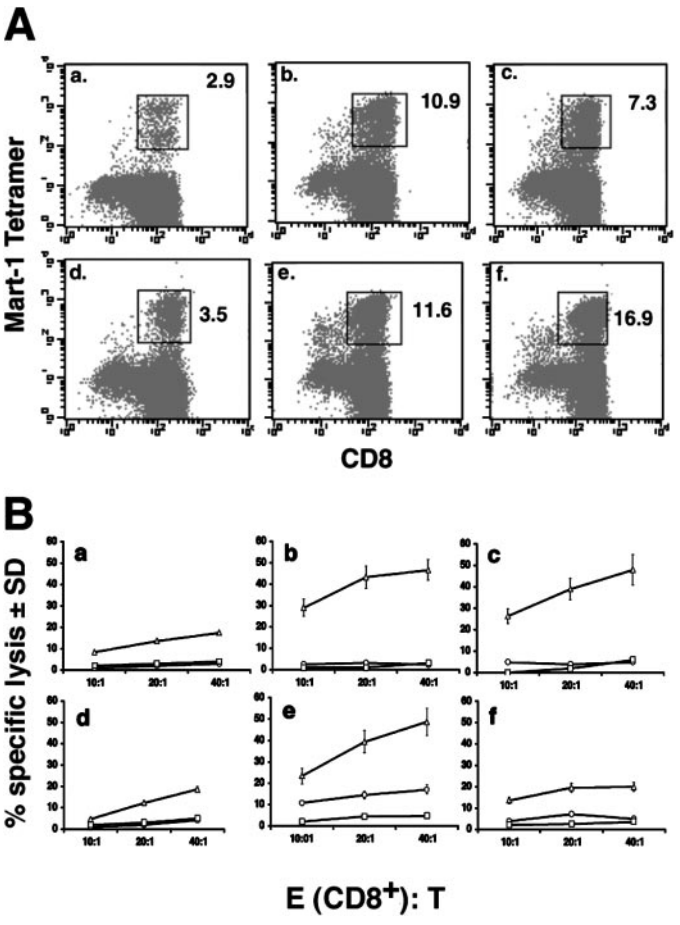

Fig. 2. Effect of secondary stimulation of the MART-$1_{27–35}$ epitope-primed CTLs, *in vitro*. *A*, number of tetramer-positive population (from normal healthy donor UC 51) after crisscross secondary stimulation of the primed $CD8^+$ T cells. *Top*, iDC-primed population: *a*, nonrestimulated; *b*, restimulated by iDCs; *c*, restimulated by actDCs; *bottom*, actDC-primed population: *d*, nonrestimulated; *e*, restimulated by iDCs; *f*, restimulated by actDCs. *B*, cytolytic response of the preprimed $CD8^+$ T cells after secondary stimulation. *Top*, iDC primed CTL: *a*, nonrestimulated; *b*, after restimulation with iDC; *c*, after restimulation with actDC. *Bottom*, actDC primed CTL: *d*, nonrestimulated; *e*, after restimulation with iDC; *f*, after resimulation with actDC. Both iDCs and actDCs were pulsed with MART-$1_{27–35}$ epitope in all experiments for priming and restimulation. Symbols representing: ○, T2 cells alone; □, T2 cells pulsed with MAGE-$3_{271–279}$ peptide; △, T2 cells pulsed with MART-$1_{27–35}$ peptide.

[4] Chakraborty, N. G., Mehrotra, S., Stevens, R., Mukherji, B., unpublished data.

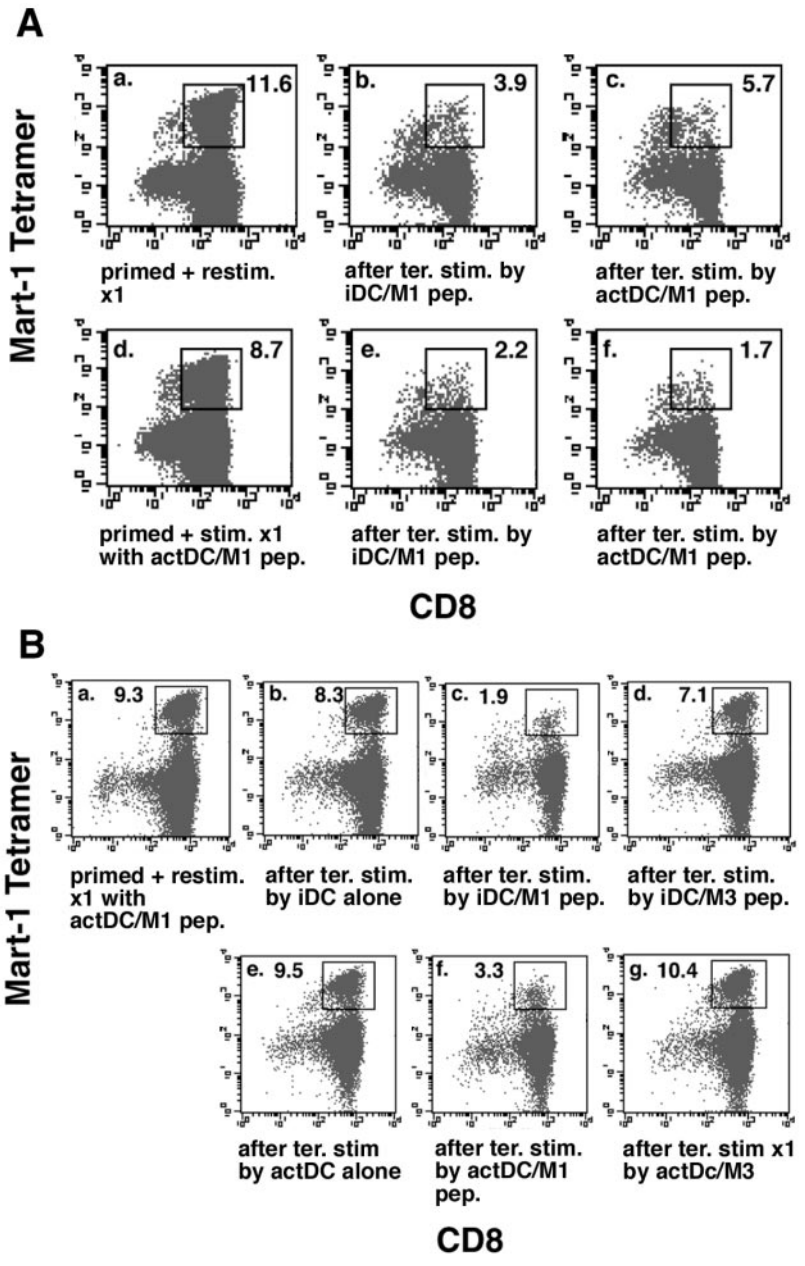

Fig. 3. Effect of tertiary stimulation of the MART-$1_{27–35}$ epitope-specific CTLs *in vitro*. *A*, number of tetramer-positive population (from normal healthy donor UC 51) after crisscross tertiary stimulation. *Top*, population primed and stimulated once with iDC: *a*, nonrestimulated; *b*, restimulated by iDCs; *c*, restimulated by actDCs. *Bottom*, population primed and stimulated once with actDC: *d*, nonrestimulated; *e*, restimulated by iDCs; *f*, restimulated by actDCs. *B*, antigen specificity in deletion on tertiary stimulation. The actDC-primed population that was stimulated in secondary stimulation was subjected to another round of stimulation (tertiary stimulation) in a crisscross manner with iDCs (*top panel*) and actDCs (*bottom panel*) pulsed with the relevant (MART-$1_{27–35}$ epitope) and irrelevant epitope (MAGE-$3_{271–279}$ epitope). Cytofluorometric analysis for the number of tetramer-positive cells was done at 24 h. Population primed and stimulated once with actDC: *a*, nonrestimulated; *b*, restimulated by iDC alone; *c*, restimulated by MART-$1_{27–35}$ pulsed iDCs; *d*, restimulated by MAGE-$3_{271–279}$ pulsed iDCs; *e*, restimulated by actDC alone; *f*, restimulated by MART-$1_{27–35}$ pulsed actDCs; *g*, restimulated by MAGE-$3_{271–279}$ pulsed actDCs.

unresponsive (Fig. 2*B*) when they were restimulated by either types of DCs in a cross-over protocol (*i.e.*, CTLp primed with iDCs and actDCs and then each population restimulated by both). The MART-$1_{27–35}$ epitope specific populations, whether primed by iDCs and actDCs, expanded further following secondary stimulation by either type of DCs (Fig. 2*A*). The number of tetramer-positive cells in the populations and the overall lytic function of some of the effector cells (in terms of total $CD8^+$ T cells) that were restimulated in cross-over manner, however, did not show perfect concordance (Fig. 1, *Ac*, *1Af* and *1Bc*, and *1Bf*). The activated effectors, nonetheless, were cytolytic in a MART-$1_{27–35}$ epitope-specific manner (Fig. 2*B*), and they continued to exhibit IFN-γ responsiveness after secondary restimulation (data not shown).

When the preprimed and restimulated CTLs were subjected to tertiary rounds (*i.e.*, three to four rounds) of restimulation, substantial deletion of the epitope-specific CTLs was observed, regardless of the type of DCs used for restimulation (Fig. 3*A*). The deletion was induced in an epitope-specific manner (Fig. 3*B*), and it was accompanied by some diminution of function (Table 2). Although the degree of deletion noted with tertiary stimulation by iDCs or actDCs differed somewhat (Fig. 3, *A* and *B*), we did not observe any major difference in the magnitude of deletion induced by either type of DCs nor did we detect any relationship between the deletion and the dose of the peptide as significant levels of deletion took place with as low as 0.1 μg/ml peptide (data not shown). This type of deletion was usually seen after three or four rounds of stimulation, and such tertiary stimulation did not always induce total deletion or complete functional loss in the effector population. Although the surviving population continued to exhibit detectable levels of IFN-γ response for some time, additional stimulation usually did not significantly prolong their life or function. When the primed population was maintained in continuous culture in low dose IL-2 (10 units/ml) without additional stimulation, the epitope-specific cells survived longer (collective data not shown). When this population was restimulated long after priming (*e.g.*, on day 80), they also expanded and exhibited functional amplification (Fig. 4).

Table 2 *Function of the MART-1$_{27–35}$-specific preprimed CTLs after tertiary stimulation*[a]

Effector population	IFN-γ (pg/ml) secreted by the CTLs when stimulated by			
	None	T2	T2/M-1 peptide	T2/M-3 peptide
Primed and restimulated by iDCs	10	7	525	10
After tertiary stimulation by iDCs	105	70	365	40
After tertiary stimulation by actDCs	25	20	210	20
Primed and restimulated by actDCs	5	5	460	7
After tertiary stimulation by iDCs	100	50	220	50
After tertiary stimulation by actDCs	125	65	90	40

[a] Dynal bead-isolated CD8$^+$ T cells primed by the MART-1$_{27–35}$ peptide pulsed iDCs or fully actDCs were restimulated for two rounds with the two DC types in a crisscross manner. Five days after the third round of stimulation, the effectors were tested for function in IFN-γ response assay against the relevant (MART-1$_{27–35}$) and irrelevant peptide (HLA-A2 restricted MAGE-3$_{271–279}$) peptide pulsed T2 cells. Supernatants (24 h) were assayed for IFN-γ in ELISA. Note that tertiary stimulation induced some nonspecific activation. M-1 peptide, MART-1$_{27–35}$; M-3 peptide, MAGE-3$_{271–279}$.

DISCUSSION

As pointed out earlier, others have shown that iDCs can activate CTL response in direct as well as in cross-presentation mode (25–27). Among these, Jonuleit *et al.* (Ref. 27 compared the immunogenicity of melanoma peptide loaded iDC- and mature DC-based vaccination, *in vivo,* and found that mature DCs induced superior results (IFN-γ-producing CD8$^+$ T cells was observed in 5 of 7 patients vaccinated with mature DCs, whereas similar response in patients vaccinated with iDCs was observed only in 1 of 7 patients). They further observed that although iDC-based vaccination resulted in some expansion of peptide-specific T cells, such vaccination did not induce peptide-specific cytolytic T cells. Our results are in agreement with the results of Jonuleit *et al.*, in part, *e.g.*, our studies show that when iDCs are made to present the MART-1$_{27–35}$ epitope directly are not inherently tolerogenic. In fact, primary stimulation with iDCs induced varied degrees of expansion as well as function (Fig. 1 and Table 1). After a second round of stimulation (and in some cases even after a third round of stimulation), whether by iDCs or actDCs, CD8$^+$ T cells primed by both types of DCs remain functional (including lytic function) and even undergo further expansion (Fig. 2). However, both iDCs and actDCs delete a large fraction of the epitope-specific CTLs on tertiary stimulation (Fig. 3). The deletion is induced in an antigen-specific manner, and it involves apoptosis (Fig. 4 and 5). This is somewhat in line with previous observations on decisive effects of the concentration of the antigen as well as longevity of antigen presentation on the nature of the response (*i.e.*, immunity *versus* tolerance) and on the quality and kinetics of the response (29–33). Although in the model of acute virus infection, the contraction is unaffected by the presence of antigen (32, 33), in our model, it appears that continued restimulation by DCs, regardless of their maturation status, after the peak has been reached can lead to an accelerated attenuation of the response. Importantly, although the restimulated population died shortly after the third or fourth rounds of stimulation, the activated effectors that were maintained without restimulation lived much longer, and they could be substantially expanded by a late round of secondary stimulation long after priming (Fig. 4).

The evidence that DCs can be "tolerogenic" has been quite compelling in animal models (10–14, 34–38), although the underlying mechanism remains poorly understood. A number of scenarios (different lineage and different states of "maturity" or differentiation) under which they function as tolerogenic APCs has been suggested (10–14, 39). To our knowledge, a "regulatory" or "tolerogenic" role of DCs, of any lineage, maturity, or differentiation, has not been critically examined in a human model of peripheral self antigen. Dhodapkar *et al.* (15) have reported a significant reduction in the influenza matrix protein-specific CD8$^+$ T cells on immunization with NP epitope-loaded iDCs. In contrast, Albert *et al.* (16) have shown that to "cross-tolerize" NP-specific T cells, DCs needed to be matured. These studies, however, dealt with memory response (*i.e.*, to influenza, a foreign and dangerous antigen), and they also differed in methodology (direct *versus* cross-presentation; *in vivo versus in vitro*). Nonetheless, the demonstration of deletion of influenza NP-specific CTL after immunization with iDCs (15) has led to concern (40) because a great deal of interest persists in DC-based immunization in human cancer with essentially "self" but tumor-associated antigens.

As pointed out earlier, the deletion on tertiary stimulation carries the hallmark of AICD, the major mechanism that maintains homeostasis in the system (41, 42). As such, this may not be equated to a "regulatory" function of DCs, *per se*. The basic phenomena of "tolerance" induction in CD8$^+$ T cells by the deletional mechanism in the transgenic animal models has, however, also been associated with activation followed by apoptosis and thus can also be viewed as AICD. Indeed, the bulk of the evidence of a "tolerogenic" role of DCs has come from studies in transgenic models with animals made to express a model antigen as self antigen ectopically, as well as made to express the relevant T-cell receptor for the model epitope or reconstituted with the epitope-specific T cells through adoptive transfers. When stimulated, these T cells undergo an aborted form of activation (*i.e.*, few rounds of proliferation followed by apoptosis). Thus, it is paradoxical that to tolerize through deletion, the T cells need to be first activated. It also raises the question of whether the fundamental process underneath these types of abortive activation represents a "tolerogenic" function of DCs, *per se*, or represents an intrinsic property of T cells that undergo an abortive form of activation,

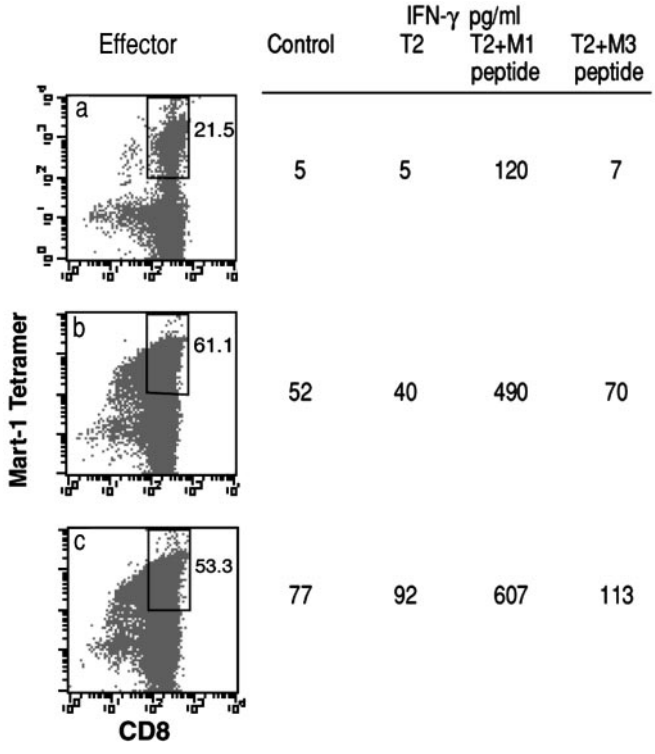

Fig. 4. Effect of secondary restimulation of the preprimed CTLs rested in long-term culture after priming. CD8$^+$ T cells from donor UC 51 primed with MART-1$_{27–35}$ epitope-pulsed actDCs were rested in low dose IL-2 (10 units/ml) in long-term culture and restimulated on day 80. The nonrestimulated and restimulated populations were then analyzed for the number of tetramer-positive cells by cytofluorometry, shown in the dot plot (*top*, nonrestimulated rested population; *middle*, restimulated by MART-1$_{27–35}$ epitope-pulsed iDCs; *bottom*, restimulated by MART-1$_{27–35}$ epitope pulsed act DCs). The IFN-γ response of the effector cells is shown on the *right* in the chart corresponding with the respective populations.

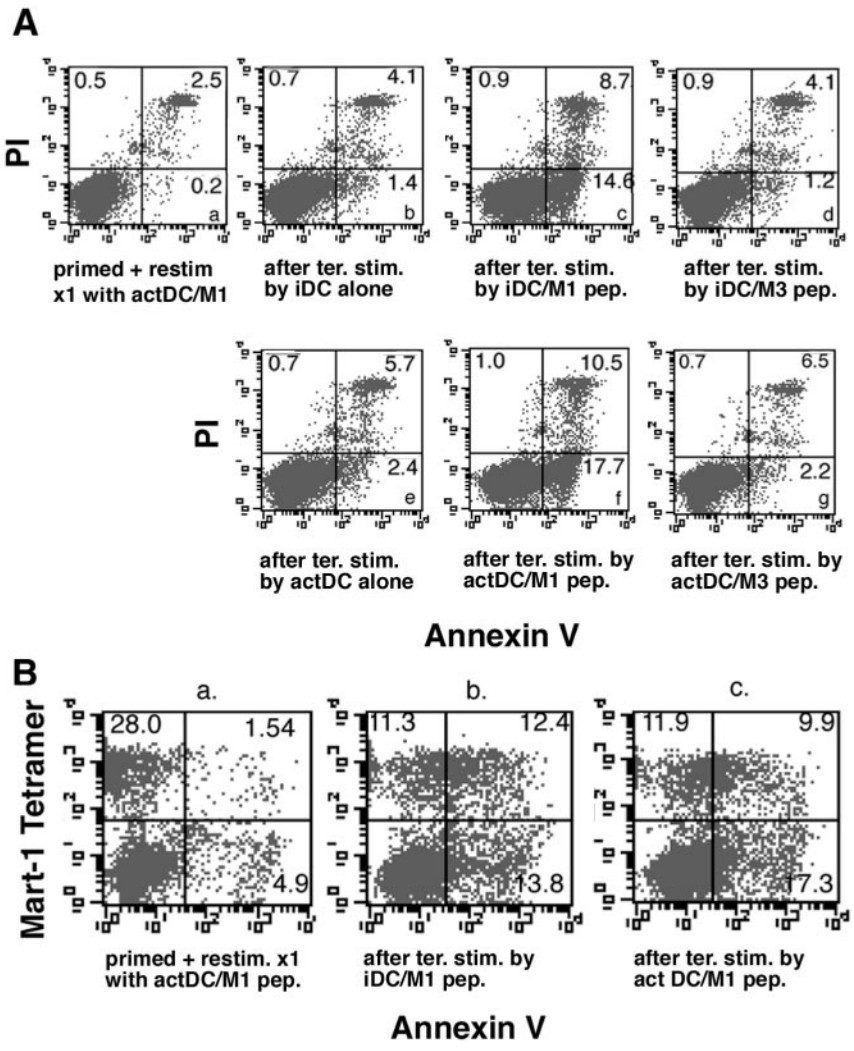

Fig. 5. Induction of apoptosis on tertiary restimulation of MART-$1_{27–35}$ epitope-specific CTLs. In *A*, $CD8^+$ T cells primed by and restimulated once with the MART-$1_{27–35}$ epitope-loaded actDCs were stimulated again (tertiary stimulation) in a criss-cross manner with the two types of DCs (*top*, iDC; *bottom*, actDC) pulsed by the relevant (MART-$1_{27–35}$ epitope) and irrelevant epitope (MAGE-$3_{271–279}$ epitope). Annexin V-FITC/PI staining was carried out 4 h later to determine the number of $annexin^+$ and PI^- cells as cells in early apoptosis. Figures represent the number of cells falling in each quadrant in population. Population primed and stimulated once with actDC: *a*, nonrestimulated; *b*, restimulated by iDC alone; *c*, restimulated by MART-$1_{27–35}$-pulsed iDCs; *d*, restimulated by MAGE-$3_{271–279}$-pulsed iDCs; *e*, restimulated by actDC alone; *f*, restimulated by MART-$1_{27–35}$-pulsed actDCs; *g*, restimulated by MAGE-$3_{271–279}$ pulsed actDCs. *B*, the same $CD8^+$ T cells primed by and restimulated once with the MART-$1_{27–35}$ epitope-loaded actDCs were stimulated again (tertiary stimulation) and then stained with Mart-$1_{27–35}$ tetramer and Annexin V to enumerate for the number of $tetramer^+/annexin^+$-positive cells. Number of tetramer/annexin-positive cells in population: *a*, non-restimulated; *b*, after tertiary restimulation with MART-$1_{27–35}$ epitope-pulsed iDCs; *c*, after tertiary restimulation with MART-$1_{27–35}$ epitope-pulsed actDCs.

perhaps resulting from receiving signal below threshold for sustained expansion or receiving signal in an improper context, such as in the absence of inflammation or "danger" (43).

The lack of "tolerogenic" property of iDCs in our system could be explained, first, if these DCs were not truly quiescent DCs. Then, presently, it is almost impossible to obtain quiescent DCs, and a suitable surrogate for quiescent DCs is also not available for this type of *in vitro* experiment in a human model. It is also unclear how truly quiescent DCs (*i.e.*, resting DCs with antigen presenting machinery unactivated) could present. Second, it could be also explained if our iDCs were not "immature" DCs or might have undergone maturation in the coculture. In this context, it should be mentioned that the various *in vitro* GM-CSF and IL-4-based myeloid DC culture protocols (consisting of serum containing *versus* serum-free medium; FCS *versus* human serum, etc.) do not necessarily generate a uniform DC phenotype, and the distinction between immature and matured DCs based on the levels of the expression of costimulatory molecules, alone, does not truly account for the enormous plasticity within the dendritic APCs (39, 44, 45). The attribute "immature" has essentially been applied to dendritic APCs to define a functional state in which they capture antigen efficiently (hence they need some degree of activation) but do not fully up-regulate the antigen presenting and costimulatory machineries and do not prime T cells efficiently. From this perspective, the iDCs in our study exhibited the phenotype of immature or "semimature" (39) DCs (CD83 negative, moderate levels of costimulatory and MHC molecules, and did not prime that efficiently). Furthermore, we believe that the likelihood of our iDCs undergoing maturation during coculture is an unlikely scenario. They were irradiated to 3000 rad. Nonetheless, we have addressed the issue of the state of "maturity *versus* immaturity" of APCs in priming *versus* tolerance induction by carrying out a number of experiments with freshly adhered monocytes and with 3–5-day-old monocytes cultured in only GM-CSF in serum-free medium (AIMS V medium; Life Technologies, Inc.) and have found that these DC precursors and immature APCs ($CD14^+$, $CD83^-$, $CD80/86^{lo}$, class I/II^+) also do not efficiently prime, but they do not delete the MART-$1_{27–35}$ epitope-specific CTLp *in vitro* either. Although the MART-$1_{27–35}$ peptide loaded "monocytes or macrophages" do not efficiently induce full functional activation of the MART-$1_{27–35}$ epitope-specific precursors, they are able to stimulate them through several rounds of proliferation, measurable in tetramer assay, even in the absence of exogenous IL-2. These proliferating effectors do not, however, survive nor become fully functional CTLs unless IL-2 is added to the culture, suggesting that these APCs deliver a type of subthreshold stimuli that, in the absence of help, induce an abortive activation.[5]

[5] Mehrotra, S., unpublished data.

Furthermore, the MART-$1_{27–35}$ epitope is a dominant epitope against which cancer patients as well as normal individuals harbor precursors at fairly high frequencies (22, 23, 46). Thus, the outcome of the presentation of this epitope on immature DCs might be explained on the basis of its "immunodominance." It is therefore possible that certain "immunodominant" epitopes might lead to CTL response even when presented by "less potent" DCs, and the observations in the MART-$1_{27–35}$ system might be viewed as an exception. However, similar patterns of CTL response to DC-based stimulation and restimulation were also observed with another self and melanoma associated but less dominant HLA-A2- restricted epitope, MAGE-$3_{271–279,}$ (collective data not shown). Similarly, the positive (expansion) as well as negative (deletion) regulatory properties of our actDCs did not solely result from being activated with a combination of strong innate and instructive signals (LPS, IFN-γ, and CD40L). Experiments carried out with iDCs further matured with IFN-γ, and LPS also revealed the same pattern of activation in primary and secondary stimulation as well as deletion or loss of function after tertiary stimulation (collective data not shown). Our study was carried out in an *in vitro* model, and it involved direct presentation of the epitope. Thus, it remains to be seen if iDCs, when made to cross-present the MART-$1_{27–35}$ antigens, could function in a tolerogenic manner. Finally, the relative inefficiency of iDCs does not equate to tolerogenicity. Few rounds of replication of the CTLp, even with some degree of functional maturation, after primary stimulation, by immature or matured DCs, does not negate a potential regulatory or tolerogenic property of either DC type in a different context. Although additional studies are needed to answer the context in which DCs are regulatory, the data presented here are relevant in the inquiry of DCs as tolerogenic APCs and have broad implications in DC-based immunization studies because self peptide and DC-based vaccine trials continue in cancer, and immature DCs are entering into the treatment of autoimmune diseases.

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