The Journal of Immunology

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J Immunol 2003; 171:2251-2261; ;
doi: 10.4049/jimmunol.171.5.2251
http://www.jimmunol.org/content/171/5/2251

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Rapid High Efficiency Sensitization of CD8$^+$ T Cells to Tumor Antigens by Dendritic Cells Leads to Enhanced Functional Avidity and Direct Tumor Recognition Through an IL-12-Dependent Mechanism[1]

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Myeloid-origin dendritic cells (DCs) can develop into IL-12-secreting DC1 or non-IL-12-secreting DC2 depending on signals received during maturation. Through rapid culture techniques that prepared either mature, CD83$^+$ DC1 or DC2 from CD14$^+$ monocytes in only 2 days followed by a single 6–7 day DC-T cell coculture, we sensitized normal donor CD8$^+$ T cells to tumor Ags (HER-2/*neu*, MART-1, and gp100) such that peptide Ag-specific lymphocytes constituted up to 16% of the total CD8$^+$ population. Both DC1 and DC2 could sensitize CD8$^+$ T cells that recognized peptide-pulsed target cells. However, with DC2, a general decoupling was observed between recognition of peptide-pulsed T2 target cells and recognition of Ag-expressing tumor cells, with peptide-sensitized T cells responding to tumor only about 15% of the time. In contrast, direct recognition of tumor by T cells was dramatically increased (to 85%) when DC1 were used for sensitization. Enhanced tumor recognition was accompanied by 10- to 100-fold increases in peptide sensitivity and elevated expression of CD8β, characteristic of high functional avidity T cells. Both of these properties were IL-12-dependent. These results demonstrate the utility of rapid DC culture methods for high efficiency in vitro T cell sensitization that achieves robust priming and expansion of Ag-specific populations in 6 days. They also demonstrate a novel function of IL-12, which is enhancement of CD8$^+$ T cell functional avidity. A new approach to DC-based vaccines that emphasizes IL-12 secretion to enhance functional avidity and concomitant tumor recognition by CD8$^+$ T cells is indicated. ***The Journal of Immunology,*** **2003, 171: 2251–2261.**

Therapeutic cancer vaccines hold remarkable promise for the treatment of malignancies. Identification of a variety of tumor-associated Ags and their representation as synthetic peptides, recombinant proteins, viral vector-expressed products, and so-called "naked" nucleic acids has allowed numerous clinical trials to proceed (1–4). Many such trials are sporadically marked by dramatic disease regressions in individual patients. Nonetheless, consistent therapeutic outcomes that would place vaccine therapy on the same footing as other cancer treatment modalities have yet to be achieved.

The failure of current cancer vaccines to induce consistently meaningful clinical responses cannot be ascribed to a failure to induce detectable immune responses against defined vaccine targets. For example, priming for delayed-type hypersensitivity, or induction of T cells that recognize professional peptide-loaded target cells, constitutes unequivocal evidence of "successful" immunization, even when each individual vaccine recipient displaying these markers does not necessarily experience tumor regression (5, 6). Indeed, an apparent property common to many candidate tumor vaccine Ags is their induction of T cells that recognize peptide-loaded target cells, but not genuine tumor cells that naturally express the same Ag (7, 8). This phenomenon has been observed both in clinical trials and preclinical investigations, and highlights a central and pervasive deficiency in current anti-cancer vaccine strategies, for if the effectors of immunity cannot be made to consistently recognize the very tumors they are intended to target, the full potential of cancer vaccines may never be realized.

A relatively recent innovation in tumor immunology has been the use of dendritic cells (DCs)[4] to generate immunity against tumor Ags both in vivo, when administered as vaccines, and in vitro for experimental sensitization schemes. The enthusiastic focus on these APCs stems from the recognition of their principal role as primary sensitizers of T cells (9–12). Of no less importance is the rapidly developing appreciation that early contact by T cells with DCs, and the signals DCs supply, has critical and lasting effects on important functional qualities of the T cell. For instance, it is known that immune responses can be broadly categorized as Th1 type, which are dominated by T cells secreting large amounts of IFN-γ and little IL-4 and IL-5, or Th2 type which conversely are comprised mostly of cells that secrete less IFN-γ and comparatively more IL-4 and IL-5 (13). These polarized response types are generally recognized to be adaptations for dealing optimally with

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Received for publication April 10, 2003. Accepted for publication June 24, 2003.

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[1] This work was supported by American Cancer Society Grant RPG-99-029-01.

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[4] Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand; CMM, cytokine maturation mixture; iDC, immature DC; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine; MIP, migration inhibitory protein; neg, negative; pos, positive.

0022-1767/03/$02.00

specific types of pathogens, and signals that skew immune responses toward one type or another can come from DCs. Therefore, DCs that skew T cell responses toward Th1 have been termed "DC1" while DCs that skew toward Th2 are termed "DC2." Although the terms DC1 and DC2 originally carried with them an ontological connotation, for myeloid-origin DCs were at first regarded as DC1 whereas lymphoid-origin cells were considered DC2 (14), we and others have demonstrated that myeloid-origin DCs possess the capacity to acquire the functional properties of either DC1 or DC2, depending on the signals supplied to DCs during maturation (15–19). The ideal DC, from the standpoint of cancer immunotherapy, would be able to sensitize highly effective tumor-recognizing T cells. However, the specific stimuli needed to generate such a DC have not been fully elucidated.

In this study, we address the issue of direct tumor recognition through a comparative study of the functional qualities of normal donor T cells sensitized in vitro by either tumor Ag-pulsed DC1 (which secrete large amounts of IL-12) or DC2 (which secrete little or no IL-12). In contrast to CD4$^+$ cells, we found that the differential effects of DC1 and DC2 on CD8$^+$ cells appeared to have relatively little to do with polarized cytokine secretion profiles. Instead, dramatic increases in functional avidity (Ag sensitivity), the capacity to recognize Ag-expressing tumor cells in cytokine release assays, and the ability to lyse Ag-expressing tumor targets were seen when DC1 rather than DC2 were used for sensitization. These T cell enhancements appeared to be largely IL-12-dependent. Therefore, this study remedies a vexing problem in tumor immunology, demonstrates new properties of IL-12, and suggests a promising, novel approach that may dramatically improve cancer vaccines.

Materials and Methods

Preparation of human PBMC fractions

Seven healthy donors (male and female age 20–30) provided informed consent and were leukapheresed. Blood product was then elutriated to obtain monocyte-rich (>94%) and lymphocyte-enriched fractions that were cryopreserved as described (20).

Reagents, Abs, and peptides

FITC-, Cy-5-, or PE-conjugated mouse anti-human CD86, CD83 (BD PharMingen, San Diego, CA), CD3, CD14, CD20, CD56, CD80, CD8 α and β, and subclass-matched controls (BD Biosciences, Mountain View, CA) were used. Cell separation studies and blocking studies used purified mouse anti-human CD45RO, CD3, CD28 (BD PharMingen) IL-12 and control Abs (R&D Systems, Minneapolis, MN). Recombinant human IL-4, IL-12, GM-CSF, TNF-α, and IFN-γ were from R&D Systems. Soluble CD40 ligand (CD40L) was the generous gift of Immunex (Seattle, WA). Peptides based on the sequences of the tumor-associated Ags MART-1 (27–35), gp100 (209m, 209–216), gp100 (154–162), HER-2/*neu* (369–377), p53 (149–157), and colon cancer Ag GA733 were generated at the University of Pennsylvania Cancer Center Protein Chemistry Laboratory (Philadelphia, PA). MART-1 tetramers were purchased from Coulter Scientific (San Diego, CA).

Tumor cell lines

Melanoma cell lines Mel 624 HLA-A2$^+$, Mel 624 A2$^-$, and MW115 (HLA-A2$^+$ MART-1/gp100 nonexpressing), as well as the nonmelanoma HLA-A2$^+$ target cell line T2, were kind gifts of Dr. S. A. Rosenberg (National Cancer Institute, Bethesda, MD). Breast cancer lines MDA-MB231 (HLA-A2$^+$, HER-2/*neu* overexpressing), MDA-MB435 (HLA-A2$^-$, HER-2/*neu* overexpressing), SKBr3 (HLA-A2$^-$, HER-2/*neu* overexpressing), MCF-7 (HLA-A2$^+$, weak HER-2/*neu* overexpression) were all obtained from American Type Culture Collection (Manassas, VA). All lines were maintained in RPMI 1640 medium supplemented with 5% FBS.

Preparation of T lymphocyte subsets

Lymphocyte-rich elutriation fractions were used to prepare either CD4$^+$CD45RO$^-$ (naive) T cells or whole populations of CD8$^+$ cells using negative depletion columns as directed by the manufacturer (R&D Systems).

Dendritic cell activation

Monocyte-rich fractions were used either fresh or thawed and plated at 1.5×10^6 cells/cc in either 24- or 48-well cluster plates (Corning, Corning, NY) in macrophage-SFM medium (Life Technologies) plus 50 ng/ml GM-CSF. After overnight culture (14–16 h) the cells were matured into DC1 or DC2. DC2 were prepared by treating with IL-4 (500 ng/ml), IFN-α (1000 U/ml), and PGE_2 (0.1 uM), and then with soluble CD40L (1 μg/ml) ~6 h later. For DC1, overnight cultures were instead treated with human IFN-γ (1000 U/cc) and TNF-α (10 ng/ml) prior to addition of CD40L 6 h later. Cytokine maturation mixture (CMM) according to the formulation of Jonuleit (5) was also used to induce DCs, with GM-CSF/IL-4 overnight cultures matured the next day using IL-1 (10 ng/ml), IL-6 (1000 U/ml), TNF-α (10 ng/ml), and PGE_2 (1 μg/ml). The CMM-DC, DC1, and DC2 were cultured a further 14–20 h prior to harvest. Cells maintained in serum-free medium with only GM-CSF and IL-4 for the entire 40-h culture period were considered immature DC (iDC). All DC and culture supernatants were harvested and analyzed after the 40-h culture/maturation period. DCs were washed two to three times prior to coculture with T cells.

FACS analysis

DCs were analyzed by multicolor flow cytometry using a BD Biosciences FACScan cytometer running CellQuest analysis software (BD Biosciences) as described previously (19). Propidium iodide staining (nonviable) cells were excluded from analysis.

Allosensitization of naive CD4$^+$ T cells

Purified allogenic CD4$^+$/CD45RA$^+$ T cells (1×10^6/well) were cocultured with iDC, DC1, or DC2 (1×10^5/well) in 48-well tissue culture plates. On day 6, the T cells were harvested and restimulated on plates coated with anti-CD3 and anti-CD28 as described previously (19). Supernatants were harvested 24 h later and analyzed by ELISA.

ELISA assays

Capture and biotinylated detection Abs and standards for IFN-γ, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-12p70 (BD PharMingen), or kits for macrophage-derived chemokine (MDC), thymus and activating-regulated chemokine (TARC), and migration inhibitory protein 1β (MIP-1β; R&D Systems) were used according to the manufacturer's recommendations and protocols.

In vitro sensitization of CD8$^+$ T cells

DCs from HLA-A2$^+$ normal donors were pulsed with MART-1 27–35, gp100 (209m, 209–217), gp100 (154–162), or HER-2/*neu* 369–377 peptides at 10 ng/ml 2 h prior to harvest. Harvested cells were washed twice and plated in fresh RPMI 1640, 5% human AB serum, and 30 IU IL-2 with purified CD8$^+$ cells at a T cell to DC ratio of 20:1. After 1 wk, the T cells were harvested and restimulated with relevant, Ag-expressing melanoma tumor cells (Mel624 A2$^+$). Also tested were negative control (Mel624 A2$^-$ and MW115, A2$^+$ tumor Ag$^-$) cell lines that express either MART-1 and gp100 Ag or HLA-A2 Ags, but not both. For the CD8$^+$ T cells sensitized to HER-2/*neu* 369–377 peptide, the CD8$^+$ T cells were tested against the breast cancer cell lines MDA-MB231 (HLA-A2$^+$, HER-2/*neu* overexpressing), MDA-MB435 (HLA-A2$^-$, HER-2/*neu* overexpressing), SKBr3 (HLA-A2$^-$, HER-2/*neu* overexpressing), MCF-7 (HLA-A2$^+$, weak HER-2/*neu* overexpression). CD8$^+$ T cells were also tested against HLA-A2 transporter (TAP)-deficient T2 cells pulsed with relevant and irrelevant peptides. Supernatants were harvested after 24 h and analyzed by ELISA.

Cytotoxicity assays

Melanoma tumor cell lines were incubated for 30 min with 25 μg/ml of the fluorescent dye calcein-AM. Dye-loaded cells were then incubated with DC-sensitized CD8$^+$ T cells at various E:T ratios in PBS with 5% FCS for 3 h at 37°C without added CO_2. Supernatants were then collected and assayed using a fluorometer. Spontaneous dye release as well as total dye release induced by detergent lysis was noted. Specific percent lysis was calculated by the formula experimental release − spontaneous release/total release − spontaneous release × 100.

Statistical evaluation of the ability of DC1 and DC2 to activate anti-tumor CD8$^+$ T cells

Each tumor Ag sensitization assay was scored positive if the IFN-γ secretion against peptide-pulsed or tumor Ag-expressing target was at least

twice that of negative control. Seven donors supplied 60 samples for assessment of $CD8^+$ cell sensitization and tumor recognition. Each donor contributed between 0 (particular Ag not tested) and 7 samples for each Ag experiment. The overall rates of $CD8^+$ cell sensitization and tumor recognition were calculated from data pooled from all donors. The Mantel-Haenszel χ^2 test (21) was used to test the association between $CD8^+$ cell sensitization (or tumor recognition) and DC1 or DC2. This test stratifies the results by donor thereby adjusting for the correlated nature of multiple observations from an individual. Wilcoxon rank sum analysis was used to compare the magnitude of $CD8\alpha\beta$ vs $CD8\alpha\alpha$ expression between T cells sensitized by DC1 and DC2. All analyses were performed in STATA version 6.0 (Stata, College Station, TX). A significance level <0.05 was considered statistically significant.

Results

Generation and characterization of DC1, DC2, and iDC

We began these studies by comparing basic phenotypic and functional qualities of iDCs, putative DC1 and putative DC2. iDC expressed virtually no detectable CD83, relatively low levels of CD80 and CD86, retained CD14 (Fig. 1*A*) and secreted virtually no detectable cytokines or chemokines except low level IL-8 (Fig. 1, *B* and *C*). In contrast, overnight monocyte cultures treated with IFN-γ, TNF-α, and CD40L developed into mature DC1 that expressed elevated levels of CD83, CD80, and CD86 and depressed CD14 (Fig. 1*A*). ELISA analysis of culture supernatants showed that DC1 secreted high levels of p70 IL-12, IL-6, IL-8, and MIP-1β, which is chemotactic for Th1 cells (Fig. 1*B*), as well as and MIP-1α (not shown). Present only at low levels was MDC and TARC, both chemotactic for Th2 (22) (Fig. 1*C*). Treatment of overnight monocyte cultures instead with IL-4, PGE_2, IFN-α, and CD40L resulted in mature, putative DC2 that expressed high levels of CD83, CD80, and CD86, with low expression of CD14, similar to DC1 (Fig. 1*A*). In contrast to DC1, the DC2 secreted little or no IL-12, IL-6, or MIP-1β. However, they did secrete IL-8 and IL-10 at levels roughly comparable to DC1 (Fig. 1*B*). DC2 were further distinguished from DC1 by their secretion of high levels of MDC and TARC (Fig. 1*C*). These results suggest that differential methods for generating DC1 and DC2 induce similar expression of common DC phenotypical markers, despite strongly polarized cytokine and chemokine expression profiles.

We next tested whether DC1 and DC2 showed characteristically polarized functional activity in T cell allosensitization experiments. DC1 stimulated naive allogeneic $CD4^+$ T cells to produce high levels of IFN-γ, with little IL-4, IL-5, or IL-10 production (Fig. 1*D*), as assessed by ELISA analysis of culture supernatants. In contrast, DC2 stimulated naive $CD4^+$ T cells to secrete much less IFN-γ and instead provoked abundant IL-4 and IL-5, although IL-10 levels were similar to DC1 and low (Fig. 1*D*). iDC stimulated negligible cytokine production by naive $CD4^+$ allogeneic T cells (Fig. 1*D*). Therefore, DC polarization into functional DC1 and DC2 types was confirmed by their respective capacity to skew

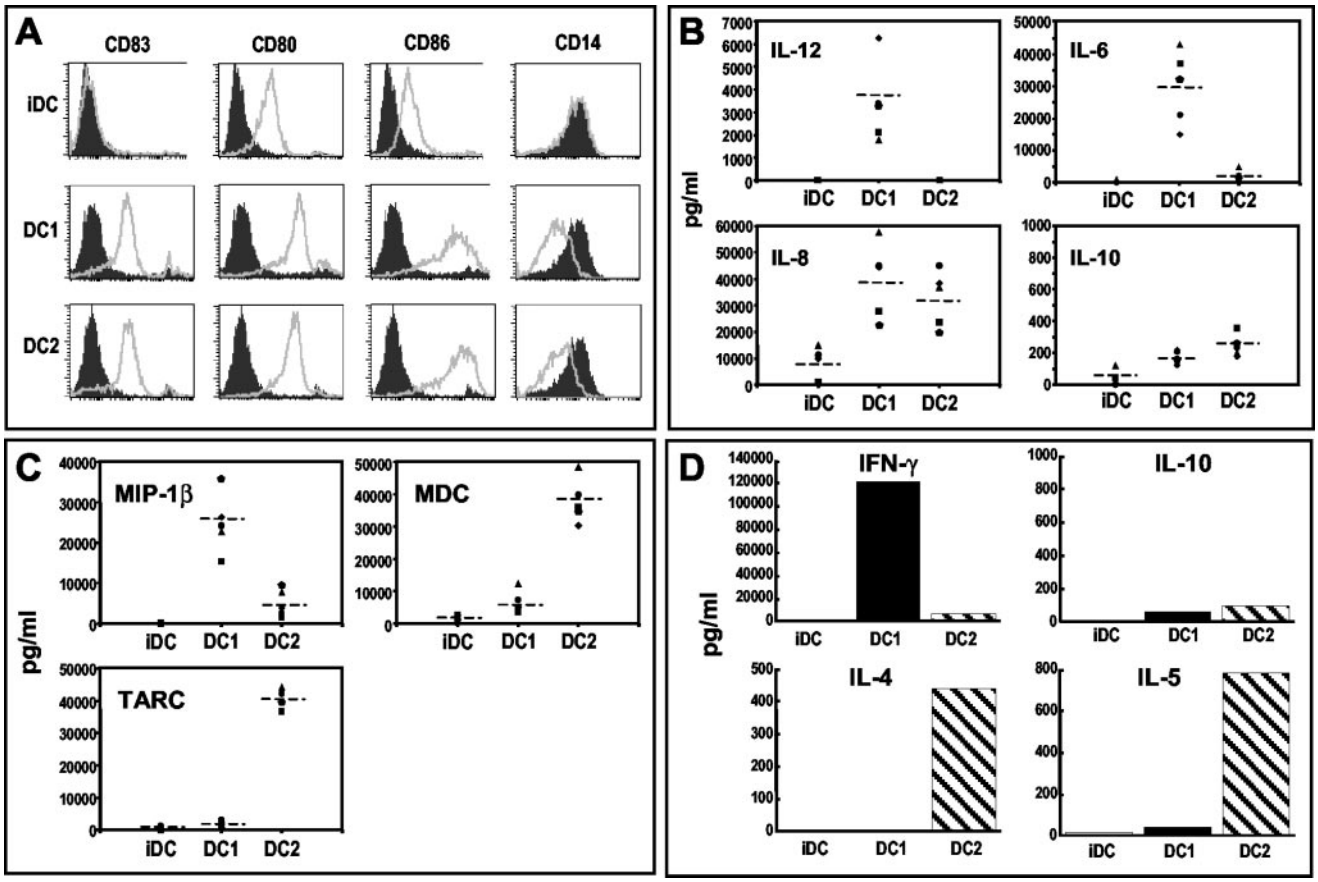

FIGURE 1. Phenotype and function of iDC, DC1, and DC2. *A*, Flow cytometry analysis of 2-day cultured DC compared to starting monocyte populations. For CD83, CD80, and CD86, filled traces represent isotype-matched negative control staining and open traces represent specific Ab staining. For CD14, filled traces represent CD14 staining of monocyte controls (starting populations), and open traces represent CD14 staining of cultured DCs. Comparative FACS analysis is representative of 10 experiments with different donors. *B*, ELISA analysis of cytokine secretion and (*C*) chemokine secretion in 2-day DC culture supernatants from five separate experiments with different donors. *D*, ELISA analysis of cytokine secretion in 24-h supernatants of $CD4^+$ T cells previously allosensitized by 6-day culture with iDCs (□), DC1 (■), or DC2 (▨) and then restimulated with plate-bound anti-CD3 and anti-CD28 Abs. Results are representative of five experiments with multiple donors.

T cell development toward types resembling Th1 and Th2 as assessed by the method described by Rissoan (14). The differential chemokine secretion patterns in DC1 and DC2, which are selectively chemotactic for memory Th1 or Th2, also suggest that polarization of immune responses could occur not only in naive lymphocytes, but also in the selective restimulation and amplification of polarized memory effector cells.

DC1 and DC2 each expand Ag-specific CD8$^+$ T cell populations

We next tested whether either DC1 or DC2 were superior for generating tetramer staining (i.e., Ag-specific) CD8$^+$ T cells in a 6-day MART-1 peptide sensitization assay using normal donor T cells. Both DC1 and DC2 induced CD8$^+$ T cells that were peptide-specific as measured by tetramer staining (Fig. 2*A*). Percentages of tetramer-positive T cells were similar whether induced by DC1 (mean 4.7%, range 2–11%, $n = 9$) or DC2 (mean 5.7%, range 0.9–16%, $n = 9$), suggesting similar potency. Intensity of tetramer staining was also similar between these two groups. Therefore, DC1 and DC2 appeared to be of comparable potency for stimulating the expansion of Ag-specific CD8$^+$ cells from normal donors.

DC1 enhance CD8$^+$ T cell recognition of some tumor-derived peptide Ags

Either DC1 or DC2, when pulsed with MHC class I binding peptides from MART-1 (27–35), modified gp100 (209m, 209–217), gp100 (154–162), or HER-2/*neu* (369–377) could usually prime CD8$^+$ T cells for appreciable Ag-specific IFN-γ release in response to peptide-pulsed T2 target cells (Fig. 2*B*, *upper panels*, and Fig. 3). This finding distinguished CD8$^+$ T cells from the CD4$^+$ cells (previously characterized in Fig. 1*D*), for capacity to secrete IFN-γ, though greater for DC1-sensitized CD8$^+$ cells, was clearly in the same high range whether sensitization was conducted with either DC1 or DC2. We also found no apparent differences in IL-4 or IL-5 secretion by CD8$^+$ cells whether sensitized by either DC1 or DC2 (which was generally either low or undetectable). Therefore, DC1 and DC2 did not induce the same degree of polarization on CD8$^+$ cells as observed with CD4$^+$ cells. However, DC2 did differ in that they were somewhat less likely to confer specific peptide recognition for some donors (compare Fig. 2*B*, both *upper left panels* for MART-1 peptide sensitization from two separate donors). Indeed, when the frequency of successful sensitization by DC1 and DC2 were compared for each peptide across seven healthy donors, it was apparent that for gp100 209m and for

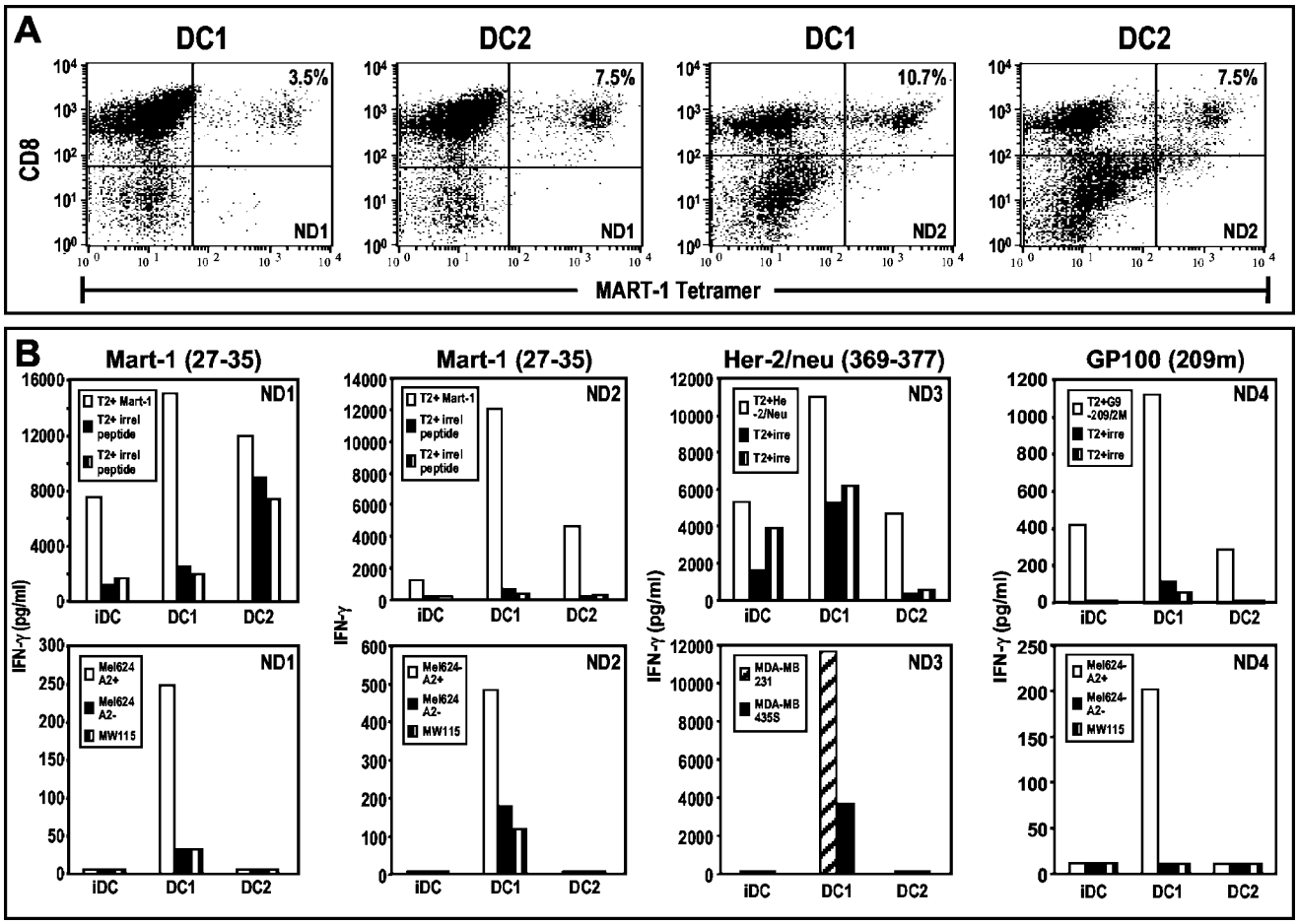

FIGURE 2. Ag specificity of CD8$^+$ T cells sensitized in vitro by DC in 6-day cocultures. *A*, Flow cytometry analysis of CD8$^+$ T cells sensitized by MART-1 (27–35)-pulsed DC1 or DC2 from two normal donors (ND1 and ND2). T cells were stained with anti-CD8 (FITC:y-axis) and MART-1 tetramer (PE:x-axis). Percentages indicate proportion of CD8$^+$/tetramer$^+$ cells (i.e., MART-1-specific T cells). Results are representative of nine experiments with multiple donors. *B*, Recall response of MART-1, HER-2/*neu*, and gp100 peptide-sensitized T cells from four separate donors to peptide-pulsed T2 target cells (*upper panels*) or tumor cells (*lower panels*). Results are representative of five experiments with MART-1 and HER-2/*neu* and three experiments with gp100 using multiple donors.

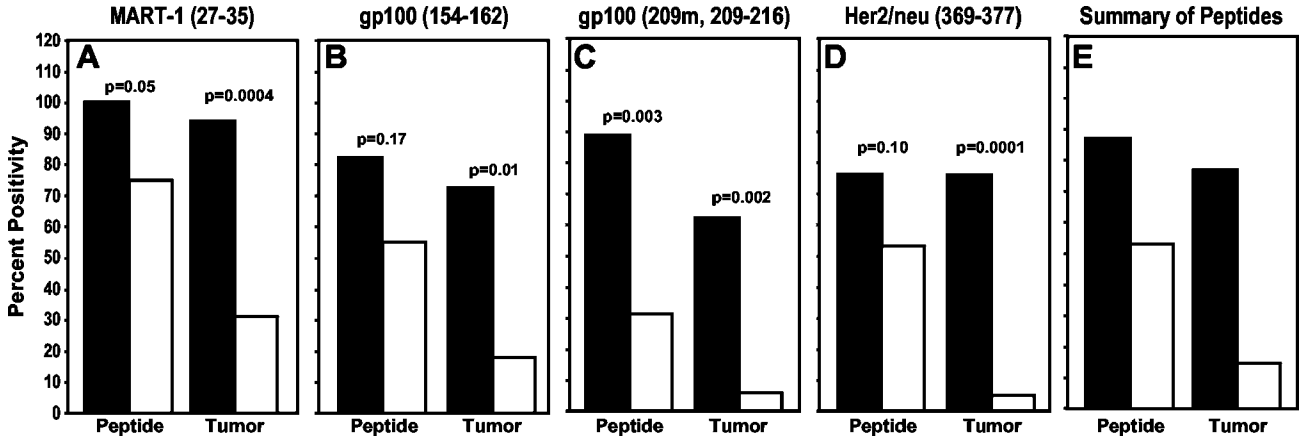

FIGURE 3. DC1 increase tendency of $CD8^+$ T cells to directly recognize tumor cells expressing endogenous Ags. Statistical summary of the results of 60 different DC-T cell cocultures from seven different donors to the Ags indicated. Normal donor $CD8^+$ T cells were incubated for 6 days with DC1 (■) or DC2 (□) pulsed with various peptides and then tested for reactivity against peptide-pulsed T2 targets or Ag expressing, HLA-$A2^+$ tumor lines. Reactivity was assessed by IFN-γ secretion in 24-h supernatants. *A*, MART-1 (27–35); *B*, gp100 (154–162); *C*, gp100 (209m 209–216); *D*, HER-2/*neu* (369–377); and *E*, summary of all cultures. A positive culture was defined as displaying at least twice the level of secreted IFN-γ compared to control cell lines or peptide. Peptide is $CD8^+$ T cells tested against the indicated peptide compared with control peptides and the tumor cells outlined in Fig. 3. The Mantel-Haenszel χ^2 test was used to test the association between $CD8^+$ cell sensitization for DC1 and DC2.

MART-1 (27–35) there was a statistically significant advantage ($p = 0.003$ and 0.05, respectively) for using DC1 to sensitize $CD8^+$ T cells to secrete IFN-γ in response to Ag (Fig. 3, *A* and *C*). For HER-2/*neu* 369–377 (Fig. 3*D*) and gp100 154–162 (Fig. 3*B*), sensitization rates also seemed higher with DC1, but these differences were not statistically significant ($p = 0.10$ and $p = 0.17$, respectively). These experiments indicate that although both DC1 and DC2 could be considered highly effective in sensitizing T cells to peptide Ag, DC1 was superior, at least for some Ags.

DC1 induce $CD8^+$ T cells to recognize tumor cells endogenously expressing tumor Ag

Perhaps the most striking advantage of DC1 became apparent when synthetic tumor Ag-sensitized T cells were tested for recognition of such HLA-$A2^+$ tumors that naturally expressed the relevant target Ags. DC1 pulsed with HER-2/*neu*, MART-1, or gp100 peptides could prime normal donor $CD8^+$ T cells for recognition of either HER-2/neu^+ breast cancer cells, or MART-1 or gp100-expressing melanoma lines, respectively (Fig. 2*B*, *lower panels*). DC1 significantly enhanced tumor recognition by $CD8^+$ T cells from seven healthy donors for all peptide Ags tested (MART-1 $p = 0.0004$, gp100 (154–162) $p = 0.01$, gp100 (209m 209–216) $p = 0.002$, and HER-2/*neu* (369–377) $p = 0.0001$ (Fig. 3, *A–D*)) compared with DC2. Of the 60 different replicate $CD8^+$ T cell cultures from the seven donors, 85% of the DC1-sensitized cultures demonstrated tumor cell recognition as assessed by specific IFN-γ secretion compared to only 15% of the $CD8^+$ T cells raised by DC2-recognized tumor cells (Fig. 3*E*). Only a few DC2 cultures induced $CD8^+$ T cells that secreted small amounts of IL-5 (<200 pg/ml) in response to tumor cells (not shown), suggesting that the apparent lack of tumor recognition induced in DC2-T cell cocultures was not merely a phenomenon of restricted IFN-γ secretion. Peptide-pulsed iDC also usually induced $CD8^+$ T cells that secrete IFN-γ in response to peptide-pulsed T2 target cells, but not tumor cells naturally presenting the same Ags (Fig. 2*B*). In addition, iDC matured with CD40L alone, CD40L plus TNF-α, or TNF-alone induced $CD8^+$ T cells that recognized tumor targets at a rate no better than that of DC2 (23). We also observed that the enhanced tumor-recognition properties conferred on T cells through sensitization with DC1 was not restricted to synthetic peptide Ags, because recombinant HER-2/*neu* intracellular and extracellular domain proteins, through an apparent cross-priming mechanism, sensitized $CD8^+$ T cells for HLA-A2-restricted recognition of breast cancer cells (S. Xu, G. Koski, M. Faries, I. Bedrosian, and B. Czerniecki, manuscript in preparation). These studies show that DC1 are unique among the DCs tested in their ability to consistently prime $CD8^+$ T cells for direct tumor recognition.

IL-12 confers enhanced tumor recognition to normal donor $CD8^+$ T cells during sensitization

Because DC1 prime T cells for tumor recognition superior to that of DC2, despite similar costimulatory molecule expression, we sought to determine whether any of the secreted factors (that vary widely between DC1 and DC2) were responsible for this functional difference. We initially focused on IL-12 (produced only by the DC1) because this cytokine has been previously linked to tumor immunity (24–26). We began by sensitizing $CD8^+$ T cells to HER-2/*neu* 369 using iDC, DC1, or DC2. As before, only DC1 primed T cells for tumor recognition (Fig. 4*A*). In contrast, when iDC or DC2 from this same experiment were used to sensitize T cells in the presence of added recombinant IL-12 at concentrations comparable to those secreted by DC1, recognition of MHC-matched, HER-2/neu^+ breast cancer cells was successfully induced (Fig. 4*B*), with similar results for MART-1-pulsed DC2 and melanoma cells (Fig. 4*C*). In related experiments, neutralizing anti-IL-12, but not control Ab, blocked induction of melanoma tumor cell recognition induced by MART-1-pulsed DC1 (Fig. 4*C*). The block of DC1-induced tumor recognition by neutralizing anti-IL-12 cannot be explained away as merely a Th2 polarization of cytokine secretion, because these T cells still abundantly secreted IFN-γ (albeit at somewhat lower levels) in response to T2 cells pulsed with optimal levels of MART-1 peptide (Fig. 4*D*), and also because IL-5 secretion in response to tumor is also blocked with anti-IL-12 (not shown). In addition, it was clear that the effect of IL-12 was primarily exerted during DC-T cell interaction (i.e., during sensitization) and not on the DC itself or on the tendency of DC to bind peptide, because only DC2 cultured with T cells in the

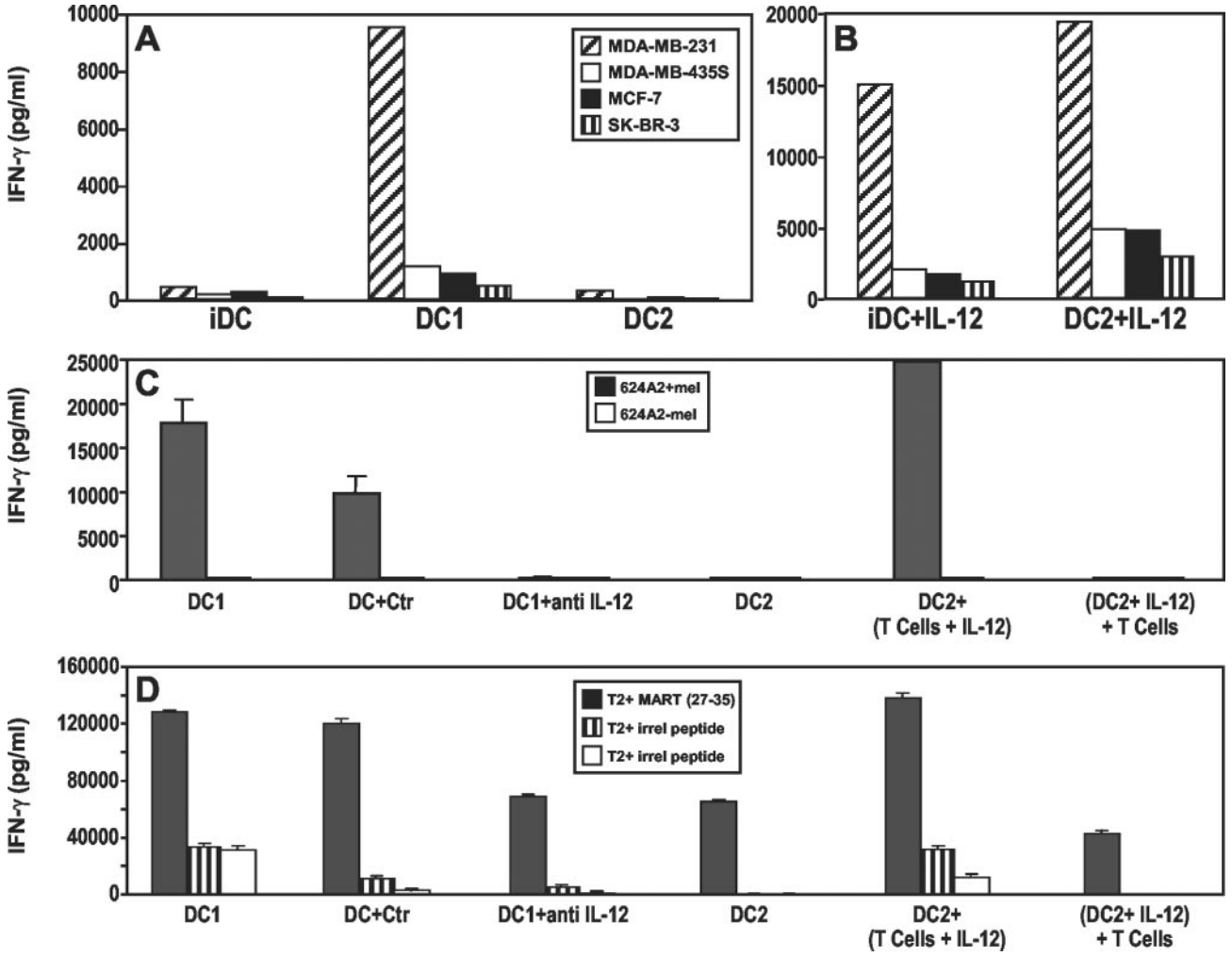

FIGURE 4. IL-12 accounts for enhanced tumor recognition by sensitized normal donor $CD8^+$ T cells. *A*, T cells were sensitized to HER-2/*neu* 369 peptide by iDC, DC1, or DC2 and tested for reactivity (IFN-γ release) to breast cancer cell lines by ELISA analysis of 24-h culture supernatants of T cell-target cocultures. Tumor targets tested expressed both HER-2/*neu* and HLA-A2 (MDA-MB231), HER-2/*neu* but not HLA-A2 (MDA-MB435s), low amounts of HER-2/*neu* with HLA-$A2^+$ (MCF-7) or cells that expressed HER-2/*neu* but not HLA-A2 (SK-BR-3). *B*, Enhanced breast cancer cell reactivity of T cells when sensitized in the presence of added rIL-12 by HER-2/*neu*-pulsed iDC or DC2. Experiments in *A* and *B* were performed three times with multiple donors. *C*, T cells were sensitized by MART-1 (27–35) pulsed DC1 in the presence or absence of neutralizing anti-IL-12 (1 μg/ml) or control Ab, or by pulsed DC2 with or without rIL-12 (10 ng/ml), and tested for reactivity (IFN-γ release in 24-h coculture supernatants) against MART-1-producing 624 melanoma cells expressing HLA-A2 Ags (624$A2^+$ *mel*), or not (624$A2^-$ mel), or MART-expressing, HLA-A2-negative MW115 cells. *D*, T cells sensitized in *C* tested against HLA-A2-expressing T2 target cells pulsed with MART-1 (27–35) peptide, or irrelevant HLA-A2-binding peptides derived from the sequences of p53 (149–157, ▥) or colon cancer Ag GA733 (□). Results of *C* and *D* are representative of five experiments with multiple donors.

presence of IL-12 (DC2 + (T cells + IL-12)) provided the enhanced effects, while DC treated separately with IL-12 prior to their washing and coculture with T cells (DC2 + IL-12) + T cells) did not increase the development of tumor-recognizing $CD8^+$ T cells (Fig. 4*C*). Therefore, addition of IL-12 to sensitizing DC2-T cell cocultures confers the $peptide^{pos}/tumor^{pos}$ recognition pattern characteristically induced by DC1, while neutralizing IL-12 in sensitizing DC1-T cell cocultures leads to the $peptide^{pos}/tumor^{neg}$ recognition pattern normally characteristic of DC2-sensitized T cells. Thus, IL-12 appears to be largely responsible for the enhanced tumor recognition.

CMM-induced DCs do not sensitize $CD8^+$ T cells for direct tumor recognition

Given the apparent advantage of DC1 over DC2 in the capacity to induce $CD8^+$ T cells that directly recognize Ag-expressing tumor cells, we undertook to compare DC1 to DCs induced by a commonly used CMM. DCs induced by this combination of cytokines, which include IL-6, IL-1β, PGE_2, and TNF-α, have been used successfully in clinical trials (5, 27) and have been shown superior to DCs induced by monocyte-conditioned medium, the method favored previously for generating DCs with optimized function (28). This mixture has also been adapted to a rapid 2-day maturation scheme that produces cells functionally identical to those produced by the "traditional" 1-wk maturation regimen (29). We found that both DC1 and CMM regimens produced DCs with comparable mature phenotype after only 2 days total culture (Fig. 5*A*). When culture supernatants from both cell types were analyzed for the presence of IL-12, DC1 produced the characteristically high levels of IL-12p70, while no IL-12 was detected in supernatants from CMM-induced DCs (Fig. 5*B*). When DC1 and CMM-matured DCs were loaded with MART-1 peptide and used to sensitize normal donor $CD8^+$ T cells in a single 6-day coculture, both DC types could induce expansion of MART-1 tetramer-staining cells (Fig. 5*C*). However, only DC1-sensitized T cells could directly recognize HLA-$A2^+$ melanoma tumors that naturally expressed

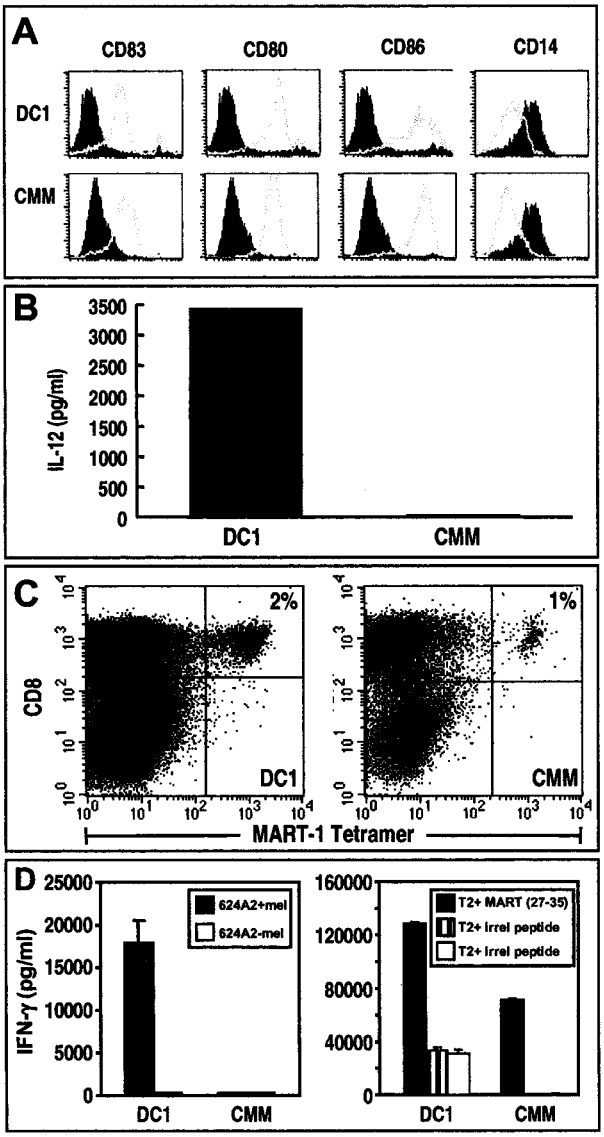

FIGURE 5. CMM-induced DCs do not prime normal donor CD8$^+$ T cells for direct tumor recognition. MART-1 peptide-pulsed DC1 or CMM-induced DCs were used to sensitize normal donor CD8$^+$ T cells in a single 6-day sensitization coculture. *A*, FACS analysis of mature DC1 and CMM-induced DCs prior to peptide pulsing. For CD83, CD80, and CD86, filled trace represents isotype-matched negative control Ab staining and open trace represents specific Ab. For CD14, filled trace represents CD14 staining of monocyte starting populations (control) and open trace represents CD14 staining of matured DCs. *B*, ELISA analysis of IL-12p70 from culture supernatants of mature DC1 and CMM-matured DCs at time of harvest. *C*, FACS analysis of CD8$^-$ and MART-1 tetramer-stained T cells after 6-day sensitization/coculture with MART-1-pulsed DC1 or CMM-induced DCs. *D*, MART-1-sensitized CD8$^+$ T cells sensitized by MART-1-pulsed DC1 or CMM-induced DCs were cocultured with HLA-A2$^+$ or HLA-A2$^-$ melanoma cell lines or with T2 target cells pulsed with MART-1 peptide or irrelevant control peptides p53 (149–157, ▥) and colon cancer Ag GA733 (□). Supernatants were harvested 24 hours later and analyzed for IFN-γ by ELISA. Experiments in *A–D* are representative of three experiments with multiple donors.

MART-1 Ag despite the fact that both DC1- and CMM-DC-sensitized T cells could secrete high levels of IFN-γ in response to MART-1 peptide-pulsed T2 target cells (Fig. 5*D*). Therefore, CD8$^+$ T cells sensitized by CMM-induced DCs possessed the same peptide^{pos}/tumor^{neg} recognition pattern previously demonstrated in DC2-sensitized cells.

DC1 enhances CTL activity of anti-tumor CD8$^+$ T cells through the action of IL-12

We next examined whether the enhanced tumor recognition of CD8$^+$ T cells conferred by DC1 through IL-12 translated into superior tumor killing activity (Fig. 6). Normal donor CD8$^+$ T cells sensitized in a single 6–7 day round of coculture with MART-1 or gp100 (209m) peptide-pulsed DC2 (◇) or CMM-DCs (●) were each capable of comparable, if relatively low, degrees of lytic activity against HLA-A2$^+$, Ag-expressing melanoma cell lines which extinguished rapidly at lower E:T ratios. In contrast, DC1-sensitized CD8$^+$ T cells (◆) displayed much higher degrees of specific lysis that tended to persist even down to E:T ratios of 1:1. In contrast, T cell sensitizations performed with DC1 in the presence of neutralizing anti-IL-12 (▲), but not isotype-matched control Ab (■), significantly inhibited the lytic capacity of the CD8$^+$ T cells down to the levels of DC2- or CMM-DC-sensitized T cells. In addition, T cells sensitized by DC2 in the presence of exogenously added rIL-12 (○), using concentrations comparable to that secreted by DC1, demonstrated enhanced lysis as compared to that achieved by DC2 without added cytokine (Fig. 6). Finally, CD8$^+$ T cells sensitized by DC1 displayed no lytic activity against tumor Ag-expressing melanoma cells that did not coexpress the HLA-A2 restriction element (MEL 624 A2$^-$, □), strongly suggesting that tumor killing was MHC-restricted, and not due to some nonspecific killing mechanism. Therefore, these results show that DC1 prime CD8$^+$ T cells for enhanced tumor cell killing, and that these effects are likewise mediated to an appreciable degree by IL-12.

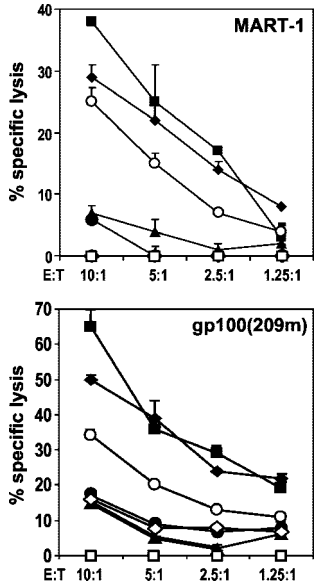

FIGURE 6. DC1 prime Ag-specific CD8$^+$ T cells for enhanced cytolysis of melanoma cells. CD8$^+$ T cells sensitized for 6 days to either MART-1 or gp100 (209m) peptides using DC1 (◆), DC2 (◇), CMM-DC (●), DC1 + anti-IL-12 (▲), DC1 + control Ab (■) or DC2 + rIL-12 (○) were cultured for 3 h with calcein-AM dye-loaded MEL 624 HLA-A2$^+$ melanoma target cells at various E:T ratios. Alternatively, DC1-sensitized CD8$^+$ T cells were similarly incubated with MEL 624 HLA-A2$^-$ cell lines as a lysis negative control (□). Culture supernatants were removed and assessed for released dye by fluorometry. Percent-specific lysis was determined by the formula experimental release − spontaneous release/total release − spontaneous release × 100. Results are representative of five experiments for MART-1 and two experiments for gp100 using multiple donors.

IL-12 induces higher functional avidity in CD8$^+$ T cells

We next explored the issue of whether the observed increase in tumor recognizing capacity induced by the presence of IL-12 was linked with an enhanced T cell functional avidity. CD8$^+$ T cells were primed by MART-1-pulsed DC1 or DC2 in the presence or absence of neutralizing anti-IL-12 Ab or exogenously added rIL-12, respectively, and tested against T2 targets pulsed at successively lower peptide concentrations. Bulk cultures of DC1, DC1 + Ab isotype control or DC2 + IL-12-primed anti-MART-1 CD8$^+$ T cells secreted half maximal levels of IFN-γ in response to HLA-A2$^+$ T2 target cells pulsed with between 10- and 100-fold lower concentrations of MART-1 peptide than T cells primed with DC2, CMM-induced DC or DC1 + anti-IL-12 (Fig. 7*A*), strongly suggesting that IL-12 plays a leading role for enhancing the functional avidity of sensitized T cells. In related experiments (Fig. 7*B*), we pulsed melanoma cell lines already expressing MART-1 protein (which could usually be recognized by T cells sensitized by DC1 but not DC2) with MART-1 peptide so that their surface expression of peptide would be enhanced. These "super-loaded" cells then could be recognized by DC2-primed T cells. This experiment suggests that the relatively low Ag density naturally found on the surface of tumors may be at least partially responsible for the lack of tumor recognition by the lower functional avidity T cells primed by DC2, and further underscores the critical advantages of high functional avidity with respect to direct tumor recognition.

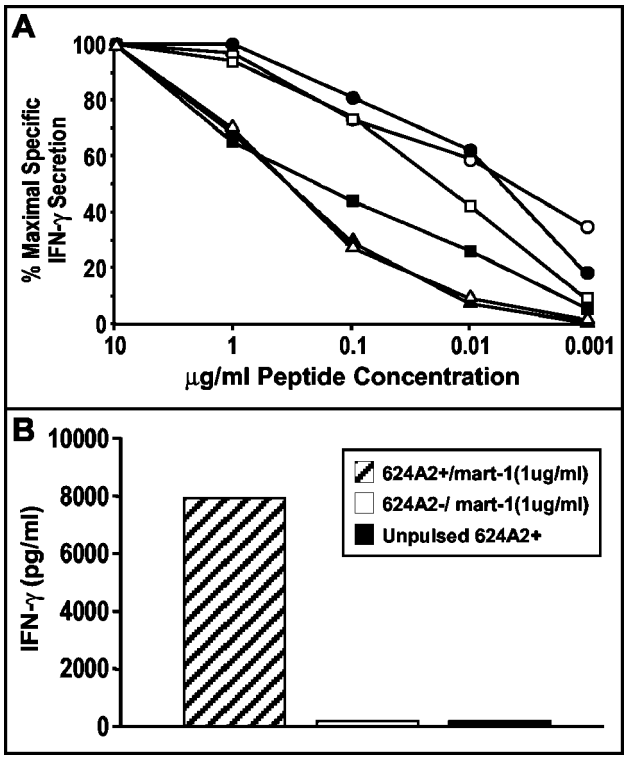

FIGURE 7. Induction of T cell high functional avidity is regulated by IL-12. *A*, T cells sensitized to MART-1 (27–35) peptide by 6-day coculture with DC1 (○); DC1 + anti-IL-12 mAb (10 μg/ml) (■); DC1 + isotype control mAb (10 μg/ml) (□); DC2 (△); DC2 + rIL-12 (5 ng/ml) (●); or CMM-DC (▲) were tested for recall against T2 targets pulsed with graded concentrations of MART-1 peptide, by IFN-γ release in 24-h culture supernatants as determined by ELISA. *B*, T cells similarly sensitized to MART-1 by DC2 were tested against both MART-1-expressing HLA-A2$^+$ and HLA-A2$^-$ 624 melanoma cells pulsed or not with synthetic MART-1 (27–35) peptide, and IFN-γ secretion in 24-h supernatants assessed by ELISA. Results in *A* and *B* are representative of three experiments each with multiple donors.

IL-12 increases expression of CD8β on T cells

We next attempted to discover any differences that might account for the enhanced Ag sensitivity of DC1-sensitized CD8$^+$ T cells. We could find no variations in expression of LFA-1, LFA-3, ICAM-1, or CD62 between T cells sensitized by DC1 or DC2, nor could we identify any alterations in TCR Vβ usage that correlated with the presence of IL-12 (not shown). It has been postulated that CD8$\alpha\beta$ heterodimers, rather than $\alpha\alpha$ homodimers ($\beta\beta$ homodimers do not exist), support more efficient TCR signaling (30) that may contribute to higher functional avidity. Therefore, we evaluated whether IL-12 present during Ag sensitization affects CD8β expression by T cells. In this representative experiment, MART-1-pulsed DC1 and DC2 were used to sensitize normal donor CD8$^+$ T cells in a 7-day coculture. FACS analysis of the MART-1 tetramer-staining subpopulation from both sensitization groups showed that DC1-sensitized MART-1-specific cells had elevated CD8β expression compared to DC2-sensitized MART-1-specific populations (Fig. 8*A*, *upper panel*). In addition, when rIL-12 was added to sensitizing DC2 cultures, a similar increase in CD8β expression was observed (Fig. 8*A*, *lower panel*). In contrast, there appeared to be no remarkable differences in CD8α expression between DC1- and DC2-sensitized or DC2- and DC2 + IL-12-sensitized groups (Fig. 8*B*). To collectively evaluate and statistically analyze the results of five such experiments, we determined the mean channel fluorescence for CD8β-stained and CD8α-stained T cells and used these values to determine relative expression ratios. A paired statistical analysis was performed using Wilcoxon rank sum analysis. T cells sensitized by DC1 had a significantly higher CD8β-CD8α ratio compared to T cells sensitized by DC2 (median ratio 1.5 (range 0.7–5.2) for DC1 and 1.0 (range 0.6–2.9) for DC2, $p = 0.04$). For DC2, addition of rIL-12 significantly enhanced the CD8β-CD8α ratio (median 1.3 (range 0.7–3.70) $p = 0.04$) and for DC1, anti-IL-12 significantly reduced the ratio (median 1.3 (range 0.6–2.4) $p = 0.04$). Isotype-matched control Abs had no statistically significant effect. To better visualize these relationships, we calculated and plotted the average percent increase in CD8β:CD8α ratio for each group relative to DC2 (Fig. 8*C*). On the average, DC1 cells induced over 60% increases in CD8β:CD8α ratios compared to DC2 (baseline ratio). The enhancing effects of addition of IL-12 to DC2 groups and the blocking effects of anti-IL-12 on DC1 groups are clearly evident. These results are consistent with a possible mechanism for enhanced Ag sensitivity through CD8$\alpha\beta$-mediated augmentation of TCR signaling.

Discussion

Most reports directly comparing the function of various DCs have done so on the basis of relative maturity (5, 31–33) which is usually assessed by surface immunophenotype. However, it is clear that comparably mature DCs can acquire diametrically polarized functional properties depending on the diverse maturation signals they may receive (15–19). We showed that putative DC1 and DC2, each matured from CD14$^+$ monocyte precursors, were virtually indistinguishable by costimulatory molecules and common surface phenotypic markers. Instead, they differed dramatically in cytokine/chemokine expression profiles. These disparate profiles had apparent profound functional consequences for the DCs, because conventional assessment of their ability to skew the cytokine secretion patterns of naive CD4$^+$ T cells by the methods described by Rissoan (14) confirmed these DCs as true functional DC1 and DC2. In contrast, CD8$^+$ T cells sensitized by either DC1 or DC2 did not fit into this classical skewing profile. This was evidenced

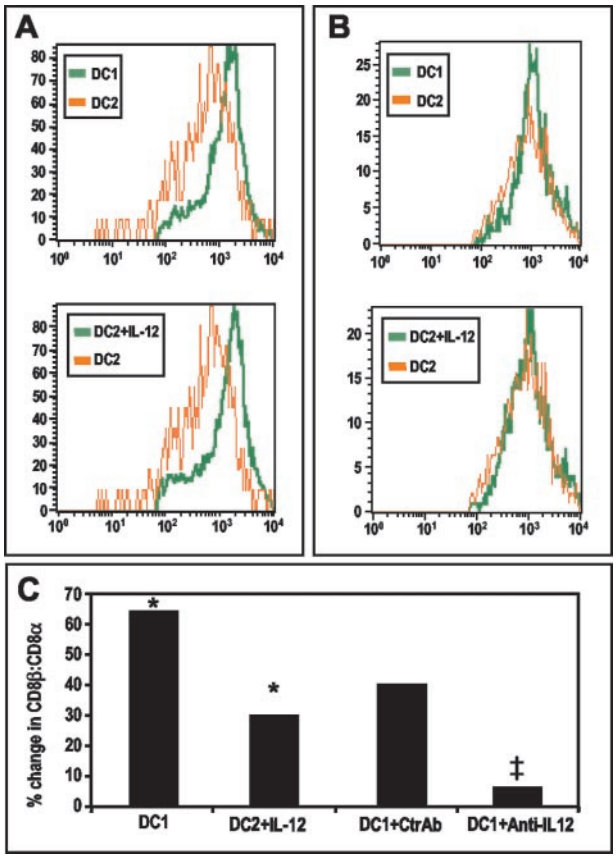

FIGURE 8. High functional avidity-inducing sensitization regimens correlate with enhanced T cell CD8β expression. CD8$^+$ T cells from normal donors were cocultured with MART-1 (27–35) pulsed DC1, DC2, or DC2 + IL-12 (10 ng/ml). The T cells were harvested on day 7 and stained with MART-1 tetramer (26–35) and counterstained with anti-CD8α or anti-CD8β. Flow cytometric analysis analyzed CD8 expression on MART-1 tetramer-staining subpopulations. *A*, Staining of CD8$^+$ cells with anti-CD8β; *B*, staining with anti-CD8α. *A* and *B* are the results from a single experiment representative of five different experiments from multiple donors. *C*, Collective analysis of results from all five experiments. Statistically significant differences in CD8β-CD8α ratios are denoted by $*$ for comparison against DC2-sensitized T cells ($p = 0.04$), while ‡ indicates statistical significance in comparison to DC1-sensitized cells ($p = 0.04$).

by the fact that CD8$^+$ cells sensitized to peptide Ag using either DC1 or DC2 could secrete large amounts of IFN-γ, but little IL-4 or IL-5, in recall responses against T2 target cells pulsed with optimal concentrations of the sensitizing peptide. Instead, the most striking feature that distinguished DC1-sensitized CD8$^+$ T cells from those sensitized by DC2 or even the CMM-induced DC was the dramatically increased sensitivity to recall Ag, a property known as high functional avidity. This finding may have great use for improving the efficacy of cancer vaccines.

It has been noted in a number of studies that T cells successfully sensitized to tumor Ags often fail to recognize or kill actual tumor cells that naturally express the same Ag (7, 8). For example, Zaks et al. (7) recently showed that breast cancer patients immunized with HER-2/*neu* 369 peptide routinely developed T cells that could recognize peptide-loaded professional target cells. However, the T cells tended not to directly recognize Ag-expressing breast cancer cells (7). Indeed, in our recent clinical study of immunotherapy for melanoma using peptide Ag-pulsed functional DC2 activated by calcium mobilizing agents, direct CD8$^+$ T cell recognition of melanoma lines was rare despite frequent sensitization to melanoma peptide Ags as determined by tetramer analysis or recognition of peptide-pulsed T2 target cells as assessed by cytokine secretion.[5]

However, the current study appears to have identified a means of rectifying this deficiency in direct tumor recognition. Starting with elutriated human peripheral blood monocytes, we used a rapid, serum-free DC maturation regimen that derived CD83$^+$ DCs in only 2 days of culture. These cells, in a remarkable display of their efficiency, could sensitize normal donor T cells to melanoma and breast cancer Ags in a single 6–7 day sensitization. Previous studies using conventionally matured DCs have reported an inability to in vitro sensitize T cells to many of these same Ags (34) or a requirement for multiple restimulations over the course of several weeks (35). DC1, which secrete generous quantities of IL-12, were particularly effective and outstripped DC2 and even the current standard CMM-induced DC in important functional categories. These categories included the capacity to secrete cytokine in response to Ag-expressing tumors, where only DC1-sensitized T cells attained this function. It also included the ability to directly lyse Ag-expressing tumors, where DC1-sensitized T cells held a clear advantage over cells sensitized by DC2 or CMM-induced DC. It appeared that the high functional avidity and concomitant enhanced capacity to directly recognize and kill bona fide tumor cells possessed by the DC1-sensitized CD8$^+$ cells were conferred by the presence of IL-12 during sensitization.

Our demonstration that the conferral of direct tumor recognition to T cells, at least in vitro, is not routinely achieved in the absence of IL-12, provides a reasonable explanation for the difficulty in consistently generating tumor-recognizing T cells in many previous preclinical studies and clinical trials. We also showed that T cells primed in the absence of IL-12 could be made to recognize natural tumors if these targets were artificially loaded with synthetic peptide Ag. This suggests that the general inability to recognize unmodified tumor is at least partly rooted in the mere absence of naturally processed target epitopes at sufficient density to trigger activation of low functional avidity T cells. Thus, priming of T cells in the presence of IL-12 probably allows recognition of tumors by lowering their Ag activation threshold to a level below the corresponding Ag density on the surface of tumors.

Biologically active IL-12 is a 74-kDa heterodimer composed of a disulphide-linked p35 β-chain and p40 α-chain (for review, see Ref. 36). IL-12 is secreted by phagocytes, DC, and B cells, and acts upon both T cells and NK cells promoting activation, proliferation, and in particular, IFN-γ secretion. IL-12 also enhances anti-tumor immunity in a variety of murine (24) and human (37) models by mechanisms that may involve activation of both T cells and NK cells (36). IL-12 has also been shown to improve CTL activity against viral Ags (38). Previous published reports have not, to our knowledge, linked these enhancing effects to increased sensitivity of T cells to Ag. Our findings, in contrast, show that the presence of IL-12 during priming greatly reduces the quantity of Ag necessary to trigger recall responses by the sensitized T cells, even to the extent of enabling direct recognition of Ag-expressing tumor. Therefore, these studies have allowed us to assign previously undescribed activities to this cytokine.

This work also recapitulated a previously reported association between high functional avidity and enhanced CD8β expression.

[5] I. Bedrosian, R. Mick, S. Xu, H. Nisenbaum, M. Faries, P. Zhang, P. Cohen, G. Koski, and B. Czerniecki. Intranodal administration of peptide-pulsed mature dendritic cell vaccines results in superior CD8$^+$ T cell function in melanoma patients. *Submitted for publication.*

Others demonstrated, using TCR transgenic mice (to rule out affinity effects), that low avidity T cells expressed CD8 molecules-composed largely of $\alpha\alpha$ homodimers, while high avidity cells had increased $\alpha\beta$ heterodimer usage (30). However, the earlier studies linked CD8 subunit usage to Ag priming dose, not IL-12 or other cytokines. Nonetheless, the remarkable correlation between T cell avidity and CD8β expression seen in both the Cawthon et al. studies (30) and our own, leads us to hypothesize that the increased functional avidity of anti-tumor T cells may result, at least in part, from an IL-12-dependent enhancement of CD8β expression.

It has been proposed that CD8$\alpha\beta$ heterodimers promote high functional avidity because only this form of the CD8 molecule will efficiently colocalize with the TCR on special membrane microdomains termed "lipid rafts" (for reviews see Refs. 39 and 40). Other signaling components also congregate at these raft sites, including p59^{fyn} and p56^{lck} (41) (42), the overall effect being the assembly of a more efficient TCR signaling apparatus. High functional avidity may have great importance in vivo, because such T cells are superior for clearing viral infections (43) and controlling tumors (44, 45). We are currently focusing on the use of IL-12 to modulate the development of both high and low functional avidity T cell lines and clones to facilitate detailed studies on differences in signal transduction properties between these distinct phenotypes.

This study demonstrated that the presence of IL-12 during T cell sensitization overcomes, probably through enhanced T cell functional avidity, lack of direct tumor recognition, thereby providing a potential remedy for a long-standing problem in human tumor immunology and ascribing new functional properties to IL-12. Therefore, an attractive new strategy for enhancing tumor vaccine effectiveness through timely induced IL-12 secretion by DCs, or timely provision of exogenous rIL-12, is strongly suggested. Such a strategy may have broad application not only for maximizing efficacy of vaccines directed against cancer, but also for infections such as HIV. We are currently determining whether DC1-based cancer vaccines can induce tumor-recognizing T cells in patients with breast cancer and melanoma.

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