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Induction of human cytotoxic T lymphocytes by artificial antigen-presenting cells

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The adoptive transfer of antigen-specific cytotoxic T lymphocytes (CTLs) is a promising therapeutic approach for a number of diseases. To overcome the difficulty in generating specific CTLs, we established stable artificial antigen-presenting cells (AAPCs) that can be used to stimulate T cells of any patient of a given human leukocyte antigen (HLA) type. Mouse fibroblasts were retrovirally transduced with a single HLA–peptide complex along with the human accessory molecules B7.1, ICAM-1, and LFA-3. These AAPCs consistently elicit strong stimulation and expansion of HLA-restricted CTLs. Owing to the high efficiency of retrovirus-mediated gene transfer, stable AAPCs can be readily engineered for any HLA molecule and any specific peptide.

Keywords: adoptive cell therapy, $CD8^+$ T cell, costimulation, dendritic cells, immunotherapy, retrovirus-mediated gene transfer.

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The infusion of antigen-specific T lymphocytes is a potential therapy against certain cancers and infectious diseases[1–5]. One limitation to its broad usage is the generation of autologous T cells directed against well-defined epitopes. The induction and expansion of antigen-specific T cells require optimal antigen presentation and T-cell costimulation[6,7]. These requirements are met by antigen-presenting cells (APCs) such as Epstein–Barr virus-transformed B cells and dendritic cells (DCs), which constitutively express high levels of costimulatory, adhesion, and major histocompatibility complex (MHC) molecules[8,9]. Despite a cumbersome generation process, the use of autologous cells to present well-defined epitopes is mandated to obviate strong allogeneic responses. Therefore, we have undertaken to generate artificial APCs (AAPCs) with a single MHC restriction that could be used for any patient sharing the same MHC molecule. Several distinct signals contribute to effectively initiate and sustain T-cell activation and proliferation. The T-cell receptor must engage the MHC–peptide complex, which provides the basis for antigen specificity[10]. Signaling through the CD28 receptor provides a powerful costimulatory signal following engagement of the B7.1 (CD80) or B7.2 (CD86) ligand[11]. The adhesion molecule ICAM-1 (CD54) provides a synergistic signal through the LFA-1 (CD11/CD18) molecule expressed on T cells, whereas other molecules, in particular LFA-3 (CD58), ligand of the T-cell molecule CD2, can also mediate costimulatory as well as adhesion functions[12,13]. These accessory molecules are expressed at high levels on DCs, which are able to induce naive T lymphocytes[8], and a major role of B7.1, ICAM-1, and LFA-3 in costimulating cytotoxic T lymphocytes (CTLs) has been reported[14–16]. These three human costimulatory and adhesion molecules were retrovirally transduced in xenogeneic mouse fibroblasts with a single human leukocyte antigen (HLA) molecule. To efficiently present MHC–peptide complexes to CTLs, single MHC class I molecules were coexpressed with human β_2-microglobulin and a single genetically encoded peptide. Starting from peripheral blood T cells harvested from HLA $A2.1^+$ donors, we demonstrate potent induction and expansion of CTLs against viral and self-peptides presented in the context of HLA A2.1. Three epitopes derived from influenza matrix[17,18], MART-1[19], and gp100[20] proteins were investigated. Cytotoxicity was highly specific and increased by restimulation with the AAPCs. Induction of CTLs was more efficient than that obtained with autologous blood-derived DCs. Cytotoxic activity induced by AAPCs encoding the MART-1 or gp100-derived peptide was elevated against HLA $A2.1^+$ (but not $A2.1^-$) melanoma cell lines that express these antigens. These findings establish that high-level cell surface expression of B7.1, ICAM-1, LFA-3, and single MHC class I–peptide complexes is sufficient to effectively induce strong antigen-specific CTL responses in human peripheral blood cells. AAPCs should be useful for the investigation of primary T-cell activation and the generation of antigen-specific T cells for adoptive cell therapies.

Results

Construction of AAPCs. To generate AAPCs restricted to the HLA class I A2.1 molecule ($AAPC^{A2}$), replication-incompetent retroviral vectors were used to sequentially transduce NIH/3T3 fibroblasts with five vectors encoding, respectively, human B7.1, ICAM-1, LFA-3, human β2-microglobulin, and HLA A2.1 (Fig. 1A). To maximize and sustain expression of a specific HLA–peptide complex, a dicistronic vector encoding an HLA-restricted epitope and puromycin-*N*-acetyltransferase was used (Fig. 1A). The expression of the peptide, targeted to the endoplasmic reticulum by the human CD8 leader, was maintained under selective pressure with puromycin. High-level and stable expression of the different transmembrane molecules was obtained (Fig. 1B). By flow cytometry analysis, the levels of expression of A2.1, B7.1, ICAM-1, and LFA-3 were comparable to those measured on mature $A2.1^+$ dendritic cells (data not shown).

Artificial APCs efficiently stimulate flu-specific cytotoxic T-cell responses. Peripheral blood T lymphocytes harvested from HLA $A2.1^+$ donors were stimulated either with primary autologous DCs pulsed with the flu peptide or $AAPC^{A2}$ genetically engineered to express the same peptide ($AAPC^{A2F}$). Highly purified populations of T cells were prepared by positive selection (sheep red blood cells

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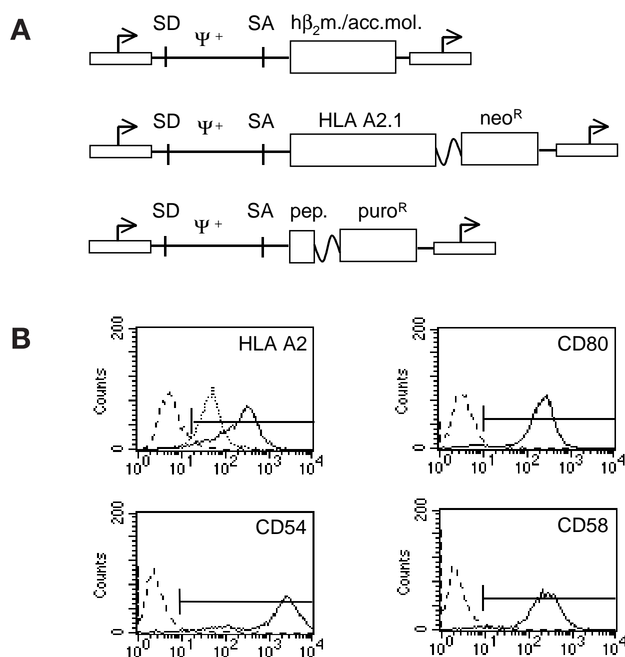

Figure 1. Generation of AAPCs from mouse fibroblasts. (A) Monocistronic retroviral vectors expressed human β_2-microglobulin (hβ_2m.) and the accessory molecules (acc.mol.) CD80, CD54, and CD58 (top). Dicistronic vectors were generated for HLA A2.1 and the peptide coding sequence (pep.), respectively linked by an internal ribosomal entry site to neomycin phosphotransferase (neo^R, middle) or puromycin-*N*-acetyltransferase (puro^R, bottom). SD, Splice donor site; SA, splice acceptor site; ψ^+, extended packaging signal. (B) Flow cytometry analysis of HLA A2.1, CD80, CD54, and CD58 expression in AAPCs. The same cells are stained for each molecule as indicated. Solid lines correspond to transduced NIH 3T3 cells and dashed lines to untransduced cells. For HLA A2.1, the dotted line corresponds to cells transduced with HLA A2.1 without human β_2-microglobulin, and the solid line to cells transduced with both cDNAs.

rosetting) and depletion of monocytes-macrophages, B cells, natural killer cells, and activated T cells (see Experimental protocol). After 8–10 days of stimulation, T lymphocytes cultured with AAPC^{A2F} exhibited strong flu-specific cytolytic activity (Fig. 2A). Typically the cytolytic activity was 1.6- to 4-fold higher than that obtained with primary dendritic cells pulsed with the flu peptide (115 and 65 lytic units, respectively, in Fig. 2A). The background on unpulsed target cells or on target cells pulsed with an irrelevant peptide was always lower than 5% (Fig. 2A). Examination of the cell surface phenotype of the CD8$^+$ cells showed a strongly activated profile, as reflected by the high level of expression of CD25 (low-affinity interleukin-2 receptor), CD69 (very early activation marker), and HLA DR (Fig. 2B). Fewer than 5% of the purified T cells expressed these markers at the start of the coculture (Fig. 2B). Furthermore, absolute cell counts of CD8$^+$ T cells on days 8–10 showed a higher cell yield following coculture with AAPCs than with primary DCs, about 2-fold higher in six different experiments ($P < 0.001$, Fig. 3). Such an expansion of CD8$^+$ T cells could not be reached with AAPCs expressing ICAM-1 and/or LFA-3 in the absence of B7.1. On the other hand, the presence of both accessory molecules increased the effect of B7.1 by a factor of 2 (unpublished observations).

Artificial APCs efficiently induce CTLs specific for self-antigens. To address whether AAPCs could induce a response against self-antigens, HLA A2.1$^+$ AAPCs encoding two peptides expressed in human HLA A2.1$^+$ melanoma cells were generated. One peptide is derived from the MART-1 protein[19] and the other from the gp100 protein including an amino acid substitution to enhance binding to HLA A2.1[20]. Highly purified T cells harvested from three HLA A2.1$^+$ donors were cultured with AAPCs expressing the MART-1 (AAPC^{A2M}) or

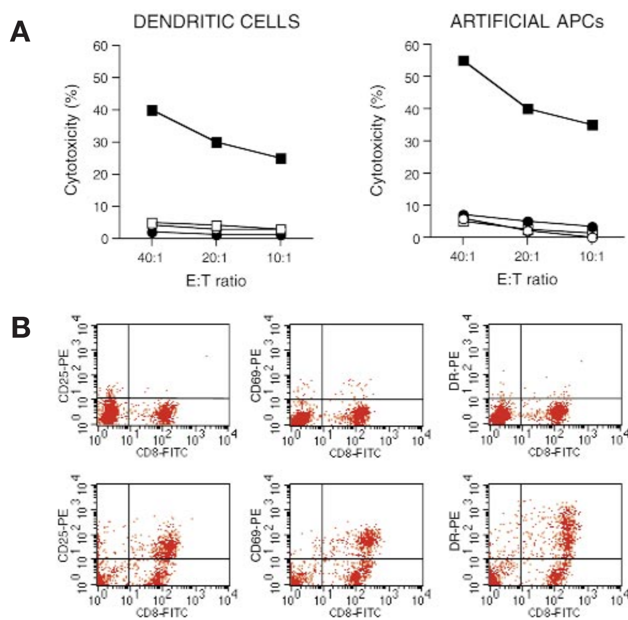

Figure 2. Stimulation of peripheral blood cytotoxic T cells against the flu peptide. (A) Cytotoxicity of T cells from HLA A2.1$^+$ donor stimulated with primary autologous dendritic cells (left panel) or AAPC^{A2F} (right panel). Standard ^{51}Cr release assays were performed using TAP-deficient A2.1$^+$ T2 target cells pulsed with the flu peptide (filled symbols) or the irrelevant MART-1 peptide (open symbols). Squares correspond to T cells stimulated against the flu peptide; circles to T cells stimulated without the relevant peptide. Y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios. (B) Flow cytometry analysis of CD8$^+$ T cells before (upper panels) and after (lower panels) cocultivation with HLA A2.1$^+$ AAPCs encoding the flu peptide. T cells were stained with a fluorescein isothiocyanate (FITC)-labeled antibody against CD8 (x-axis) and, from left to right, phycoerythrin–labeled antibodies against CD25, CD69, and DR (y-axis). Results are from one of six experiments with one representative donor.

gp100 (AAPC^{A2G}) derived peptide, using AAPC^{A2F} as control. After the first stimulation, as expected, a high response was obtained against the flu peptide in all three donors. In one donor, we readily detected a measurable CTL response against the MART-1 peptide (Fig. 4). After restimulation with the respective AAPCs, a readily detectable cytolytic response was obtained against all three peptides while the flu response further increased (Fig. 4). After restimulation, the response against the MART-1 peptide was of comparable magnitude to that obtained against the flu peptide after the first stimulation. The cytolysis obtained

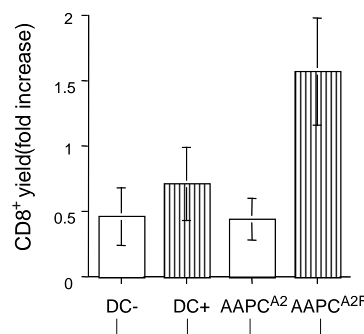

Figure 3. Expansion of primary CD8$^+$ T cells stimulated with AAPC^{A2F} or flu peptide-pulsed autologous dendritic cells. CD8$^+$ T-cell yield (fold increase, mean ± s.d) is indicated on the y-axis, corresponding to six independent experiments with the same donor. The yield was significantly greater with AAPC^{A2F} than with flu peptide-pulsed DCs ($P < 0.001$, Student's *t*-test). Similar results were obtained with two other donors. Open bars, stimulation without relevant peptide; hatched bars, stimulation against the flu peptide.

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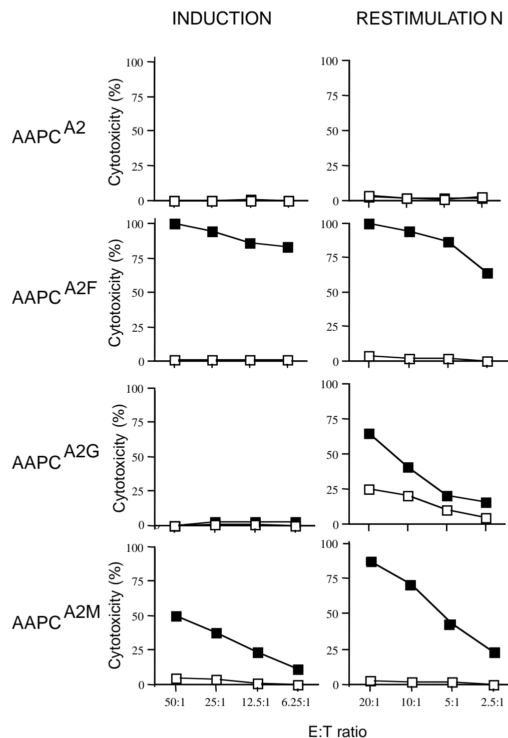

Figure 4. Artificial APCs induce cytotoxic T-cell responses against tumor antigens. Cytotoxicity was measured after the first stimulation (left panels) or after restimulation with the same AAPCs (right panels). Four HLA A2.1+ AAPCs were used: AAPC^{A2} without peptide (AAPC^{A2}), AAPC^{A2} expressing the flu peptide (AAPC^{A2F}), the gp100-derived peptide (AAPC^{A2G}), or the MART-1-derived peptide (AAPC^{A2M}). Cytotoxicity assays were performed with T2 cells as targets. Filled symbols correspond to target cells pulsed with the relevant peptide; open symbols to target cells pulsed with an irrelevant peptide (MART-1 peptide for CTLs stimulated with AAPC^{A2F}, flu peptide for CTLs stimulated with AAPC^{A2}, AAPC^{A2G} or AAPC^{A2M}). Y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios.

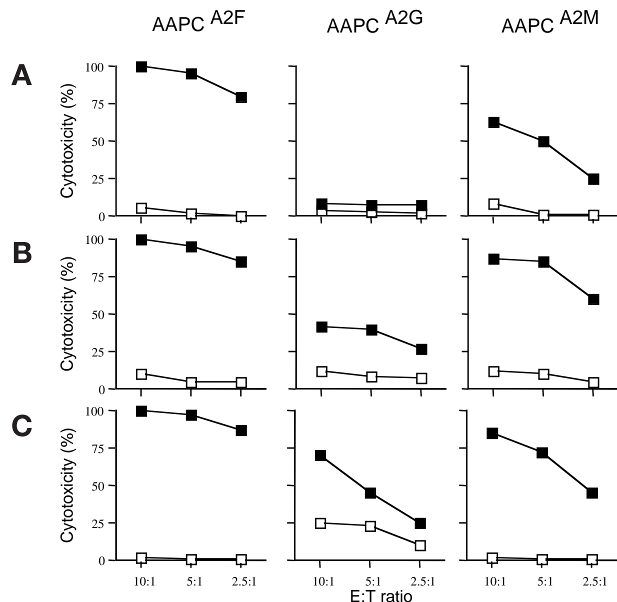

Figure 5. Cytotoxic T-lymphocyte induction against tumor antigens in different HLA A2.1+ donors. T cells purified from three HLA A2.1+ donors (A, B, C) were stimulated twice by AAPC^{A2F}, AAPC^{A2G}, or AAPC^{A2M}. Cytotoxicity stimulation was performed on T2 cells as described in Figures 2 and 4. y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios.

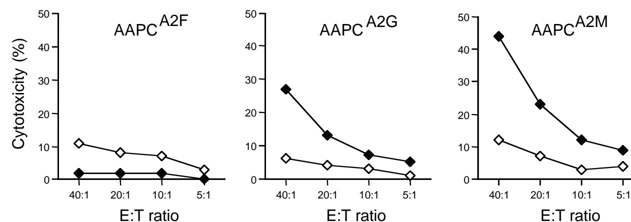

Figure 6. HLA-restricted cytolysis of melanoma cells by CTLs induced by AAPC^{A2G} and AAPC^{A2M}. Cytotoxicity of T cells of donor C (Fig. 5) induced by AAPC^{A2F}, AAPC^{A2G}, or AAPC^{A2M} against SK-MEL23 (HLA A2.1$^+$, filled symbol) and SK-MEL28 (HLA A2.1$^-$, open symbol). Y-axis, percentage of specific ^{51}Cr release; x-axis, effector:target (E:T) ratios. Cytotoxic T lymphocytes induced by AAPC^{A2M} and AAPC^{A2G} efficiently lysed SK-MEL23. The same low level of cytotoxicity was obtained against SK-MEL28 whether the CTLs were activated on AAPC^{A2F}, AAPC^{A2M}, or AAPC^{A2G}. Similar results were obtained with donor B of Fig. 5 (data not shown).

after two stimulations for the three peptides in three HLA A2.1$^+$ donors is shown in Figure 5. All three donors showed strong responses against the MART-1 peptide, and two out of three significantly responded to the gp100 peptide. Results obtained with these three donors in terms of cellular expansion and antigen specificity for all three peptides investigated in this study are summarized in Table 1. After two rounds of stimulation with AAPC^{A2F}, CD8$^+$ T-cell yields increased 25- to 80-fold. After two rounds of stimulation with AAPC^{A2G} or AAPC^{A2M}, CD8$^+$ T-cell yields increased 8- to 30-fold. CD8$^+$ T cells were highly activated, as indicated by their elevated expression of CD25, CD69, and HLA DR (with phenotypic profiles similar to those shown in Figure 2B).

Cytotoxic T lymphocytes induced by AAPC^{A2} that encode the MART-1 or gp100-derived peptide specifically lyse HLA A2.1$^+$ melanoma cells. To address whether T cells induced by AAPCs recognize and lyse melanoma cells in an HLA-restricted manner, cytotoxicity assays were performed using HLA A2.1$^+$ and HLA A2.1$^-$ melanoma cells as targets. The SK-MEL23 and SK-MEL28 cell lines both express MART-1 and gp100 proteins and are, respectively, A2.1$^+$ and A2.1$^-$ (ref. 21). T cells induced by AAPC^{A2G} or AAPC^{A2M} effectively lysed SK-MEL23 cells, showing, respectively, 30 and 45% lysis at the 40:1 effector:target ratio (Fig. 6). These T cells were HLA restricted as they failed to lyse SK-MEL28. On the other hand, T cells stimulated by AAPC^{A2F} failed to lyse SK-MEL23, demonstrating their high specificity. The low-level cytoxicity against SK-MEL28 was comparable whether the T cells had been previously stimulated by AAPC^{A2F}, AAPC^{A2G}, or AAPC^{A2M} (Fig. 6).

Discussion

Xenogeneic fibroblasts expressing retrovirally transduced HLA class I–peptide complexes along with CD80, CD54, and CD58 efficiently stimulate peripheral blood T cells of donors sharing the same HLA molecule. The AAPCs express a human tripartite complex comprising one HLA molecule, human β_2-microglobulin, and one encoded peptide. The total yield of CD8$^+$ T cells obtained by stimulation with AAPCs is higher than that achieved with peptide-pulsed autologous dendritic cells, albeit under distinct culture conditions. Several factors may contribute to the high efficiency of the AAPCs. The level of cell surface expression of HLA A2.1, CD80, CD54, and CD58 is elevated, comparable to that of mature primary HLA A2.1$^+$ DCs. The density of the specific HLA–peptide complex may also play an important role. Artificial APCs endogenously express under selective pressure the relevant peptide, which is targeted to the endoplasmic reticulum where peptides are loaded onto nascent HLA class I complexes[22,23]. Expression of the specific complex is therefore maintained irrespectively of the turnover of these complexes at the cell membrane, which is not the case with peptide-pulsed APCs, including artificial APCs derived from *Drosophila* cells[24]. Another advantage of using mouse fibroblasts com-

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Table 1. Expansion and antigen specificity of CD8+ T cells after one or two AAPC stimulation[a]

	AAPC^{A2F} A	AAPC^{A2F} B	AAPC^{A2G} A	AAPC^{A2G} B	AAPC^{A2M} A	AAPC^{A2M} B
Donor 1						
CD8+ T-cell number[b]	2	25	0.3	8	0.45	9
Specific cytotoxicity	40	98	3	7.5	10	60
Donor 2						
CD8+ T-cell number[b]	5	80	0.9	22.5	0.8	30.5
Specific cytotoxicity	85	95	5	35	10	75
Donor 3						
CD8+ T-cell number[b]	2.9	39.2	1.2	11.1	0.9	8.1
Specific cytotoxicity	80	99	1	45	25	80

[a]Between 6 and 18 million HLA A2.1+ donor T cells were plated on the different AAPCs on day 0. Cells were counted and stained for CD8, CD25, CD69, and HLA DR expression after the first (A) and second (B) stimulations. T-cell numbers correspond to a starting number of 1×10^6 CD8+ T cells. Specific cytotoxicity measured against T2 cells pulsed with the immunizing peptide (as in Figs 4 and 5) is shown as the 10:1 E:T ratio. Background activity measured at the same ratio against an irrelevant peptide (as in Figs 4 and 5) was subtracted.
[b]$\times 10^6$

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pared to *Drosophila* cells is their stability in culture and ease of manipulation. The low ability of fibroblasts to process and load peptides onto MHC molecules, as compared to professional APCs, may also contribute to enhance the expression of the specific HLA–peptide complex by decreasing simultaneous presentation of irrelevant peptides[25,26]. Furthermore, primary APCs, like DCs, express six HLA class I alleles and concomitantly present a greater diversity of HLA–peptide complexes. Cytotoxic T lymphocytes of other HLA–peptide specificities are therefore stimulated. In contrast, AAPCs express a single HLA class I molecule efficiently loaded with the relevant peptide.

Vigorous CTL responses were induced against two peptides expressed in melanoma, one derived from the MART-1 and the other from the gp100 antigen. After two rounds of T-cell stimulation, specific CTLs were induced in three out of three donors for MART-1 and two out of three for gp100. These findings are concordant with studies in melanoma patients and normal donors, suggesting that MART-1 elicits a greater immune response than gp100[27–29]. These results demonstrate that AAPCs can induce strong responses against autoantigens and suggest that they do not only recall primed CTLs—as is the case for the flu response—but also activate naive T cells present at a very low frequency in the peripheral blood of healthy donors. T cells induced by AAPCs against autoantigens specifically kill tumor cells that overexpress these antigens in an HLA class I-restricted manner. This strongly suggests that AAPCs may be used to expand CTLs for clinical purposes. Artificial APCs are stably transduced and thus obviate the need to generate autologous primary cells to effectively induce populations of antigen-specific T cells for each patient. Artificial APCs can easily be generated for different MHC–peptide combinations, and could be modified to stimulate T helper cells if MHC class II–peptide complexes are expressed. Additional costimulatory and/or adhesion molecules may further augment their capacity to promote the expansion of antigen-specific T-cell populations.

For adoptive immunotherapy using antigen-specific T cells, cell doses in the range of 10^9 are typically infused[1–5]. Based on a conservative estimation of 8-fold expansion obtained with AAPC^{A2G} or AAPCA^{2M} after two stimulations (Table 1), generation of 10^9 CD8+ T cells would require about 1.2×10^8 peripheral blood CD8+ T cells as the starting material, thus requiring 250–500 ml of blood. If additional cells were needed or if the starting cell number was less, a third round of stimulation or further nonspecific activation using, for example, beads coated with anti-CD3 and anti-CD28 antibodies[30] could be envisaged. Currently, virally infected B cells or DCs can be used to generate T cells for adoptive cell therapies[3–5,31,32]. Transduced mouse fibroblasts provide an alternative cellular system that is very effective in activating B lymphoma cells[33], restimulating genetically modified T cells[34,35], or activating and expanding human primary T cells as shown here. The use of viral vectors should facilitate the generation of AAPCs for other HLA molecules and peptides, starting from other cell types if necessary. Artificial APCs are therefore versatile and useful to study T-cell activation and to induce antigen-specific T cells for clinical purposes.

Experimental protocol

Vector construction. cDNAs were cloned into the *Nco*I and *Bam*HI sites of the SFG vector backbone[36]. A dicistronic vector encoding neomycin phosphotransferase 3′ of the encephalomyocarditis virus internal ribosomal entry site[37] was constructed to express HLA A2.1 (kind gift of Drs. S.Y. Young and N. Cereb). A dicistronic vector encoding puromycin-*N*-acetyltransferase was used for the minigenes encoding the different peptides used in this study. The human CD8α leader was fused to the peptide antigens to target the endoplasmic reticulum. Monocistronic vectors were constructed for the human β_2-microglobulin (kind gift of Dr. S.Y. Young), CD80 (ref. 35), CD54, and CD58 (kind gift of Dr. M. Dustin).

Gene transfer procedures. 293GPG packaging cells[38] were transfected with each plasmid by calcium chloride method as described[39]. A total of 5×10^4 NIH 3T3 cells (ATCC, Manassas, VA) were plated in a 6 cm plate and cultured in Dulbecco's modified Eagle medium (DMEM; Mediatech, Herndon, VA) with 10% heat-inactivated donor calf serum (DCS; Hyclone, Logan, UT), penicillin at 100 U ml^{-1}, and streptomycin at 100 μg ml^{-1}. They were infected the day after with cell-free retroviral supernatant (0.45 μm filtration, Acrodisc; Pall Corporation, Ann Arbor, MI) in the presence of polybrene (Sigma, St. Louis, MO) at 8 μg ml^{-1} for 16 h. Geneticin (Sigma) was added at 1.2 mg ml^{-1} to the medium for two weeks to select the cells expressing A2.1. Puromycin (Sigma) was added at 3 μg ml^{-1} to the medium for one week to select cells expressing the vector-encoded peptide. After transduction with a monocistronic vector, if gene transfer was extremely efficient (>95%), no cell purification was required. If gene transfer was less efficient, transduced cells were purified by using magnetic beads (Dynal, Oslo, Norway) or flow cytometry (Becton Dickinson, San Jose, CA).

Generation of dendritic cells and T-cell purification. Peripheral blood was obtained from normal HLA A2.1+ donors in heparinized tubes. HLA typing was performed by PCR in the HLA laboratory at MSKCC. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on lymphocyte separation medium (Accurate Chemical & Scientific Corporation, Westbury, NY). Dendritic cells were generated as described[40,41]. Briefly, the T-cell-depleted (ER−) population was prepared by rosetting with sheep red blood cells (Colorado Serum Company, Denver, CO) as described[42]. Two million ER− cells were plated per well in six-well plates. Gramulocyte-macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA) and Interleukin-4 (R&D Systems, Minneapolis, MN) were added at 1,000 U ml^{-1} every second day for eight days. Conditioned medium (CM) was prepared by adding 50×10^6 ER− cells on Petri dishes coated with human γ-globulins (Sigma) at 10 mg ml^{-1}. Nonadherent cells were removed and the CM, collected after 24 h, was added (a half or a third of the final volume) to the cells for four days to get fully mature DCs. After four days with CM, the cells had a phenotype of fully mature DCs: they had lost the expression of CD14, expressed high levels of CD40, CD80, MHC class I and class II molecules, and had acquired the expression of the specific marker CD83 (data not shown). T cells were purified as described[43]. Briefly, the T-cell-enriched (ER+) population was collected from the same donors. After lysis of the sheep red blood cells and three washes in phosphate-buffered saline (PBS) with 2% heat-inactivated fetal calf serum (FCS, Hyclone), B cells, natural killer cells, monocytes-macrophages, and activated T cells were depleted. This was accomplished by incubating cells with mouse IgG monoclonal antibodies directed against CD11b, CD16, and HLA DP, DQ, DR (Pharmingen, San Diego, CA) at 1 μg per million cells for 30 min, followed by a panning on Petri dishes coated with goat anti-mouse IgG (Caltag, Burlingame, CA) as described[44]. After three washes in PBS with 2% FCS, the T cells were resuspended at a final concentration of 10 million cells/ml. Dendritic cells were maintained in RPMI 1640 (Mediatech) with 10% FCS. T lymphocytes were maintained in AIM V medium (Life Technologies, Rockville, MD) without serum. Penicillin at 100 U ml^{-1} and streptomycin at 100 μg ml^{-1} were added to all the cultures.

Flow cytometry analysis. To analyze the phenotype of the AAPCs, we used antibodies against human β_2-microglobulin, A2.1 (kind gifts of Dr. S.Y. Young), B7.1 (Pharmingen), ICAM-1, and LFA-3 (Becton Dickinson). Anti-CD14, CD80, CD40, HLA DR (Becton Dickinson), and anti-CD83 (Immunex, Marseilles, France) antibodies were used to evaluate the level of

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maturation of the DCs. To verify the purity of the preparations of T cells and to study the phenotype of these T cells, we stained cells with antibodies anti-CD19, CD14, CD56, CD16, CD3, CD4, CD8, CD25, CD69, and HLA DR (Becton Dickinson).

Stimulation of specific CTLs. Dendritic cells were pulsed with the peptide (10 μM) for 2 h at room temperature in RPMI without serum. Coculture with T cells was established at the ratio 10 T lymphocytes to 1 DC in 24-well plates, with 1 million T cells per well for 8–10 days, in RPMI with 10% FCS. Artificial APCs were irradiated (1,500 Gy) and plated the day before in 24-well plates at the concentration 10^5 cells/ml in AIM V medium with 5% DCS, 500 μl per well. T cells were resuspended in AIM V medium at the concentration of 2×10^6 cells/ml, added to AAPCs at 500 μl per well, and cultured for 8–10 days. Interleukin-2 (IL-2; Chiron, St. Louis, MO) was added to the cultures after seven days (20 IU ml^{-1}, every third day). To restimulate the T cells 10–14 days after induction, T cells were cocultured with AAPCs following the same procedure, with 10^5 T cells per well for 10–14 days. Every third day, Il-2 at 20 IU ml^{-1} were added.

Cytotoxicity assays. Standard chromium release assays were performed, usinga as target cells Transfer associatred with antigen processing (TAP) protein-deficient HLA $A2.1^+$ T2 cells (kind gift of Dr. J.W. Young), loaded with the different peptides (10 μM, 1 h at room temperature, in RPMI without serum) before pulsing with ^{51}Cr for 1 h at 37°C. We used 5,000 T2 cells per well in 96 V-bottom plates at different effector:target cell (E:T) ratios for 4 h. We also used SK-MEL23 and SK-MEL28 cells as targets (kind gifts of Dr. P. Chapman). They are, respectively, HLA $A2.1^+$ and HLA $A2.1^-$ melanoma cell lines that express MART-1 and gp100 antigens[21]. SK-MEL cells were pulsed with ^{51}Cr as for the T2 cells. We performed 16-h cytotoxicity assays with 1,000 target cells per well. Specific ^{51}Cr release was calculated using the formula ((^{51}Cr release - spontaneous release)/(maximum release - spontaneous release)) × 100. Lytic units (LU) were calculated according to equation (12) in Bryant and coworkers[45].

Peptide synthesis. All the peptides were synthesized in the Peptide Synthesis Facility at MSKCC, resuspended in 50% (vol/vol) RPMI–dimethylsulfoxide (Sigma), and stored at -20°C. The following peptides were used in this study: the influenza matrix protein-derived peptide$_{58–66}$ GILGFVFTL (flu peptide); the MART-1 protein-derived peptide$_{27–35}$ AAGIGILTV; the gp100-modified peptide$_{209–217}$ IMDQVPFSV (g209-2M, which efficiently induces CTLs against the natural gp100 peptide[20]).

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