Extensive Replicative Capacity of Human Central Memory T Cells[1]

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To characterize the replicative capacity of human central memory (T_{CM}) CD4 T cells, we have developed a defined culture system optimized for the ex vivo expansion of Ag-specific $CD4^+$ T cells. Artificial APCs (aAPCs) consisting of magnetic beads coated with Abs to HLA class II and a costimulatory Ab to CD28 were prepared; peptide-charged HLA class II tetramers were then loaded on the beads to provide Ag specificity. Influenza-specific DR*0401 CD4 T_{CM} were isolated from the peripheral blood of normal donors by flow cytometry. Peptide-loaded aAPC were not sufficient to induce resting CD4 T_{CM} to proliferate. In contrast, we found that the beads efficiently promoted the growth of previously activated CD4 T_{CM} cells, yielding cultures with >80% Ag-specific CD4 cells after two stimulations. Further stimulation with peptide-loaded aAPC increased purity to >99% Ag-specific T cells. After in vitro culture for 3–12 wk, the flu-specific CD4 T_{CM} had surface markers that were generally consistent with an effector phenotype described for CD8 T cells, except for the maintenance of CD28 expression. The T_{CM} were capable of 20–40 mean population doublings in vitro, and the expanded cells produced IFN-γ, IL-2, and TNF-α in response to Ag, and a subset of cells also secreted IL-4 with PMA/ionomycin treatment. In conclusion, aAPCs expand T_{CM} that have extensive replicative capacity, and have potential applications in adoptive immunotherapy as well as for studying the biology of human MHC class II-restricted T cells. *The Journal of Immunology,* **2004, 172: 6675–6683.**

Recently, it has become apparent that memory T cells exist in at least two subsets (1–3). First, there are central memory T cells (T_{CM})[4] that circulate through the lymphoid system similar to naive T cells specific for the Ag, but in greater numbers and with a more rapid response rate. Second, there are effector or tissue memory T cells (T_{EM}) that can carry out immunosurveillance in the tissues where they might meet Ag at the site of infection. The differentiation of these memory subsets of cells remains unclear, but in the mouse, T_{CM} appear to arise from T_{EM} (4). This is controversial, because other studies suggest that CD8 T_{EM} appear to arise from T_{CM} (1, 5–7). The strongest data to suggest that the T_{CM} cell population may be an intermediate between naive T cells and T_{EM} cells is the observation of shorter telomere lengths for memory T cells that are $CCR7^{neg/dull}$ compared with those that $CCR7^{high}$ (8). Murine Th1 CD4 T_{EM} appear to derive from T_{CM}, because the T_{EM} do not survive upon transfer (9, 10).

Ag presentation by dendritic cells can be defective in the setting of cancer, either due to problems in access or processing of Ag (11), or in the maturation process of dendritic cells after capturing Ag (12, 13). Furthermore, Ag presentation by dendritic cells is a complex process, which in some cases may result in specific T cell tolerance (14). One method of overcoming the problems of in vivo Ag presentation is with adoptive immunotherapy of effector cells. For this purpose, efficient culture systems to activate and expand human T cell subsets ex vivo are required.

Immunotherapy studies in both humans and in mouse models of cancer and infectious diseases have shown that $CD4^+$ T cells have a major role in priming the immune system and eliciting meaningful clinical responses (15–20). Our laboratory has focused on developing various artificial APCs (aAPCs) to stimulate and propagate human T cell subsets in culture. Our initial approach was to engineer a bead-based aAPC that would stimulate polyclonal $CD4^+$ T cells; our anti-CD3/anti-CD28-coated beads (CD3/28 beads) activate and promote the expansion of a full repertoire of purified $CD4^+$ T cells for several months ex vivo in the absence of any other feeder cells (21). These expanded polyclonal $CD4^+$ T cells have been infused as therapy in pilot clinical trials for patients with HIV and cancer (22–24). In this study, we aimed to expand a population of Ag-specific CD4 T_{CM} cells ex vivo. Using MHC tetramer technology, we engineered Ag-specific aAPCs to activate and expand highly enriched populations of human class II-restricted CD4 T_{CM} cells. We found that T_{CM} have extensive replicative potential and that the T cells were functional. These aAPCs may have clinical use in expanding large numbers of Ag-specific T cells for eventual use as adoptive immunotherapy alone or in combination with $CD8^+$ T cells.

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[4] Abbreviations used in this paper: T_{CM}, central memory T cell; T_{EM}, effector memory T cell; aAPC, artificial APC; HA, hemagglutinin; DC-flu, dendritic cell pulsed with flu HA peptide.

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Materials and Methods

Bead and tetramer preparation

Epoxy-activated magnetic beads, 4.5 μm in diameter, were purchased from Dynal Biotech (Lake Success, NY); 200 million beads were coated with a total of 75 μg of protein overnight at 4°C in a 0.1 M borate buffer at pH 7.2. Excess uncoated protein was removed by three 10-min washes and one overnight wash at 4°C in Bead Wash Buffer (PBS, 3% human AB serum, 0.5 M EDTA, and 1% sodium azide). In each preparation, 37.5 μg of anti-class II (clone TU36; Caltag Laboratories, Burlingame, CA) or anti-CD3 (OKT3) and 37.5 μg anti-CD28 (clone 9.3) were coated on the beads. HLA-peptide tetramers consisting of HLA DR*0401 with flu hemagglutinin (HA) peptide PKYVKQNTLKLAT in its groove were prepared as described (25). Tetramers that had been conjugated with streptavidin-PE were used in FACS staining protocols, whereas tetramers loaded onto beads were conjugated with streptavidin only. Loading of tetramers was accomplished by washing the beads twice into culture medium (or FACS buffer), then incubating 3 μg of tetramer per 10×10^6 beads for 20 min at 4°C, and then washing the beads once more with culture medium (or FACS buffer) before incubation with T cells (or flow cytometric analysis, respectively).

Cell preparation, stimulation, and culture

$CD4^+$ T cells were purified by elutriation and negative selection from a leukopheresis product of normal donors with the HLA type DR*0401 as previously described (26). PBMC were prepared by Ficoll gradient centrifugation of a portion of the leukopheresis product. Dendritic cells were generated by 7-day incubation of the adherent cell fraction of PBMC with 800 IU/ml GM-CSF (Immunex, Seattle, WA) and 500 IU/ml IL-4 in AIM-V medium (Life Technologies, Grand Island, NY). On day 7, dendritic cells were matured by overnight stimulation with GM-CSF, IL-4, TNF-α (R&D Systems, Minneapolis, MN), and TRANCE (PeproTech, Rocky Hill, NJ). Dendritic cell preparations were irradiated (3000 rad) and pulsed with 50 μg/ml flu HA peptide for 2 h at 37°C before stimulation of T cells. Feeder cells consisted of autologous irradiated (3000 rad) PBMC. T cell cultures were maintained in AIM-V supplemented with 3% heat-inactivated human AB serum (BioWhittaker, Walkersville, MD) and 20 IU/ml human IL-2 (Chiron Therapeutics, Emeryville, CA). Population doublings are calculated with the equation $A_t = A_0 2^n$, where n is the number of population doublings, A_0 is the input number of cells, and A_t is the total number of cells.

Immune synapse studies

T cells were incubated for 20 min with either flu/28 beads or with EBV-transformed B cells that had been pulsed overnight with flu HA peptide. APC/T cell conjugates were then settled for an additional 5 min at 37°C onto poly-L-lysine-coated coverslips, fixed in 3.7% formaldehyde, and stained with anti-LFA-1 (FITC conjugated; BD PharMingen, San Diego, CA) and anti-CD3 (OKT3; biotinylated). Cy-3/streptavidin (Sigma-Aldrich, St. Louis, MO) was used to visualize staining with anti-CD3. After staining, the coverslips were mounted onto microscope glass slides (Fisher Scientific, Hanover Park, IL) with GelMount (Biomeda, Hayward, CA) and stored at 4°C until analysis. Immunofluorescence and corresponding Nomarski images of the cells and T cell/APC conjugates were recorded by a digital fluorescence microscopy system (Intelligent Imaging Innovations, Denver, CO) consisting of a Zeiss Axioplan microscope (Carl Zeiss, Thornwood, NY) fitted with a xenon light source and a Sensicam charge-coupled device camera (Cooke, Auburn Hills, MI). Slidebook software (Intelligent Imaging Innovations) was used for image analysis and three-dimensional volume rendering. A constrained iterative deconvolution algorithm was used to remove out-of-focus haze. Within each experiment, all images were renormalized to the same range of intensity.

Flow cytometry

aAPC beads were stained with anti-HLA DR allophycocyanin (BD PharMingen) in FACS buffer (PBS, 3% FBS, 0.02% sodium azide) for 20 min at 4°C. The beads were then washed twice in FACS buffer and analyzed on a FACSCalibur (BD Biosciences, Mountain View, CA). For analysis of Ag-specific cells and for cell sorting, cells were stained with anti-CD4 allophycocyanin (BD PharMingen) and HLA DR*0401-flu HA tetramer labeled with PE for 20 min at 4°C in FACS buffer. Cells were then washed twice in FACS buffer and analyzed on a FACSCalibur or sorted on a MoFlo cell sorter (Cytomation, Fort Collins, CO). Other Abs used in surface phenotyping analysis include Abs to CD3, CD11a, CD25, CD28, CD69, TCRab, CD45RA, and CD45RO (BD Biosciences), and CCR7, CD27, and CD62L (BD PharMingen). All flow cytometry data were analyzed by uploading .fcs files into FlowJo software (TreeStar, San Carlos, CA).

Intracellular cytokine staining

T cells were washed in AIM-V medium and incubated with either flu/28 or CD3/28 beads, or 0.05 ng/ml PMA and 2 ng/ml ionomycin (Sigma-Aldrich) for 6 h in 0.1 μg/ml brefeldin A (Sigma-Aldrich). Cells were washed in PBS and stained with surface Ab to CD4, and then fixed and permeabilized with the Fix & Perm kit (Caltag Laboratories). Intracellular staining was performed at room temperature for 20 min before flow cytometry analysis. We used Abs to IL-2, IFN-γ, TNF-α, IL-4, (BD Biosciences), and rat anti-IL10 (Caltag Laboratories) along with their respective isotype controls.

ELISPOT analysis

Plates (MultiScreen-IP; Millipore, Bedford, MA) were coated overnight at 4°C with 50 μl/well mAb anti-human IFN-γ (Endogen, Woburn, MA) at 10 μg/ml in sodium carbonate buffer (2.93 g of sodium bicarbonate, 1.59 g of sodium carbonate, and 0.2 g of sodium azide, in a final volume of 1 liter of distilled water). The plates were washed three times in sterile PBS, and then blocked with AIM-V/3% AB serum for 1 h at room temperature. One thousand T cells were incubated with stimulator cells or beads overnight. Cells and beads were removed by washing with PBST (PBS containing 0.1% Tween 20), and then anti-human IFN-γ biotinylated detection mAb (1 μg/ml; Endogen) was added to each well for 2 h in PBST containing 0.5% human serum albumin. After additional washing, extravidin/alkaline phosphatase at a 1/10,000 dilution in PBS (Sigma-Aldrich) was added and incubated for 1 h at room temperature. After washing, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added for 20–30 min at room temperature in the dark. The reaction was stopped by adding 100 μl/well 1 M sodium phosphate solution. The spots were scanned and counted by computer-assisted ELISPOT image analysis (Hitech Instruments, Edgemont, PA).

Results

Creation and evaluation of a class II-restricted aAPC

We sought to develop Ag-specific aAPCs to characterize the replicative capacity of human CD4 T_{CM}. Our first Ag-specific aAPCs were constructed by coating recombinant peptide-loaded HLA DR*0401 complexes directly onto beads; however, we found that these aAPCs did not elicit IL-2 production in Ag-specific T cells (27). Therefore, we explored different approaches to construct Ag-specific aAPCs, i.e., by coating HLA monomers and tetramers directly or indirectly (via an Ab) to paramagnetic beads. We found that the optimal aAPC to induce IL-2 production involved indirectly immobilized tetramers, prepared by covalently coating beads with a nonblocking Ab to MHC class II, followed by exogenous loading of HLA-peptide tetramers. The initial experiments to optimize the aAPCs were performed with T cell lines that had been cultured extensively and did not require costimulation for IL-2 production; in these experiments, where we used fresh resting lymphocytes, we added an Ab to CD28 to the beads to provide costimulation (Fig. 1*A*). Next, we optimized the exogenous loading of HLA-peptide tetramers onto the beads by titration. Various amounts of tetramers (0–5 μg) were loaded onto a fixed number of beads (10×10^6); after washing, bound tetramer was assayed by flow cytometry using an anti-HLA DR Ab (Fig. 1*B*). We found that optimal loading occurred with 2–3 μg of tetramer per 10×10^6 beads. An isotype control for HLA DR Ab demonstrated no increase in nonspecific staining when increasing amounts of tetramer were added to the beads (data not shown). We used these peptide-HLA DR*0401-tetramer-loaded anti-class II/anti-CD28 beads, referred to as flu/28 beads for simplicity, in the all of the following experiments. Each batch of beads was tested and optimized for tetramer loading in this fashion, with all batches yielding consistent results with the flu HA tetramers.

We first examined whether flu/28 beads could bind to human $CD4^+$ T cells in an Ag-specific manner. Cells from a highly en-

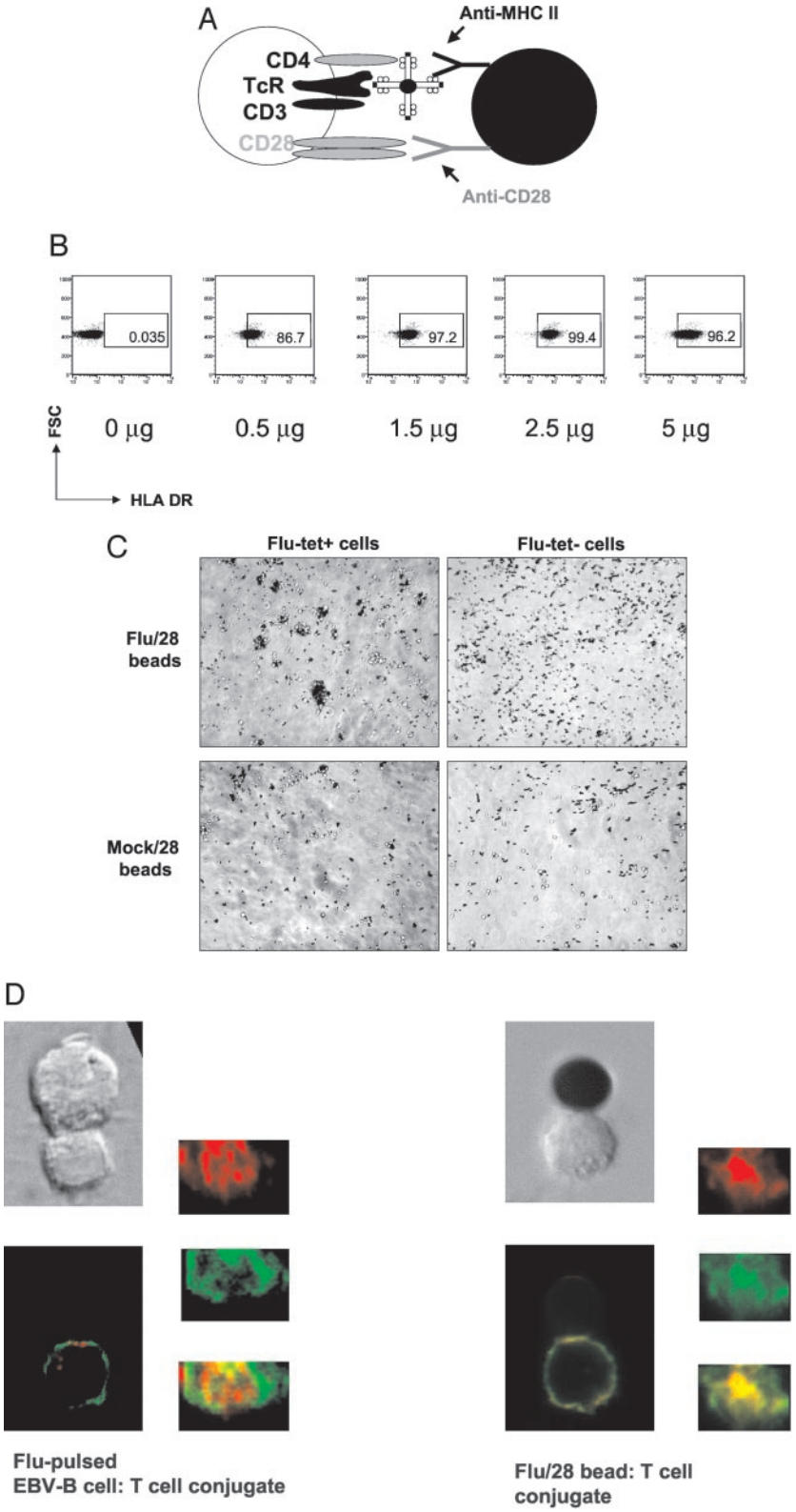

FIGURE 1. Design and testing of an Ag-specific aAPC. *A*, Model of bead-based Ag-specific APC interacting with $CD4^+$ T cell via stimulation of the TCR with indirectly loaded peptide-loaded HLA tetramer and anti-CD28. *B*, Titration of tetramer loading on aAPCs. Ten million beads were loaded with 0–5 μg of peptide-loaded HLA tetramers, and then washed and stained with anti-HLA DR to assess bound HLA. Plots shown are gated on size to exclude doublets. *C*, T cell conjugate formation with aAPCs after 2-h incubation with flu-specific T cells or polyclonal CD4 T cells; $\times$100 magnification with phase-contrast light microscopy. *D*, Flu/28 beads bind and induce clustering of CD3 (red) at the contact site (*right*), but do not segregate LFA-1 (green) as natural peptide-pulsed APCs do (*left*). Nomarski images of APC:T cell conjugates are shown in *top panels*; single-color and overlay en face images are shown at *right*.

riched (>99% pure) population of flu HA $tetramer^+$ CD4 T cells, generated by a combination of tetramer-guided FACS sorting and repeated stimulation with peptide-pulsed autologous dendritic cells, were incubated with flu/28 beads for 2 h to assess aAPC:T cell interactions. We found that binding occurred only with the combination of flu/28 beads and $tetramer^+$ cells; no significant binding of flu/28 beads occurred with a bulk polyclonal population of $CD4^+CD28^+$ T cells from the same donor, indicating that ligation of CD28 alone or nonspecific ligation of MHC tetramers to TCRs was not sufficiently strong to induce aAPC:T cell conjugate formation (Fig. 1*C*); after longer periods of incubation, the beads did bind to polyclonal T cells by CD28, but the large clusters observed with flu/28 beads and Ag-specific T cells were not observed. Similarly, tetramer loading of beads was required for conjugate formation, because mock/CD28 beads did not bind $tetramer^+$ cells or bulk $CD4^+$ cells.

Further studies revealed that flu/28 beads induced clustering of TCRs and formation of lipid rafts (28) at the aAPC contact site, indicated by fluorescent staining of CD3 (Fig. 1*D*) and the glycosphingolipid GM1 (data not shown). However, unlike natural APCs, such as peptide-pulsed EBV-transformed B cells, T cells bound to flu/28 beads did not exclude LFA-1 from the contact site (Fig. 1*D*), indicating incomplete formation of the immunological synapse (29). Thus, physical evidence indicates that these aAPCs could be loaded with MHC class II tetramers, and biologic evidence shows that the flu/28 aAPCs could form peptide-specific conjugates with Ag-specific T cells.

aAPCs promote the growth of previously activated high-affinity CD4 T_{CM}

To determine whether flu/28 beads were sufficient to activate resting Ag-specific $CD4^+$ memory T cells, peripheral blood T cells from influenza immune donors was used as a source of CD4 T_{CM}. We first isolated $CD4^+$ T_{CM} cells from normal HLA DR*0401 donors by negative selection with magnetic beads to remove non-CD4 cells, and then further isolated the flu HA-specific T cells by FACS. The purity of the sorted population immediately afterward is difficult to determine precisely because of the relatively low numbers of sorted cells (<10,000); however, we reacquired an aliquot of sorted cells, which revealed a homogeneous population (data not shown). The sorted flu HA $tetramer^+$ cells were stimulated with anti-CD3/28 beads alone, flu/28 beads alone, or flu/28 beads in the presence of autologous irradiated PBMC feeder cells (Fig. 2*A*). All cultures were maintained in low dose (20 IU/ml) recombinant human IL-2. After 3 wk of culture, we found that sorted cells that had been stimulated with CD3/28 beads had expanded (Fig. 2*B*), but that the culture had not maintained Ag specificity (*A*). Although resting CD4 T_{CM} stimulated with flu/28 beads alone failed to proliferate, sorted cells stimulated with flu/28 beads in the presence of irradiated autologous feeder cells proliferated vigorously and were greatly enriched for flu-specific T cells (Fig. 2*A*). The flu-specific T_{CM} proliferated for 2 mo using this protocol, and doubled at least 10 times during the first 3 wk of culture (Fig. 2*B*); this is a minimal assumption of cell division, given that we assumed 100% plating efficiency of the sorted cells. Thus, Ag-specific expansion of sorted resting CD4 T_{CM} cells required flu/28 beads and feeder cells.

Because resting T cells have more stringent activation requirements than cycling cells, we next wanted to determine whether flu/28 beads could stimulate previously activated T_{CM}. We used the same scheme to sort the flu-specific $CD4^+$ cells, but first stimulated them with autologous dendritic cells pulsed with flu HA peptide (DC-flu) (Fig. 3*A*). After 10 days, the culture was split and restimulated with either DC-flu or flu/28 beads alone. In these experiments, the initial sort and DC-flu stimulation yielded a purity of 7.3% flu HA-specific T_{CM} cells after 10 days; however, restimulation with DC-flu or flu/28 beads significantly enhanced the purity of the Ag-specific culture after 1 wk, with 60.2% flu $tetramer^+$ cells in the DC-flu-stimulated culture and 77.9% flu $tetramer^+$ cells in the flu/28 bead-stimulated culture (Fig. 3*A*). Interestingly, the mean fluorescence intensity of tetramer staining of CD4 T_{CM} cultured with flu/28 beads was consistently higher than the same CD4 T_{CM} cells cultured using DC-flu-pulsed APCs. To rule out differences in TCR expression as a mechanism to explain this difference in tetramer staining, we analyzed TCR surface expression and found that each cell population expressed similar levels of TCR (data not shown). Some groups have correlated tetramer staining with TCR affinity (30, 31), whereas others have not necessarily seen this correlation (32, 33). Therefore, until further biochemical analysis can be performed using cells expanded by these two APCs, the ability of the flu/28 aAPCs to selectively expand high-affinity T cells will remain speculative in nature.

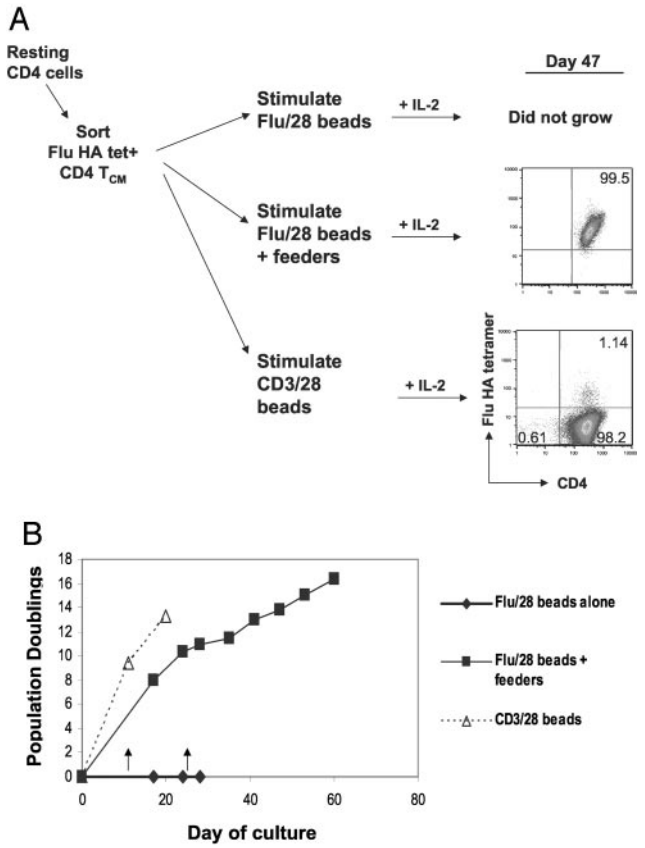

FIGURE 2. Resting CD4 T_{CM} require feeder cells in addition to flu/28 beads for expansion. Resting CD4 T cells were stained with DR*0401-flu HA tetramer, and flu-specific CD4 T cells were enriched by FACS sorting. Sorted cells were then immediately stimulated with flu/28 beads alone, flu/28 beads plus autologous irradiated PBMC feeder cells, or CD3/28 beads, and cultured for 60 days. *A*, Experimental schematic and tetramer stains of resting cells as indicated. Plots shown are gated on size and $CD3^+$ lymphocytes. *B*, Growth curve of cells stimulated with aAPCs. Arrows indicate restimulation with APCs.

Although we were unable to activate resting CD4 T_{CM} with flu/28 beads, the beads were similar in efficiency to DC-flu in promoting the growth of previously activated T_{CM} (Fig. 3*B*). Furthermore, after 30 days in culture, the flu/28 bead culture consisted of >99% $CD4^+$ flu $tetramer^+$ cells, whereas the DC-flu culture had been enriched to 73.2% $CD4^+$ flu HA $tetramer^+$ cells (data not shown).

Use of aAPCs to propagate Ag-specific CD4 T_{CM} cells in the absence of feeder cells

The above results indicated that sequential culture of CD4 T_{CM} in autologous DC and aAPC is a promising approach to obtain highly enriched memory cells in a relatively short time. Because of the potential clinical and regulatory advantages of a T cell culture system that is free from feeder cells, we next asked whether the flu/28 aAPC could promote the growth of cells previously activated with CD3/28 beads rather than with DC-flu or PBMC feeder cells. In a two-step approach, resting CD4 cells from an HLA DR*0401 donor were first polyclonally expanded with aAPCs comprised of CD3/28 beads (Fig. 4*A*). At day 10 of culture, the flu-specific CD4 T_{CM} were FACS sorted, and 21,000 total $CD4^+$ $tetramer^+$ cells were recovered and split into three groups

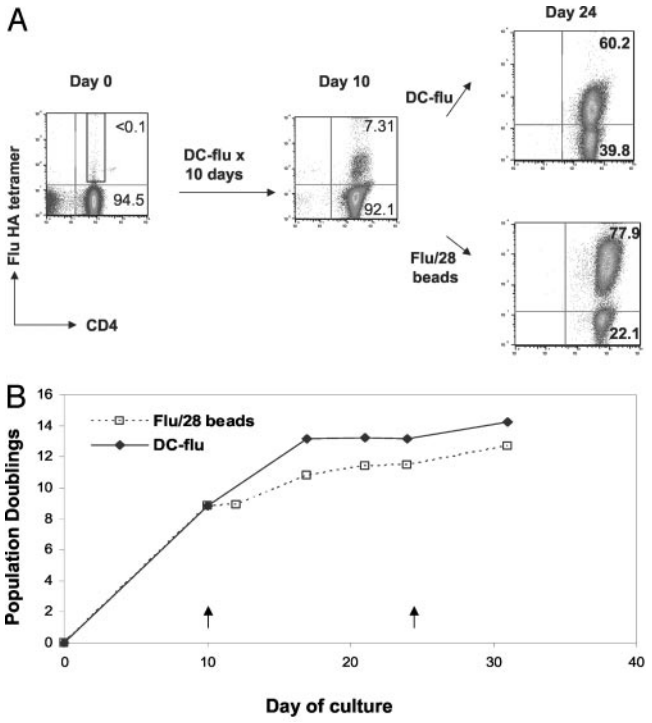

FIGURE 3. Flu/28 beads stimulate CD4 T_{CM} previously activated with peptide-pulsed dendritic cells. *A*, Experimental protocol and tetramer stains of cells on day 0, after one stimulation with DC-flu, and after a second stimulation with either DC-flu or flu/28 beads. *B*, Growth curve of DC-flu- and flu/28 bead-stimulated cell cultures. Arrows indicate restimulation with the respective APCs.

(Fig. 4*B*). The first group was stimulated with flu/28 beads, the second with DC-flu, and the third with CD3/28 beads. On day 25 of culture, all three cultures were restained with anti-CD4 and flu HA tetramer. The cells cultured with flu/28 beads were the most pure, with 81.8% of the cells being flu-specific T cells, whereas the culture stimulated with autologous DC-flu contained 36.5% flu HA-specific cells. In contrast, the sorted cells stimulated with CD3/28 beads contained only 0.71% flu-specific cells, indicating that tetramer FACS sorting was not sufficient to prevent the outgrowth of Ag-nonspecific cells after expansion on CD3/28 beads. The decrease in tetramer staining could not be accounted for by decreased levels of TCR expression, because they were equivalent in flu/28 bead-, CD3/28 bead-, and DC-flu-stimulated T cell cultures (data not shown).

The cultures stimulated with flu/28 beads and autologous DC-flu were continued with periodic restimulation of the T cells at ~14-day intervals (Fig. 4*C*). Under these conditions, we found that the flu/28 aAPC were similar to flu-loaded DC in promoting the growth of CD4 T_{CM} cells. Using the two-step experimental approach shown in Fig. 4*A*, the replicative capacity of the CD4 T_{CM} was found to be extensive (*C*). Human CD4 T_{CM} achieved 20–40 population doublings in the course of 4 mo. The purity of the cultures increased with time, reaching >99% tetramer staining cells after five stimulations.

Surface phenotype analysis of human CD4 T_{CM}

Using the above approach to propagate CD4 T_{CM}, we performed surface phenotyping analysis of each culture, comparing the resting T_{CM} to those stimulated with DC-flu or flu/28 aAPCs (Fig. 5). Gating on the flu HA tetramer-positive cells at baseline demonstrated a phenotype consistent with CD4 T_{CM}, because they were $CCR7^+$, $CD11a^{low}$, $CD27^+$, and $CD28^+$. The cells were heterogeneous for $CD45RA^+CD45RO^-$ (52%) and $CD45RA^-CD45RO^+$ (20%). After 2 mo of culture with either flu-DC or flu/28 beads, >99% of both cultures consisted of $CD3^+CD4^+$ lymphocytes. Of these, 95.6% were flu HA $tetramer^+$ in the DC-flu-stimulated culture, and 99.3% were flu HA $tetramer^+$ in the flu/28-stimulated culture. Both cultures were also stained for CCR7, CD62L, CD11a, CD28, CD45RA, CD45RO, HLA DR, CD27, CD25, and CD69. Flu-specific cells in both cultures were $CCR7^-$, $CD11a^{high}$, $CD28^+$, $CD45RA^-$, $CD45RO^+$, HLA DR^+, and $CD27^-$, which, except for the maintenance of CD28 expression, is consistent with an effector memory phenotype for T cells (1, 5). Interestingly, in some experiments, cells stimulated with DC-flu had re-expression of the lymph node-homing receptor CD62L, whereas repeated flu/28 stimulation resulted in a homogeneous population of cells that did not express CD62L. Lane and colleagues (34) have shown that memory CD4 T cells expressing CD62L are smaller, proliferate well in response to Ag, and have longer telomeres. Expression of CD25 and CD69 varied with time from the last stimulation, and was similar between the cultures stimulated with DC-flu and those stimulated with flu/28 beads (data not shown). In summary, the CD4 T_{CM} lost CCR7 and CD27 expression during culture, increased CD11a expression, and maintained CD28 expression. This phenotype was consistent in several donors and stable over several months of in vitro culture (data not shown), indicating that loss of CCR7 and CD27 expression did not correlate with loss of proliferative capacity in CD4 memory cells.

Functional analysis of human CD4 T_{CM}

To assess the functional status of the flu-specific cell cultures, we performed intracellular cytokine staining for IL-2, IFN-γ, TNF-α, and IL-10. After 2 mo in culture, each culture was either left unstimulated or was stimulated with flu/28 beads, CD3/28 beads, or PMA/ionomycin for 6 h in the presence of brefeldin A. Nearly all of the cells in both cultures produced IL-2 and IFN-γ when stimulated with PMA/ionomycin (72–91%), and ~20–40% produced cytokine when stimulated with Ag, despite the purity of the cultures (>95% flu HA $tetramer^+$) (Fig. 6, *A* and *B*). CD3/28 bead stimulation induced a greater number of cells to produce IL-2 and IFN-γ (40–70%) in both DC-flu- and flu/28-stimulated cultures (Fig. 6*A*). Cultures that had been stimulated for 2 mo on flu/28 beads had an equal number of cells produce IFN-γ when stimulated with flu/28 beads or CD3/28 beads for 6 h (~40%), suggesting that the tetramer was equivalent to anti-CD3 for inducing cytokine effector function. Results for intracellular staining of TNF-α were similar to those with IL-2 and IFN-γ, in that essentially all of the cells in both cultures produced TNF-α when stimulated with PMA and ionomycin, and 50–90% produced TNF-α when stimulated with flu/28 beads (Fig. 6*C*). In contrast, only a small percentage of cells produced IL-10 (Fig. 6*D*). The relative lack of IL-10 secretion, in conjunction with the preservation of IL-2 and IFN-γ secretion, suggests that regulatory T cells are not generated in significant numbers by aAPC using these culture conditions. Thus, ex vivo expansion of CD4 T_{CM} with either flu/28 beads or DC-flu generated functional Ag-specific T cells that preferentially produced Th1-type cytokines.

Given that ~60% of the cells did not secrete IFN-γ after Ag stimulation, we assessed the cultures for Th2 cytokines. Two-color intracellular cytokine analysis was performed on cells from flu/28- and DC-flu-stimulated cultures. Twelve to 18% of cells secreted IL-4 after PMA/ionomycin stimulation (Fig. 7), whereas Ag did not elicit significant IL-4 secretion (data not shown). The majority of cells producing IL-4 also secreted IFN-γ, indicating that, in humans, some flu-specific CD4 memory T cells are capable of producing Th2 cytokines in addition to Th1-type cytokines, i.e.,

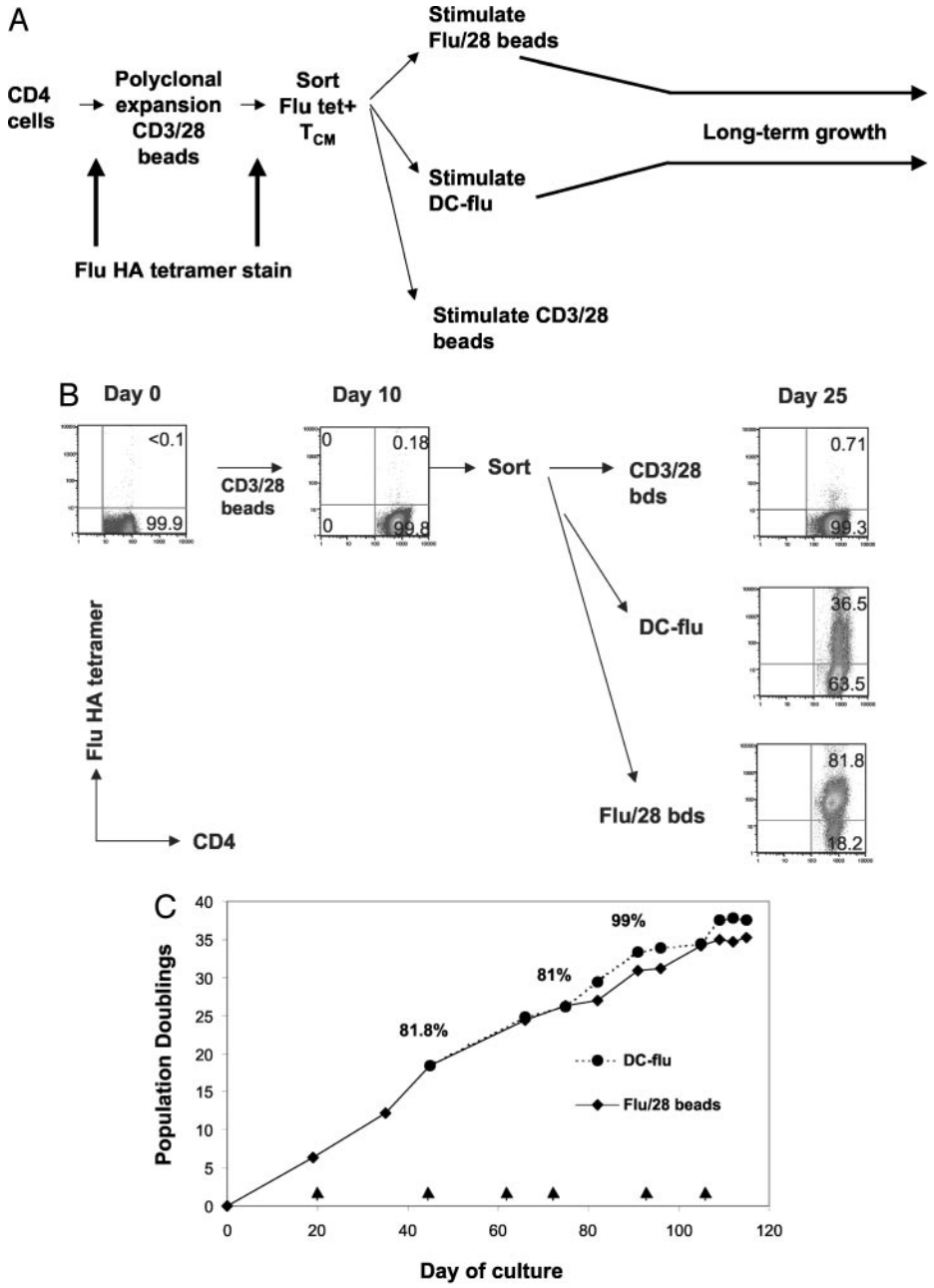

FIGURE 4. Sequential stimulation of CD4 T_{CM} with CD3/28 and flu/28 beads generates large numbers of Ag-specific T cells. *A*, Experimental protocol of cell preparation, FACS sorting, and cell culture with APCs. *B*, Assessment of Ag specificity by tetramer stains of input cells, after polyclonal expansion with CD3/28 beads, and Ag stimulation with flu/28 beads or peptide-pulsed dendritic cells. Plots are gated on size and $CD4^+$ lymphocytes. *C*, Growth curve of cells stimulated first with CD3/28 beads, and then either DC-flu or flu/28 beads. Arrows indicate restimulation with APCs. Percentages shown are the percentage of $CD4^+$flu HA $tetramer^+$ lymphocytes in the flu/28 bead culture. Data are representative of six experiments with two donors.

both IL-4 and IFN-γ, even after 60 days of culture. Although very few cells produced only IL-4 after stimulation with PMA and ionomycin, nearly all of the cells from both cultures produced IFN-γ. In summary, there was no substantial difference in cytokine production profiles between memory T cells that had been grown in flu/28 beads or flu-DC; both types of APCs generated Ag-specific $CD4^+$ T cells with a dominant Th1-like cytokine production profile, and retained a substantial percentage of flexible cells capable of producing both Th1 (IFN-γ)- and Th2 (IL-4)-type cytokines.

Discussion

We have described an efficient culture system for the enrichment and propagation of human memory CD4 T cells. Using this system, in combination with a single flow-cytometric sort, a >1000-fold enrichment of MHC II-restricted CD4 T_{CM} cells from the peripheral blood was routinely achieved within 2–3 wk of culture. The replicative capacity of human CD4 T_{CM} cells was found to be extensive using this culture system. The memory T cells retained effector function as judged by cytokine secretion for several months in continuous culture.

To construct efficient class II-restricted beads, we first tested several different approaches for the construction of tetramer-loaded beads to serve as aAPCs. Previously, we found that exogenous loading of tetramer onto beads indirectly via an Ab induced optimal production of IL-2 in an Ag-specific T cell culture (27). Next, we verified that HLA tetramer loading was titratable on these flu/28 beads, and that Ag-loaded beads bound specifically to Ag-specific T cells.

Using this novel class II-restricted aAPC culture system, we have found that CD4 T_{CM} have an extensive replicative capacity of at least 20–40 mean population doublings, representing a 10^6- to 10^{12}-fold expansion. Given this replicative capacity, clinical-scale adoptive immunotherapy appears feasible. Our use of tetramers to quantify CD4 T_{CM} cell division of input Ag-specific CD4 T cells with a limited repertoire (35) is likely to reveal a more accurate representation of T cell clonal life span than when bulk cultures are

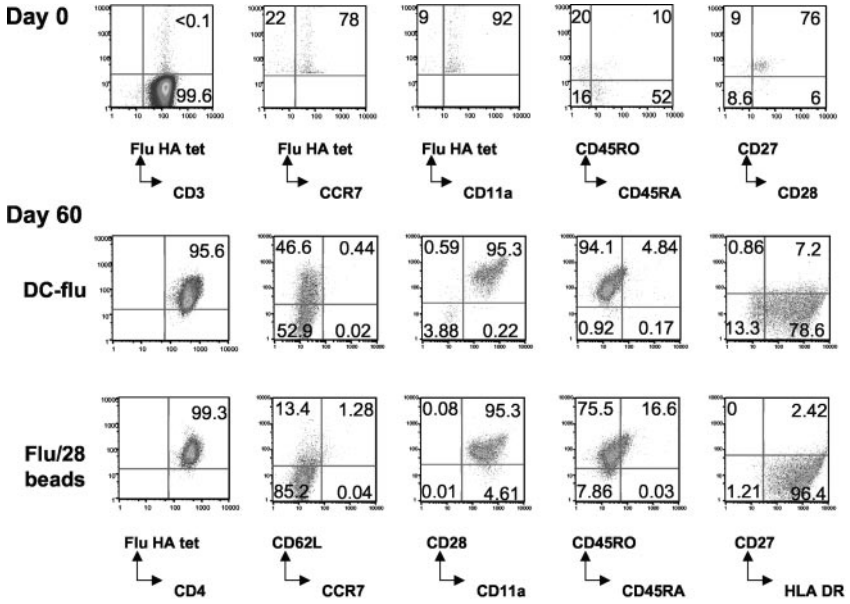

FIGURE 5. Characterization of surface phenotype analysis of flu-specific CD4 T_{CM} before and after expansion with peptide-pulsed dendritic cells or flu/28 aAPCs. *Top row*, All plots shown are gated on size and $CD4^+$ lymphocytes. All except first plot on *left* are gated on flu HA $tetramer^+$ staining as well. Percentage of gated cells is shown in quadrants. *Second* and *third rows*, Surface phenotyping of Ag-specific cells after 2 mo of culture. Plots shown are gated on size and $CD3^+CD4^+$ lymphocytes.

used (36). We are not aware of previous studies that have characterized the proliferative potential of human memory CD4 T cell subsets. In previous studies conducted before memory T cell subsets were defined, we found that polyclonal human CD4 T cells had a 10^9- to 10^{11}-fold expansion potential (21), and that memory CD4 T cells had shorter telomeres and a less extensive replicative potential than naive CD4 T cells (37). However, those studies were based on the use of CD45 isoforms to identify memory and naive populations, and were therefore subject to contamination due to incomplete separation of subsets based on the current understanding of lymphocyte differentiation (2, 38).

One of the main advantages of aAPC expansion methods is their potential for clinical applications. aAPCs can be engineered under the constraints of good manufacturing practices, large batches can be manufactured simultaneously, and product quality is easily verified. Although polyclonal CD3/28 beads have already been developed for use in the clinic, these beads were not suitable for maintaining the purity of an Ag-specific T cell population. One possibility is that the ratio of anti-CD3 to anti-CD28 on these beads has been optimized for naive T cells, and memory or previously stimulated cells require a different ratio of costimulation. For at least 2 wk, however, CD3/28 beads maintained the proportion of Ag-specific cells when stimulating bulk populations of CD4 cells, indicating that there was no inherent inability of the flu-specific cells to respond to CD3/28 beads. Alternatively, cells that have not previously divided have a higher replicative capacity than the Ag-specific memory T cells, and perturbations of the repertoire occur only after the several weeks that are required to generate pure Ag-specific T cell cultures. Finally, it is possible that, once Ag-specific stimulation is provided, either in the form of tetramer staining before FACS sorting or by stimulation with natural APCs, the Ag-specific cells acquire a growth disadvantage, due to down-regulation of the TCR, for example. In this case, even a relatively small number of T cells that have not been stimulated with Ag could rapidly outgrow the Ag-specific cells upon stimulation with CD3/28 beads, because even a minimal growth disadvantage can translate into major alterations in the repertoire of expanded cells during the extended time these cells are in culture. HLA tetramer sorting before CD3/28 stimulation may be particularly devastating to the Ag-specific population, because only the labeled cells receive TCR signaling without costimulation, down-regulate the TCR, and then are subjected to high-pressure cell sorting. These disadvantages compared with contaminating, unlabeled cells are overcome when only Ag-specific stimulation is provided. For expansion of CD4 T_{CM}, the bead-based aAPC had an efficiency of growth similar to that of mature autologous peptide-loaded DC. However, there were three notable differences between the culture systems. First, the apparent affinity of the T cells propagated by the bead-based system was consistently higher than the DC cultures. The reason for this is not clear. It is possible that the tetramers on the beads are only able to effectively signal the T cells with higher affinity TCRs. Alternatively, it is possible that the negative signaling from CTLA-4 that is expected in a DC-based culture system from B7:CTLA-4 interaction, would not occur in the bead-based system that uses anti-CD28 rather than B7 as the costimulatory signal. The CTLA-4 signal has been proposed to function to maintain a broad repertoire by terminating the signaling of the T cells with high-affinity TCRs (39). The ability of the bead-based aAPC to propagate high-affinity CD4 T_{CM} could be desirable for immunotherapy, because studies in mice show that high-affinity CTL are preferable to low-affinity CTL for adoptive tumor immunotherapy (40). The second difference between the aAPC- and DC-based culture system was that the bead-based system was not able to activate resting T_{CM}, and required previous activation with DC or CD3/CD28-coated beads. One possibility is that beads do not provide sufficient costimulation for resting cells; however, we have found that CD3/28 beads stimulate >95% of input resting polyclonal $CD4^+$ T cells (21, 41), indicating that TCR and CD28 stimulation provided on a bead scaffold is generally sufficient to activate resting cells. Thus, the failure of the flu/28 beads to activate resting T_{CM} likely represents differences in the signal delivered by beads prepared with class II tetramers rather than anti-CD3. This difference can be overcome by the addition of autologous feeder cells. At this time, it is unclear whether feeder cells quantitatively alter the signal delivered by flu tetramer ligation or provide a qualitatively distinct signal that can cooperate with flu tetramer signal to induce resting T cells into the cell cycle. The third difference uncovered between the aAPC and DC was in the quality of the immune synapse that was formed. The immunological synapse has been described as the accumulation of TCR, coreceptor, and CD28 in the contact area, and the exclusion of molecules such as the CD45 phosphatase and the integrin LFA-1 (29). Although initial

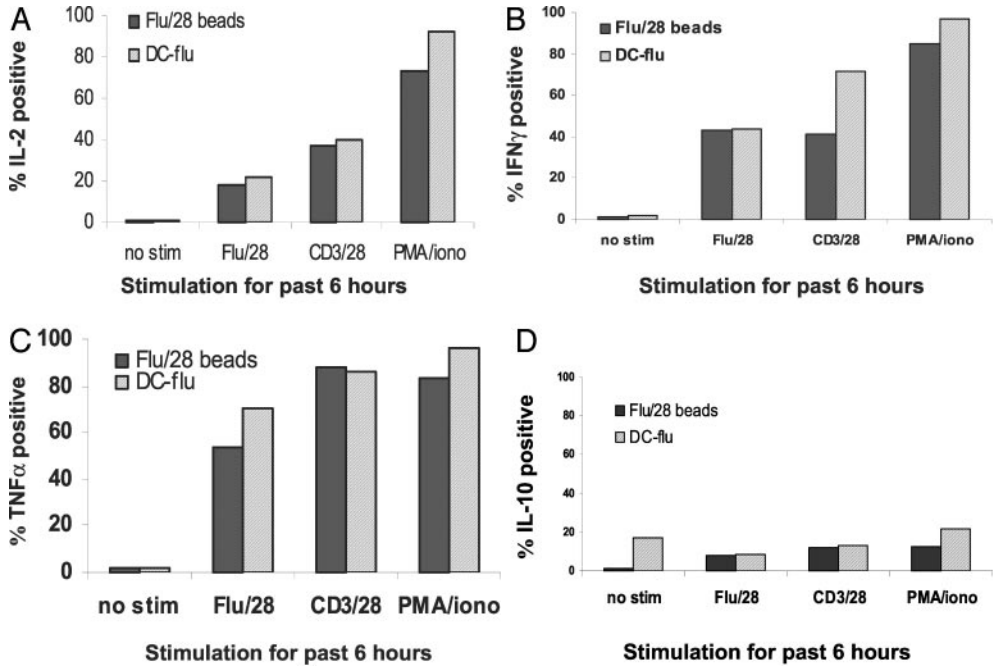

FIGURE 6. Characterization of cytokine secretion of flu-specific CD4 T_{CM} after expansion with peptide-pulsed dendritic cells or flu/28 aAPCs for 2 mo. Cytokine production was measured by intracellular cytokine staining after a 6-h stimulation of Ag-specific CD4 T cells. Both DC-flu- and flu/28 bead-stimulated cultures consisted of >95% $CD4^+$ flu HA tetramer-reactive cells. *A*, FACS staining for IL-2 secretion, with percentage of IL-2-positive cells shown on *y*-axis, and different stimuli shown on *x*-axis. *B*, FACS staining for IFN-γ secretion. *C*, FACS staining for TNF-α production. *D*, FACS staining for IL-10 secretion.

studies suggested that formation of the immunological synapse was required for T cell activation (42), recent evidence has shown that T cell activation signaling events precede formation of the synapse (43). In previous studies, we also found that flu/28 beads induced the formation of lipid rafts at the contact site (28), which is also thought to be required for T cell activation (44). Thus, formation of the complete immunological synapse (i.e., with exclusion of LFA-1) is not required for signaling or growth of Ag-specific human $CD4^+$ T cells, because flu/28 beads induced IL-2 production and T cell propagation. However, our data do not exclude the possibility that formation of a complete immunological synapse is required for the activation of naive or resting T cells, given that flu/28 beads were unable to activate resting T_{CM}.

Functional analysis of expanded Ag-specific CD4 T_{CM} revealed that, in the absence of polarizing treatment (i.e., anti-IL-12 or anti-IL4 Abs), both natural and artificial APCs tended to polarize the

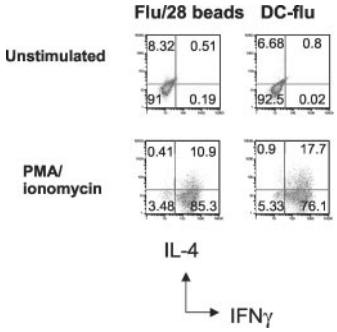

FIGURE 7. Th1/Th2 cytokine production in Ag-specific CD4 T cells. T_{CM} cells were cultured for 60 days by repeated stimulation with either flu/28 beads or DC-flu and were >95% flu HA $tetramer^+$. Cytokine production was measured by intracellular staining after 8 h of stimulation with PMA/ionomycin or medium. Plots shown are gated on size and $CD4^+$ lymphocytes.

T_{CM} toward Th1 cytokine production. However, unlike the situation described in mouse cells, where chromatin remodeling can permanently skew cytokine production (45), it appears that human cells are able to maintain the ability to produce both IFN-γ and IL-4. This has been described before in human T cell clones (46, 47), and more recently in polyclonal populations stimulated with anti-CD3 and anti-CD28 (48). The latter study demonstrated that, even when cells were stimulated under polarizing conditions, most human CD4 T cells retained flexible cytokine gene expression, because they switched their cytokine profile when stimulated under the opposite polarizing conditions. Recent studies have suggested that human CD8 T_{CM} and T_{EM} have largely distinct repertoires, raising the question whether the subsets arise from a direct and linear differentiation pathway (49). It will be interesting to use the aAPC that we have developed to determine the repertoire of human CD4 central and effector memory subsets.

The lack of outgrowth of regulatory T cells after extended culture of T_{CM} using aAPC is encouraging for future clinical applications for chronic viral infections and cancer. In addition, the extended ex vivo life span of human T_{CM} is encouraging for engraftment and long-term persistence of the cells after adoptive transfer. One of the main advantages of aAPC expansion methods is their potential for clinical applications. Unlike dendritic cells that require individual preparation and have batch-to-batch variation in potency, aAPCs can be engineered under the constraints of good manufacturing practices, large batches can be manufactured simultaneously, and product quality and consistency is easily verified. In summary, flu/28 aAPCs promote the expansion of high-affinity memory T cells, generating large numbers of Ag-specific CD4 T cells in a relatively short amount of time. The widespread application of class II Ag-specific aAPCs will be expedited by two major developments: technical progress in manufacturing a library of HLA class II-soluble molecules, and the discovery of corresponding class II-restricted viral and tumor Ags (50). In the meantime, class

II-restricted aAPCs will be a useful tool in studying the basic biology of human class II-restricted T cell activation and differentiation.

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