The effect of artificial antigen-presenting cells with preclustered anti-CD28/-CD3/-LFA-1 monoclonal antibodies on the induction of *ex vivo* expansion of functional human antitumor T cells

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ABSTRACT

Background
Adoptive cell therapy with *ex vivo* expanded autologous antitumor cytotoxic T lymphocytes represents an important therapeutic option as an anticancer strategy. In order to identify a reliable method for producing adequate amounts of functional antitumor cytotoxic T lymphocytes with a potentially long *in vivo* lifespan, we tested the T-cell expansion efficiency of a new artificial antigen-presenting cell-based system.

Design and Methods
Our artificial antigen-presenting cells were generated with activating (anti-CD3), co-stimulating (anti-CD28) and adhesion (anti-LFA-1) biotinylated monoclonal antibodies preclusterted in microdomains held on a liposome scaffold by neutravidin rafts. The co-localization of T-cell ligands in microdomains and the targeting of an adhesion protein, increasing the efficiency of immunological synapse formation, represent the novelties of our system. The activity of our artificial antigen-presenting cells was compared with that of anti-CD3/-CD28 coated immunomagnetic microbeads and immobilized anti-CD3 monoclonal antibody (OKT3 clone), the only two commercially available artificial systems.

Results
Our artificial antigen-presenting cells expanded both polyclonal T cells and MART-1-specific $CD8^+$ T cells in a more efficient manner than the other systems. Stimulation with artificial antigen-presenting cells allows for the generation of viable T cells displaying an immunophenotype consistent with *in vivo* potential for persistence, without increasing the frequency of regulatory T cells. The starting specificity of anti MART-1 $CD8^+$ T cells was preserved after stimulation with artificial antigen-presenting cells and it was statistically greater when compared to the activity of the same cells expanded with the other systems. Finally, our artificial antigen-presenting cells proved to be suitable for large-scale application, minimizing the volume and the costs of T-cell expansion.

Conclusions
Our artificial antigen-presenting cells might represent an efficient tool to rapidly obtain a sufficient number of functional T cells for adoptive immunotherapy in patients with cancer.

Key words: cytotoxic T cells, T-cell activation, T-cell differentiation, co-stimulation, tumor immunity.

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The online version of this article contains a supplementary appendix.

Introduction

Manipulation of the immune system by adoptive transfer of tumor-specific lymphocytes, activated and expanded *in vitro* or through anti-tumor vaccination, holds promise for the treatment of cancer.[1-5] However, both strategies have some drawbacks.[6] In fact, active immunotherapy has produced the most significant clinical results only in patients treated while in complete response after conventional chemotherapy.[2,3] Adoptive immunotherapy on the other hand has induced objective clinical responses even in patients with metastases and a conspicuous tumor burden,[7] but this strategy is technically demanding, as it requires the generation of large amounts of functional anti-tumor T cells for every patient. Improvement of this immunotherapeutic intervention requires strategies to boost the rapid expansion of anti-tumor T cells. One option is to use natural antigen-presenting cells (APC), usually dendritic cells or virally infected B cells, to activate and expand T cells for adoptive immunotherapy. However, this is extremely time-consuming as it may take months for T-cell expansion in response to stimulation with dendritic cells to reach the target amount of lymphocytes to be transferred into patients.[8-10] Moreover, after long-term *ex vivo* expansion, T cells are expected to have a limited capacity to replicate and persist once infused into patients.[11] Finally, other important obstacles to the widespread use of natural APC include the requirement for application only in the autologous setting and the lack of standard protocols for *in vitro* generation of dendritic cells with similar, reproducible phenotypes and immunostimulatory functions.[8,12]

These problems led to the production of artificial systems that express relevant molecules for T-cell expansion. In this way, the functional activity of artificial APC can be modulated by modifying the composition of the expressed molecules. Although several artificial systems have been produced,[13-24] only the following systems are suitable for clinical use, having been generated under conditions of *Good Manufacturing Practice*: immunomagnetic beads coated with anti-CD3 and anti-CD28 monoclonal antibodies (mAb)[25,26] and anti-CD3 mAb immobilized on culture plates. Although this technology can efficiently support the expansion of $CD4^+$ T cells,[27] the long-term growth of purified $CD8^+$ T cells is hampered.[28] One potential constraint of the artificial APC currently approved for clinical use is the suboptimal interaction of these systems with T cells.[29,30] In fact, these artificial APC lack a fluid membrane, which would allow ligands to cluster and activate T cells efficiently.[31]

The aim of this study was to engineer an artificial APC-based system with the properties of a fluid cellular membrane and the flexibility derived from an artificial structure that could be tailored to carry the desired immunostimulatory molecules. Recent evidence indicated that preclustering of MHC-peptide complexes in membrane microdomains on the APC surface affects the efficiency of immune synapse formation and the related T-cell activation.[32,33] Building on these data, we modified the liposome-based artificial APC recently described by Albani *et al.*,[34,35] which contained class II HLA molecule-peptide complexes associated with co-stimulatory molecules on the liposome rafts. In our artificial APC, the HLA-peptide complex was replaced by anti-CD3 mAb, and we added anti-LFA-1 mAb to allow an efficient artificial APC-T-cell interaction.

Here we show that, when compared to the other clinically approved artificial techniques, these artificials APC can expand more efficiently polyclonal T cells and antigen-specific cytotoxic T lymphocytes with immunophenotypic characteristics that suggest a potential long-term persistence *in vivo*.

Design and Methods

Generation of artificial antigen-presenting cells

In order to prepare artificial APC, we used the approach described by Albani *et al.* for the identification of rare antigen-specific T cells in autoimmune disorders[34-36] with the following modifications. Our artificial APC consisted of a scaffold made of GM1-enriched liposomes, which anchor microdomains where mouse anti-human CD3 (clone UCHT1, isotype IgG1k; BD PharMingen, San Diego, CA, USA), CD28 (cloneCD28.2, isotype IgG1k; BD PharMingen) and LFA1 (clone 38, isotype IgG2a; Biodesign, Saco, Maine, USA) triggering mAb had been preclustered (Figure 1A). To this end, biotinylated anti-CD3, anti-CD28 and anti-LFA1 mAb were combined with biotinylated cholera toxin B (Sigma-Aldrich Inc.) in a 3:1 molar ratio for 5 min at room temperature, as previously described.[34] Subsequently, neutravidin (Pierce Biotechnology, Rockford, IL, USA) was added at a ratio of 1 mol per four biotinylated moieties. After 15 min of incubation at room temperature, the ganglioside GM1-enriched liposomes (kindly provided by Dompè Biotec Spa, L'Aquila, Italy) were added. Liposomes were formed by detergent removal for 72 h through dialysis at 4°C against PBS in a 10K Slide A Lyzer (Pierce Biotechnology, Rockford, IL, USA). After 90 min of incubation, while mixing, the artificial APC solution was washed in PBS at 14000 rpm for 10 min. The pellet with artificial APC was resuspended in culture medium and used for T-cell expansion. As controls, only the liposomal formulation (liposome alone) or the biotinylated mAb on neutravidin rafts without liposomes (microdomains alone) were used in T-cell cultures in the same conditions of complete artificial APC (Figure 2). To validate the efficacy of the association of the three mAb on artificial APC, initial experiments were performed using APC whose microdomains contained only one type (anti-CD3, or anti-CD28, or anti-LFA1) or two types (anti-CD3 and anti-CD28, anti-CD28 and anti-LFA1, anti-CD3 and anti-LFA1) of biotinylated mAb. CFSE (CFDA, SE; Molecular Probe Inc. Eugene, OR, USA) stained peripheral blood mononuclear cells from healthy donors were stimulated with artificial APC containing one of the following combinations of mAb: anti-CD3/-CD28/-LFA1 (hereafter named standard artificial APC), anti-CD3/-CD28, anti-CD3/LFA1, anti-CD28/-LFA1, anti-CD3 alone, anti-CD28 alone, anti-LFA1 alone. For each combination of mAb, artificial APC were made by progressively reducing the concentration of each mAb using the two-fold serial dilution approach (range 1/1 to

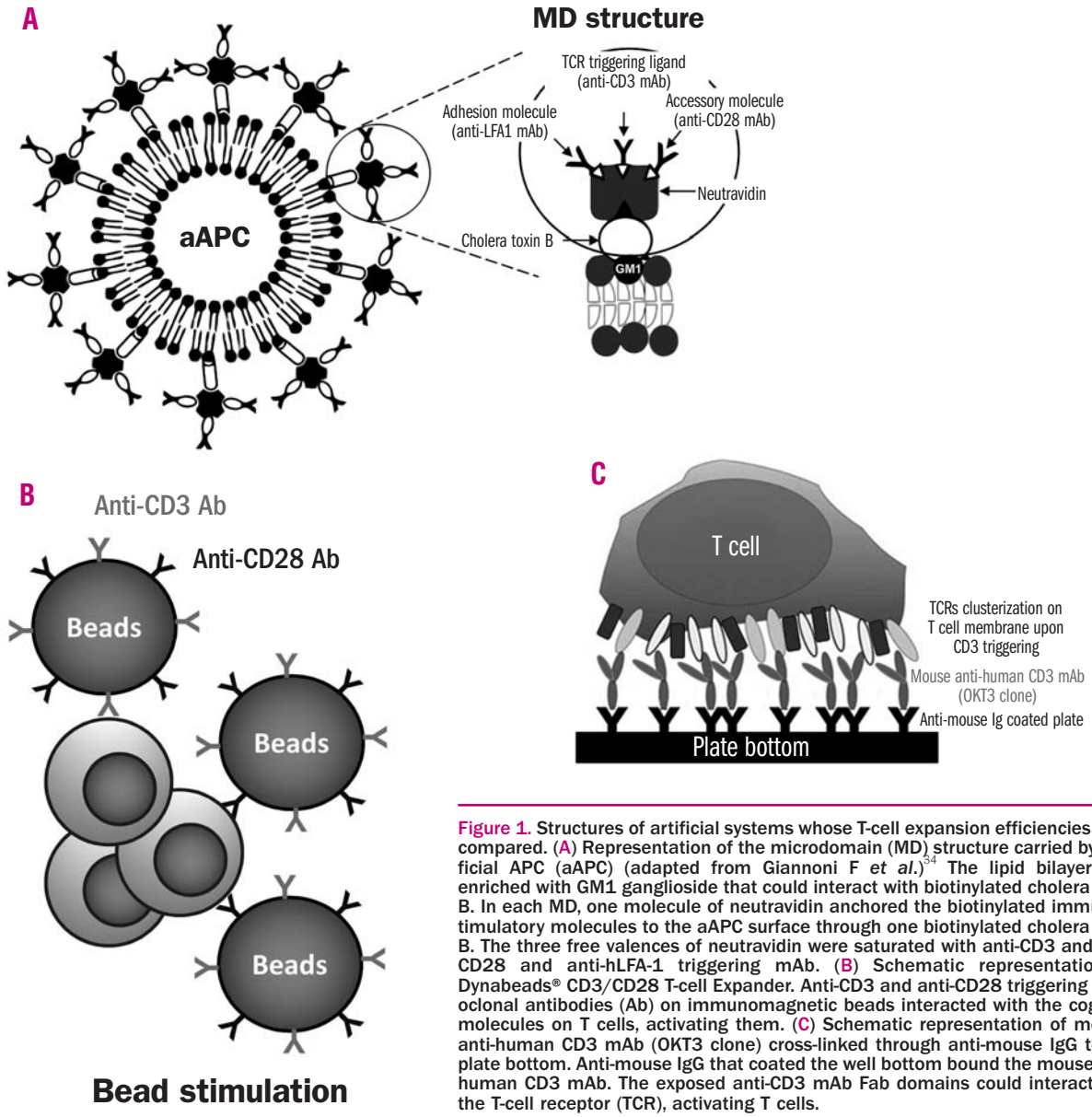


Figure 1. Structures of artificial systems whose T-cell expansion efficiencies were compared. (A) Representation of the microdomain (MD) structure carried by artificial APC (aAPC) (adapted from Giannoni F *et al.*)[34] The lipid bilayer was enriched with GM1 ganglioside that could interact with biotinylated cholera toxin B. In each MD, one molecule of neutravidin anchored the biotinylated immunostimulatory molecules to the aAPC surface through one biotinylated cholera toxin B. The three free valences of neutravidin were saturated with anti-CD3 and anti-CD28 and anti-hLFA-1 triggering mAb. (B) Schematic representation of Dynabeads® CD3/CD28 T-cell Expander. Anti-CD3 and anti-CD28 triggering monoclonal antibodies (Ab) on immunomagnetic beads interacted with the cognate molecules on T cells, activating them. (C) Schematic representation of mouse-anti-human CD3 mAb (OKT3 clone) cross-linked through anti-mouse IgG to the plate bottom. Anti-mouse IgG that coated the well bottom bound the mouse anti-human CD3 mAb. The exposed anti-CD3 mAb Fab domains could interact with the T-cell receptor (TCR), activating T cells.

1/64). CFSE dilution was assayed after 4, 7 and 10 days using a FACSCalibur flow cytometer and results were analyzed with CellQuest software (Becton Dickinson). In addition, artificial APC lacking the anti-LFA-1 antibody and standard ones were compared in 14-day cultures for the expansion of $CD3^+$ T cells in the presence of high-dose recombinant human (rh) interleukin (IL)-2 plus IL-15.

Human cells and culture conditions

Written informed consent was obtained from healthy donors and melanoma patients whose material was used in this study after Institutional Review Board approval. Two sources of T cells were tested for the expansion: (i) $CD3^+$ lymphocytes, negatively isolated from peripheral blood mononuclear cells obtained from the heparinized blood of healthy donors after Ficoll-Hypaque density gradient separation, using a Pan T cell isolation kit with a MiniMACS device (Miltenyi Biotech, Gladbach, Germany), according to the manufacturer's protocol and (ii) lymphocytes from metastatic lymph nodes were cultured for 2 weeks with the HLA-A*0201+ T2 cell line loaded with 10 μg/mL of Melan-A/MART-1 27-35 (modified sequence 27–35: ELAGIGILTV; PRIMM s.r.l., San Raffaele Biomedical Park, Milan, Italy)[37,38] as described previously.[39] Lymphocytes from melanoma-associated lymph nodes were analyzed for the frequency of $tetramer^+$ T cells in the $CD8^+$ fraction before and at the end of culture. T cells were cultured at a concentration of 0.2×10^6 cells/mL in a 96 flat-bottomed well plate with 250 μL/well of RPMI 1640 (Cambrex, Verviers, Belgium) containing 10% human serum, 1% PenStrep (Cambrex), 1% Glutamine (Cambrex) and 1% Hepes buffer (Cambrex). T-cell expansion was performed with a single administration of artificial APC or immunomagnetic microbeads (Figure 1B) coated with mouse anti-human CD3 and CD28 mAb (Dynabeads® CD3/CD28 T Cell Expander or

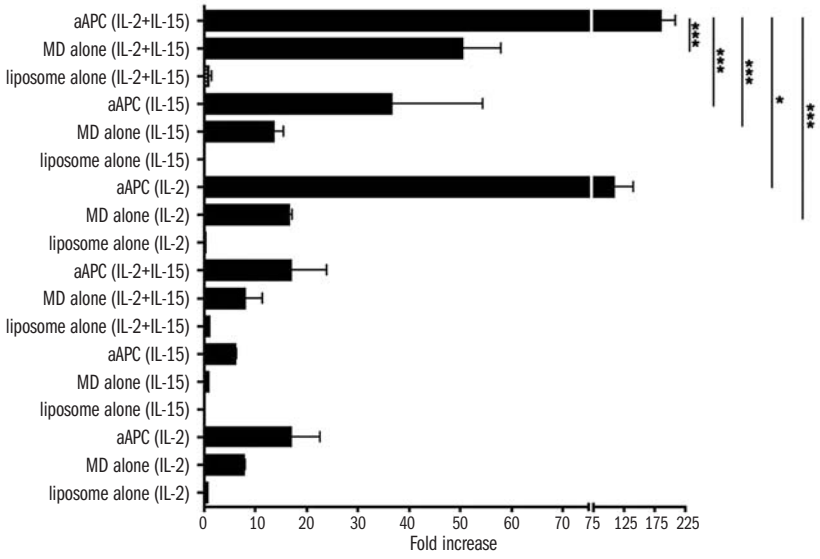


Figure 2. Activity of complete artificial APC (aAPC) compared to their single components. Purified polyclonal CD3+ T cells freshly isolated from healthy donor peripheral blood were stimulated with aAPC or microdomains (MD) or liposomes and cultured for 14 days with low-dose IL-2, or IL-15, or the combination of high-dose IL-2 and IL-15. After 7 and 14 days of culture, expanded T cells were counted using the trypan blue dye exclusion test and the fold increase in T-cell number was calculated for each condition. Results are representative of four independent experiments with cells from different donors. Error bars indicate standard deviation of the mean. Statistically significant differences, calculated using two-tailed *t* test, are reported (*: $p \leq 0.05$; ***: $p \leq 0.01$).

Dynabeads® ClinExVivo CD3/28, Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's instruction, or mouse anti-human CD3 mAb (functional grade purified anti-hCD3, OKT3 clone, eBioscience, San Diego, CA, USA) immobilized on the well bottom. Anti-CD3 mAb was cross-linked (Figure 1C) to the well bottom of 96-flat bottomed well plates precoated with anti-mouse IgG (whole molecule, SIGMA). To this end, plates were incubated overnight at 4°C with anti-mouse IgG at 10 μg/mL in PBS (Cambrex). Plates were then washed three times with PBS and anti-CD3 was added at a concentration of 0.5 μg/mL of RPMI 1640 (50 mL/well) and the plates were incubated for 45 min at 4°C. The incubation was stopped by blocking the plates with 20% human serum-supplemented RPMI 1640 (50 μL/well). These cultures were carried on with the following combination of γ-chain cytokines according to previous results:[40] (i) low-dose rhIL-2 (300 U/mL, Proleukin, Chiron, Emeryville, CA, USA); (ii) rhIL-15 (Peprotech Inc., Rocky Hill, NJ, USA) 10 ng/mL; (iii) high-dose rhIL-2 (3000 U/mL) plus rhIL-15 10 ng/mL. Complete culture medium with cytokines was replaced every 2 days during the 14-day expansion after the artificial stimulation. The artificial APC/T-cell ratio was reduced by diminishing mAb concentrations for the generation of the APC or by increasing the starting amount of T cells in culture with the APC produced as previously described. In the first case, the biotinylated mAb were added at 1/10 or 1/100 of the standard dose. These artificial APC were used for 14-day expansion of 0.05×10^6 CD3+ purified T cells in association with high-dose IL-2 and IL-15 and were compared to the standard ones in the same culture condition. In addition, standard artificial APC were tested for the expansion of 10- or 100-fold increased numbers of starting T cells. Briefly, 0.5×10^6 or 5×10^6 CD3+ purified T cells were cultured in 1 or 4 mL of culture medium in 48- or 12-well plates, respectively, with standard artificial APC in the presence of high-dose IL-2 plus IL-15. Culture medium with cytokines was replaced every 2 days during the 14-day expansions.

Flow cytometry analysis

Flow cytometry analysis of expanded T cells was performed on a FACSCalibur using the CellQuest software (Becton Dickinson). Data were subsequently analyzed using FlowJo software. T-cell maturation and activation phenotype were evaluated by staining with the following mouse-anti-human mAb in different combinations: FITC-labeled anti-CD3, PE-labeled anti-CD25 (Miltenyi Biotech, Gladbach, Germany), FITC-labeled anti-CD62L, FITC-labeled CD27, PE-labeled anti-CD45RA, PE- or PerCP-labeled anti-CD4, anti-CD69 and anti-CD3, APC-labeled anti-CD8 and anti-CD4 (BD Biosciences, San Jose, CA, USA), APC labeled anti-CCR7 (R&D systems, Minneapolis, MN, USA). To detect antigen-specific T cells directed to Melan-A/MART-1, lymphocytes were stained with PE-labeled tetramers of HLA-A 0201 containing Melan-A/MART-1$^{27-35}$ peptide (Beckman Coulter Inc., Fullerton, CA, USA). Surface staining was performed by incubating mAb at 4°C for 30 min. Regulatory T-cell expansion was assayed by intracellular hFoxP3 staining using the FITC-labeled anti-human FoxP3 staining kit (eBioscience, San Diego, CA, USA) in association with PE-labeled anti-CD25, PerCP-labeled anti-CD8 and APC-labeled anti-CD4 surface mAb, according to the manufacturer's protocol. Apoptosis assays were performed by staining with FITC-labeled annexin V and propidium iodide (rh Annexin V/FITC Kit, Bender MedSystem, Vienna, Austria), according to the manufacturer's protocol. To detect intracellular perforin or granzyme B in T cells after 14 days of stimulation with artificial APC, microbeads or immobilized anti-CD3, expanded T cells were permeabilized with Cytofix/Cytoperm (BD Biosciences) and then stained with mouse-anti-human FITC–labeled perforin (BD Biosciences), or PE–labeled granzyme B (CLB, Amsterdam, The Netherlands) in the presence of Perm/Wash solution (BD Biosciences). To demonstrate that artificial APC were retained on the T-cell surface during expansion, 7 and 14 days after stimulation, T cells were stained with FITC-labeled goat-anti-mouse IgG mAb (Jackson ImmunoResearch Laboratories

Inc., West Grove, PE, USA) in order to detect the mouse-anti-human mAb loaded on microdomains. To assess the sensitivity of this flow cytometry-based assay a fixed amount of human CD3+ T cells was incubated with decreasing concentrations of anti-CD3, anti-CD28 and anti-LFA-1 mAb, followed by staining with a FITC-labeled anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc.). The mean fluorescence intensities (MFI) of T cells were then plotted against the known concentrations of anti-CD3, anti-CD28 and anti-LFA-1 mAb to obtain a titration curve.

Cytotoxic assay

The cytotoxic activity of expanded specific MART-1 T cells was assessed through a ^{51}Cr-release assay using an HLA-A*0201+ lymphoblastoid cell line (LCL 9742) loaded with Melan-A/MART-1$_{27-35}$ peptide as the target. Negative controls were unloaded LCL or LCL loaded with Influenza A (Flu) Matrix$_{58-66}$ (GILGFVFTL) or HIV (ILKEPVHGV) produced by PRIMM s.r.l., San Raffaele Biomedical Park, Milan Italy. Synthetic peptides were ≥95% pure.[39] Results are expressed as follows:

% Lysis = (experimental release (cpm) – spontaneous release (cpm)) / (maximum release (cpm) – spontaneous release (cpm)) × 100

where spontaneous release was assessed by incubating target cells in the absence of effectors and maximum release was determined in the presence of 1% Nonidet P40 detergent (BDH Biochemical, Poole, UK).

Statistical analysis

Statistical significance was determined using the two-sided Student's t test. Regression analysis was conducted using GraphPad Prism version 5 for Apple Macintosh software (GraphPad Software, La Jolla, CA, USA).

Results

Artificial antigen-presenting cells efficiently bind and expand human polyclonal T cells

As shown in Figure 1, artificial APC consist of a scaffold made of GM1-enriched liposomes, which anchor microdomains of preclustered activating biotinylated mAb (anti-CD3, anti-CD28, and anti-LFA1) through biotinylated cholera toxin B subunit and neutravidin. The highest proliferation rate of selected polyclonal CD3+ T cells (mean purity 90±3%) was obtained when all the three activating molecules were combined in the same APC. In fact, artificial APC built with anti-CD3 and anti-CD28 mAb, but lacking anti-LFA-1 mAb, were less efficient, in terms of T-cell expansion at both 14 and 28 days compared with those made with anti-CD3/CD28/LFA-1, (*Online Supplementary Figure S1*). In the subsequent experiments APC containing the mixture of all three mAb were tested in association with low-dose IL-2, or IL-15 or high-dose IL-2 plus IL-15 for their ability to expand *in vitro* human polyclonal CD3+ T cells. As controls, T cells were cultured with liposomes or with microdomains alone. After 7 days of stimulation, the greatest efficiency was observed when complete artificial APC were associated with any cytokine combination. However, after 2 weeks, artificial APC combined with exogenous high dose IL-2 plus IL-15 resulted in the greatest fold increase in T cells, which was statistically significant when compared to the other conditions (Figure 2).

Microdomains alone provided a positive stimulus for T-cell proliferation, since they consisted of the biotinylated activating molecules grouped on neutravidin, but putting them into a lipid membrane allowed their activity to be efficiently oriented, increasing the final stimulus for T-cell expansion. Liposomes alone did not provide stimulation for T-cell expansion (Figure 2).

Artificial antigen-presenting cells stimulation enhances survival of human polyclonal T cells

The activity of our artificial APC was compared with that provided by the other commercially available artificial systems for T-cell expansion (anti-CD3 and anti-CD28 coated immunomagnetic microbeads, Dynabeads® CD3/CD28 T Cell Expander and immobilized anti-CD3 mAb, clone OKT3). Freshly purified polyclonal CD3+ T cells were stimulated with one of these systems and expanded in the presence of IL-2 and/or IL-15. At 2 weeks of culture, microbeads and artificial APC in combination with high-dose IL-2 plus IL-15 gave the best results, and showed comparable efficacy of polyclonal T-cell expansion (Figure 3A). However, when apoptosis of expanded T cells was evaluated, our artificial APC preserved the highest cell viability in culture compared to that afforded by the other artificial systems (Figure 3B). In the bead-stimulated culture, only 46% of expanded T cells were viable (annexin V-/propidium iodide-), whereas 83% of T cells were still alive 14 days after the stimulation with artificial APC. Thus, our artificial APC provided the highest expansion of viable T cells among the artificial systems tested (Figure 3C). As a further control, anti-CD3/-CD28 microbeads were compared to artificial APC lacking anti-LFA-1 mAb, to evaluate the efficiency of T-cell expansion of the two systems, both generated to target CD3 and CD28 molecules on T cells. Even in these conditions, our system was able to expand a higher number of T cells without affecting their viability (*Online Supplementary Figure S2*). These results demonstrated the advantages provided by the presence of a fluid membrane on our artificial APC as a scaffold carrying the stimulating molecules. However, the addition of anti-LFA-1 mAb allowed a further increase in the efficiency of our membrane-based APC (*Online Supplementary Figure S2*).

Immunophenotype of expanded polyclonal T cells

Fourteen days after stimulation with artificial APC or beads, T cells displayed a naïve (CCR7+CD45RA+ CD62L+) or an effector (CCR7-CD45RA+ CD62L-) phenotype (Figure 4, *Online Supplementary Figure S3* and *Online Supplementary Table S1*). By contrast, the naive population was greatly reduced in T cells expanded with immobilized anti-CD3 mAb (Figure 4). T cells expanded with artificial APC or with microbeads expressed CD27 at high levels (*Online Supplementary Figure S3*). In all experimental conditions a large fraction of T cells expressed the activation marker CD69 (Figure 4A). Expression of the homing

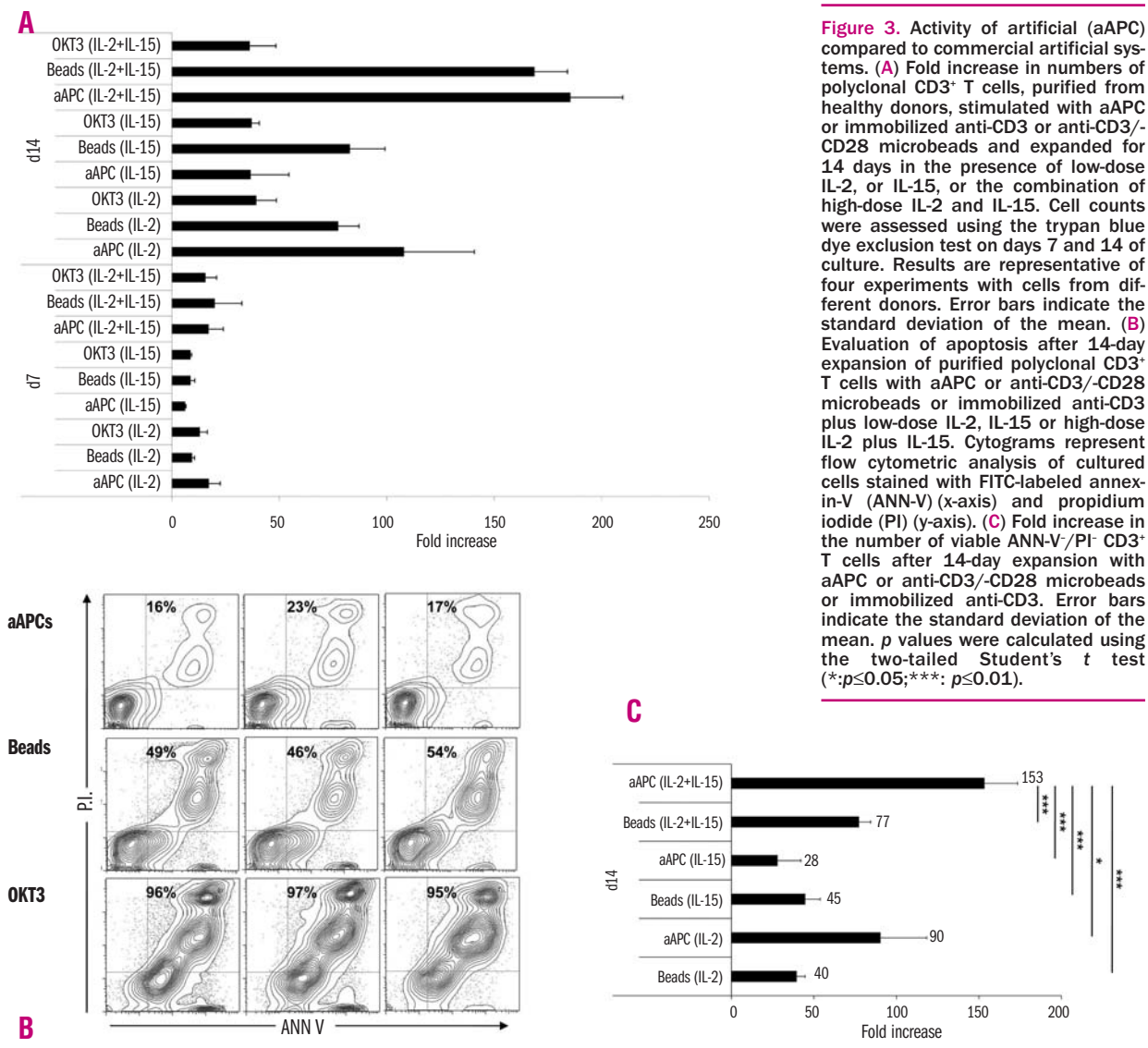


Figure 3. Activity of artificial (aAPC) compared to commercial artificial systems. (A) Fold increase in numbers of polyclonal CD3+ T cells, purified from healthy donors, stimulated with aAPC or immobilized anti-CD3 or anti-CD3/-CD28 microbeads and expanded for 14 days in the presence of low-dose IL-2, or IL-15, or the combination of high-dose IL-2 and IL-15. Cell counts were assessed using the trypan blue dye exclusion test on days 7 and 14 of culture. Results are representative of four experiments with cells from different donors. Error bars indicate the standard deviation of the mean. (B) Evaluation of apoptosis after 14-day expansion of purified polyclonal CD3+ T cells with aAPC or anti-CD3/-CD28 microbeads or immobilized anti-CD3 plus low-dose IL-2, IL-15 or high-dose IL-2 plus IL-15. Cytograms represent flow cytometric analysis of cultured cells stained with FITC-labeled annexin-V (ANN-V) (x-axis) and propidium iodide (PI) (y-axis). (C) Fold increase in the number of viable ANN-V-/PI- CD3+ T cells after 14-day expansion with aAPC or anti-CD3/-CD28 microbeads or immobilized anti-CD3. Error bars indicate the standard deviation of the mean. *p* values were calculated using the two-tailed Student's *t* test (*:$p \leq 0.05$;***: $p \leq 0.01$).

adhesion molecule CD62L on naive, expanded T cells should make the cells capable of trafficking through lymph nodes. On the other hand, the high expression of a marker associated with T-cell receptor engagement (CD69) suggests that such cells are acutely activated and could exert immediate effector functions. In some experiments, artificial APC or microbeads, or adherent anti-CD3 mAb-stimulated T cells were cultured for 4 weeks. As shown in *Online Supplementary Table S2*, T cells expanded for 27 days following stimulation with artificial APC expressed CD62L at diminished levels and showed an increased percentage of CD45RA+CD62L-CCR7- effector cells compared to the same cultures after 14 days of expansion. Moreover, long-term T-cell expansion led to the down-regulation of CD25 expression, suggesting the exhaustion of T-cell activation. Similar results were obtained for microbead- or adherent anti-CD3 mAb-stimulated T cells (*data not shown*). Collectively, these observations suggested that 2 weeks of culture was the optimal time window to expand a large amount of T cells with suitable immunophenotypic characteristics for adoptive immunotherapy. In addition, when our artificial APC were used, CD8+ T cells were predominantly expanded, while CD4+ T cells were preserved at a low level. In contrast, anti-CD3/-CD28 microbead and immobilized anti-CD3 stimulation preferentially gave rise to CD4+ lymphocyte expansion (Figure 4 A-B). Finally, the risk of also expanding regulatory T cells was evaluated by assessing their frequency in the culture of polyclonal T lymphocytes stimulated with the different artificial systems. Two weeks after the stimulation with artificial APC, the frequencies of CD4+FoxP3+ T cells and CD8+FoxP3+ T cells were significantly lower than after stimulation with microbeads or anti-CD3 (Figure 4A-B). When regulatory T cells were analyzed, both CD25+ and CD25- FoxP3+ CD4+ were considered, given the recent evidence that FoxP3 expression can be found independently of CD25.[41]

By comparing the mean fluorescence intensity (MFI) for granzyme B staining on the expanded CD8+ or CD4+ T cells, our artificial APC resulted in the lowest up-regulation of granzyme B expression among the artificial stimulation systems tested (Figure 4C, left panel). Moreover, using our artificial APC, perforin expression was kept at the lowest level and was confined to minimal fractions of

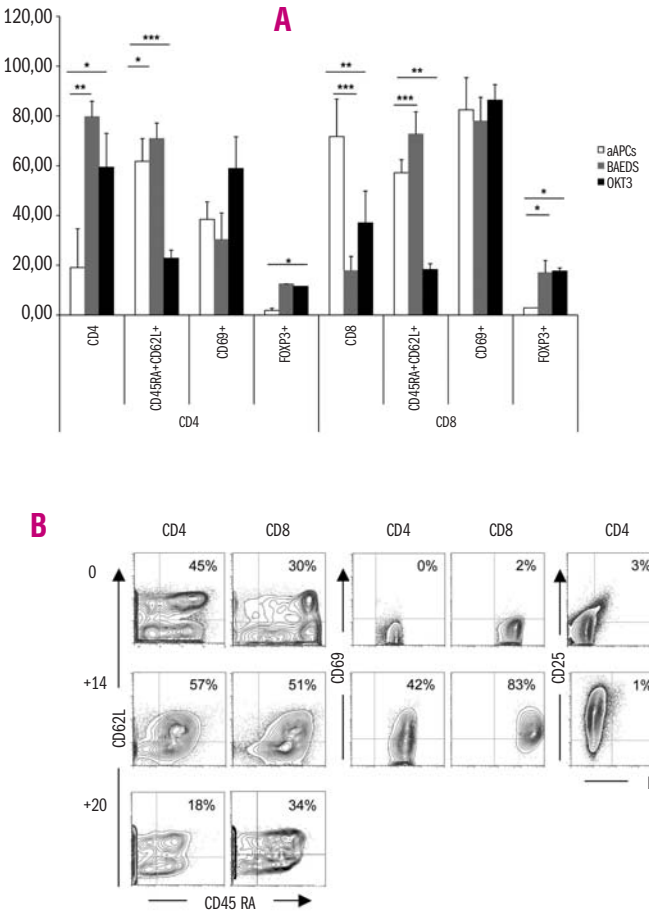


Figure 4. Phenotypic characteristics of polyclonal human T cells expanded with artificial APC (aAPC) or other artificial systems. (A) Histogram of the frequencies of the most relevant T-cell subsets in cultures expanded with high-dose IL-2 plus IL-15 and aAPC or OKT3 or anti-CD3/-CD28 microbeads. Results are representative of four independent experiments with cells from different donors. Error bars indicate the standard deviation of the mean. Statistical analyses of aAPC versus microbeads and of aAPC versus immobilized anti-CD3 ones were performed using the two-tailed Student's t test (*: $p \leq 0.05$;**:$p \leq 0.02$; ***: $p \leq 0.01$). (B) Representative example of T-cell immunophenotype after 14-day expansion with aAPC stimulation and high-dose IL-2 plus IL-15. Multiparametric FACS analysis was performed by gating on $CD4^+$ cells versus side scatter and $CD8^+$ cells versus side scatter populations. (C) Histogram plot representation of intracellular expression of granzyme B (left panel) and perforin (right panel) in $CD8^+$ T cells (upper panel), identified by gating on the $CD3^+CD8^+$ T-cell subset, and in $CD4^+$T cells (lower panel), identified by gating on the $CD3^+CD4^+$ T-cell subset. For each condition, the mean fluorescence intensity of granzyme B and perforin expression is reported.

the expanded CD8+ and CD4+ T cells (Figure 4C, right panel). The reduced differentiating properties of our artificial APC were confirmed even analyzing the expression of perforin and granzyme B in the CCR7+ and CCR7− subpopulations in both CD4+ and CD8+ cell subsets (*Online Supplementary Figure S4*). These observations suggest that T cells expanded in the presence of our artificial APC might exert a cytolytic function, since they express granzyme B, but they require further activation, due to the limited induction of perforin. This phenotype is consistent with the high expression of CD62L and with the increased survival of T cells expanded with artificial APC.

Specific cytotoxic activity of T cells efficiently expanded by artificial antigen-presenting cells is preserved

Since the artificial APC rapidly activated and expanded polyclonal T lymphocytes, we also tested the effect of the APC on the expansion of human anti-melanoma cytotoxic T lymphocytes enriched for MART-1 specificity. To this end, lymphocytes from tumor-invaded lymph nodes of HLA-A*0201+ melanoma patients were activated by culture with the MART-1 peptide-loaded HLA-A*0201+ TAP-deficient T2 cell line. After 2 weeks of culture with peptide-loaded T2 cells, the resulting T-cell lines were restimulated for 2 more weeks with antigen or with standard artificial APC, or immobilized anti-CD3, or anti-CD3/-CD28 microbeads in the presence of high-dose IL-2 and IL-15. The stimulation with the cognate antigen exhibited a limited expansion efficacy (Figure 5A) while maintaining a high percentage of MART-1 tetramer+ T cells (73.5%). The highest efficiency in T-cell expansion was obtained using artificial APC (Figure 5A), which also preserved T-cell viability better than did either microbeads or anti-CD3 (Figure 5B). In addition, anti-MART-1 CD8+T lymphocytes stimulated with artificial APC showed significantly greater cytotoxic activity against MART-1-loaded T2 cells compared to beads or anti-CD3-stimulated T cells (Figure 5C). The trend of artificial APC in preferentially supporting CD8+ T-cell expansion in the polyclonal setting was confirmed in the anti-MART-1 CD8+ T-cell culture (*Online Supplementary Figure S5*) and could explain the capacity of these cells to preserve the highest specific activity of expanded anti-MART-1 CD8+ T cells (Figure 5C).

Immunophenotype of expanded anti-MART-1 cytotoxic T-lymphocytes

After 2 weeks of culture with the cognate antigen, MART-1-specific T cells showed an effector memory immunophenotype characterized by low expression of CCR7 and CD45RA as a consequence of the continuous T-cell receptor stimulation (*Online Supplementary Figure S5*). Fourteen days after the additional aspecific stimulation, artificial APC-expanded T cells showed a higher frequency of MART-1 specific CD8+ T cells compared to the microbead-expanded cultures (*Online Supplementary Figure S5*). Following the 14-day expansion provided by artificial APC or microbead stimulation, the maturation level of MART-1-specific CD8+ T cells remained similar to that observed before the artificial stimulation (*Online Supplementary Figure S5*). However, as shown in Figure 5D, CD8+ and CD4+ expanded T cells still expressed high levels of the lymph node homing molecule CD62L. On the other hand, the widespread expression of CD69 suggested that the stimulation by artificial APC could activate specific cytotoxic T-lymphocytes as efficiently as polyclonal T cells (Figure 5D). In addition, after 14 days of culture with artificial APC plus high-dose IL-2 and IL-15, the frequency of FoxP3+ regulatory T cells was not increased for either total CD8+ or anti-MART-1-specific CD8+ T cells, or for the less frequent CD4+ T cells (Figure 5D).

Optimization of the artificial antigen-presenting cells-based system for T-cell expansion

In order to verify the feasibility of the procedure in the light of large–scale clinical application, several experiments were performed. First, the results obtained by comparing our APC with Dynabeads® CD3/CD28 T Cell Expander were corroborated by a further comparison with clinical grade anti-CD3/-CD28 coated microbeads (Dynabeads® ClinExVivo CD3/CD28) (*Online Supplementary Figure S6*). The research product Dynabeads® CD3/CD28 T Cell Expander and the clinical version of anti-CD3/CD28 microbeads displayed comparable activity in their extent of T-cell expansion. In order to evaluate the possibility of using our artificial APC in compliance with Good Manufacturing Practice requirements, the persistence of artificial APC components in expanded cultures was assessed 7 and 14 days after stimulation by flow cytometry. First, we derived a regression line to evaluate the sensitivity of mouse mAb detection on a fixed number of T cells (5×10^4) on the basis of MFI values using a FITC-labeled anti-mouse IgG antibody (*Online Supplementary Figure S7A*). Using the assay, we could then extrapolate the amount of mouse anti-human mAb carried on artificial APC microdomains and the amount remaining on T cells after 7 or 14 days of culture (*Online Supplementary Figure S7B*). Both these values (0.08 and 0.04 pmol at 7 and 14 days, respectively, *Online Supplementary Figure S7A*) were at the lowest left end of the regression line, indicating a reduction of more than two logs in mAb concentration compared to the starting mAb concentration (represented by the highest value on the right of the titration curve). Finally, to determine the conditions that would reduce the cost of the procedure, while preserving the efficiency of the T-cell expansion, we tried two alternative strategies: reducing the concentration of mAb used to produce the artificial APC and decreasing the APC/T-cell ratio. In the first strategy, the dose of anti-CD3, anti-CD28 and anti-LFA-1 mAb used for the preparation of the artificial APC was lowered by one or two logs. This modification did, however, lower the probability of obtaining microdomains with neutravidin sites completely saturated by biotinylated mAb. Such APC were less efficient than the standard ones in expanding T cells when the APC/T-cell ratio was unchanged (Figure 6). In contrast, efficient results were achieved when reducing the APC dose/T-cell ratio by one ort two logs (i.e. by keeping the amount of artificial APC constant and increasing the starting T-cell number by 10- to 100-fold) (Figure 6A). These conditions preserved the maturation level and the potential capability of *in vivo* persistence,

based on the high CD62L expression (Figure 6B). In addition, using a one log decrease in the APC dose/T-cell ratio, 10 billion antitumor T cells could be generated in a reduced volume with a limited cost, making this procedure suitable for clinical applications (Figure 6C).

Discussion

This study demonstrates the advantages for T-cell expansion conferred by a fully artificial system capable of mimicking natural APC membrane reorganization during immune synapse formation. Our artificial APC: (i) generated large numbers of T cells starting from polyclonal $CD3^+$ T cells; (ii) preserved the highest cell viability in culture compared to the other commercial artificial systems; (iii) limited the differentiation of expanded T cells enriching the fraction of T cells expressing a naïve or effector phenotype; (iv) prevented the increase of $CD25^{high}$ $FoxP3^+$ regulatory T cells; (v) expanded functionally competent anti-MART-1-specific $CD8^+$ T cells; and finally (vi) proved suitable for scaling up for clinical application.

To engineer our artificial system, we exploited the structure of the artificial APC developed by Albani *et al.*[34-36] By substituting the HLA-peptide complexes, which originally covered one site on microdomains, with the ubiquitous T-cell receptor triggering signal conferred by anti-CD3 mAb and by adding co-stimulatory and adhesion molecules needed for productive T-cell activation (CD28 and LFA-1), we obtained new artificial APC capable of efficiently stimulating and expanding T cells irrespective of their antigen specificity. Our artificial APC differ from the other clinically approved acellular artificial systems by the presence of a fluid membrane that allows free movement of microdomains and their effective orientation after T-cell contact for the productive formation of immunological synapses. Other advantages of our system are the possibility to create in advance a first level of clustering by grouping the triggering molecules for T-cell activation on microdomains and the presence among them of the anti-LFA-1 mAb, which acts as the most important adhesion molecule on natural APC (ICAM-1). In fact, by favoring a more stable interaction with T cells, this strategy increases the possibility of immune synapse formation, the essential prerequisite for T-cell activation. Thus, our artificial APC could be useful for generating T

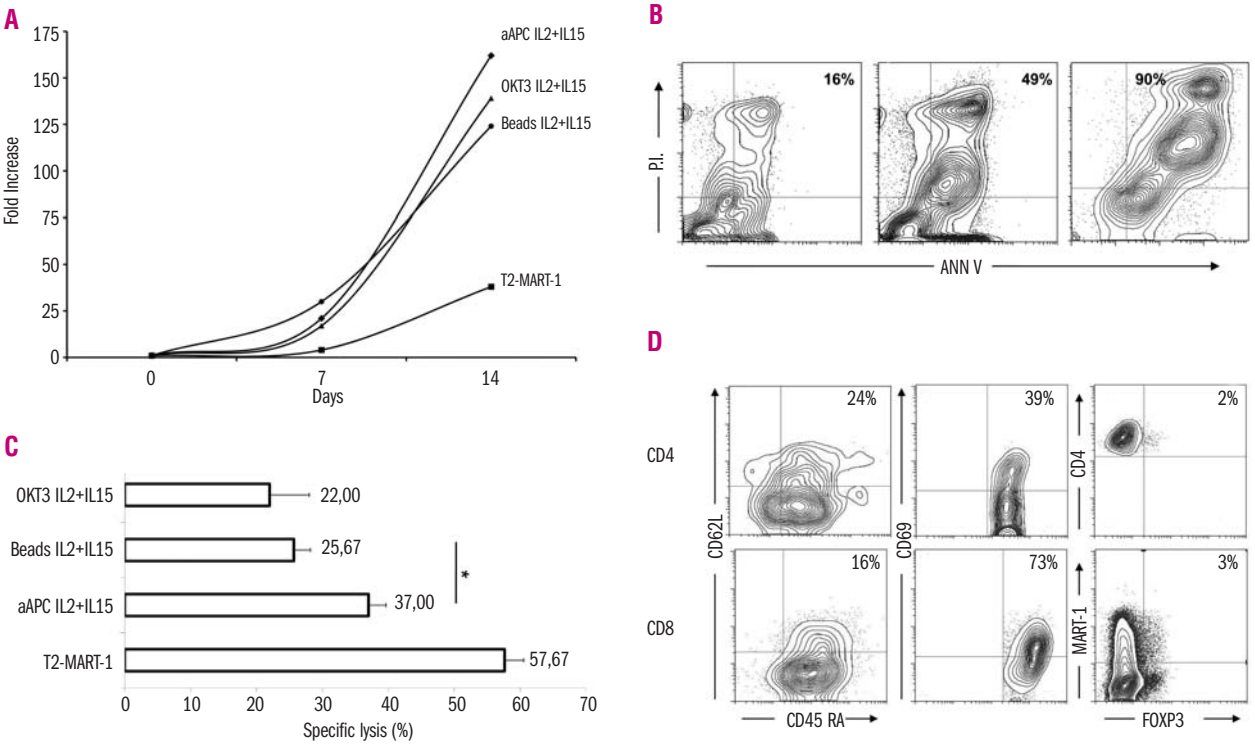
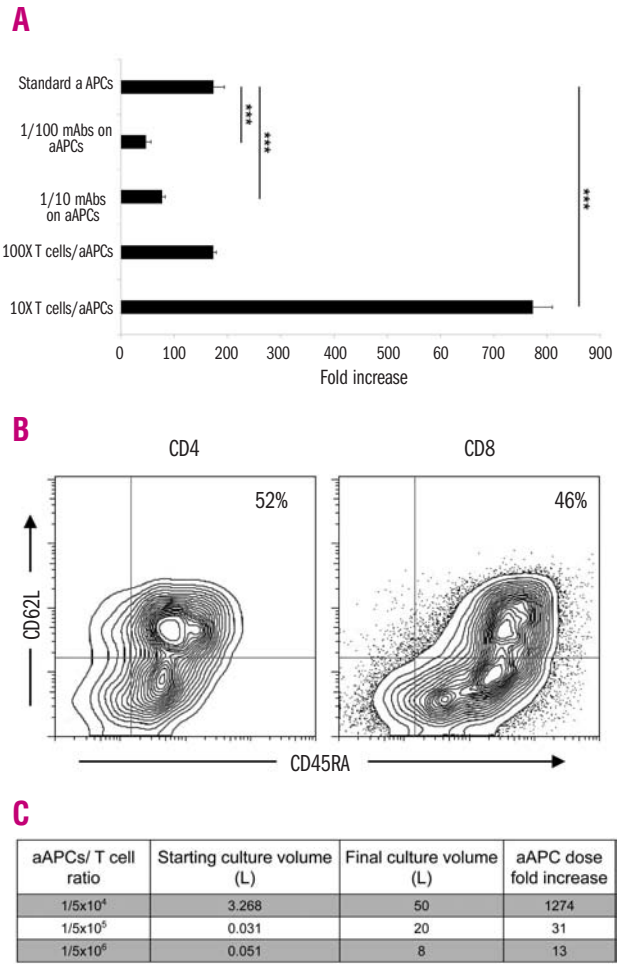


Figure 5. Expansion of anti-MART-1 human specific T lymphocytes. (A) After 7 and 14 days of stimulation with anti-CD3/-CD28 microbeads or immobilized anti-CD3 or our artificial APC (aAPC) in the presence of high-dose IL-2 and IL-15, anti-MART-1 specific human $CD8^+$ T lymphocytes were counted using the trypan blue dye exclusion test to evaluate the fold increase in their numbers. X-axis, days in culture; y-axis, fold increase. (B) Evaluation of apoptosis in cultures of anti-MART-1-specific human T cells after 14 days of expansion. Cytograms represent flow cytometric analysis of cultured cells stained with FITC-labeled a Annexin V (ANN-V) (x-axis) and propidium iodide (PI) (y-axis). (C) Cytotoxicity of anti-MART-1-specific human T cells 14 days following stimulation with aAPC or anti-CD3/-CD28 microbeads or immobilized anti-CD3 plus high-dose IL-2 and IL-15, or with MART-1 loaded T2 cell line (T2-MART-1) was tested by the ^{51}Cr release assay using LCL target cells pulsed with the specific MART-1 antigen. The difference between the lysis of LCL presenting the MART-1 peptide and the lysis of LCL alone or loaded with unrelated Flu or HIV peptides indicated the specific lysis. Results are representative of three independent experiments. Error bars indicate the standard deviation of the mean. Statistical analyses were performed using the two-tailed *t* test (*: $p \leq 0.05$). (D) Representative example of anti-MART-1 T-cell immunophenotype 14 days after aAPC stimulation. FACS analyses were performed on $CD4^+CD3^+$, $CD4^-CD3^+$ (CD8 cell subset) and $MART\text{-}1^+CD4^-CD3^+$ gated subpopulations. The expression of CD62L versus CD45RA, CD69 and of FOXP3 was evaluated to define, respectively, the maturation and activation level and regulatory T-cell frequency in expanded cultures.



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aAPCs/ T cell ratio	Starting culture volume (L)	Final culture volume (L)	aAPC dose fold increase
1/5x10^4	3.268	50	1274
1/5x10^5	0.031	20	31
1/5x10^6	0.051	8	13

Figure 6. Optimization of the artificial APC (aAPC) protocol for T-cell expansion. (A) Fold increases in T-cell numbers provided by aAPC in standard conditions, or by aAPC with one or two log lower mAb concentration, or by increasing the starting T-cell number per aAPC by one or two logs. Freshly isolated polyclonal CD3$^+$ T cells were stimulated with these systems and were cultured in the presence of high-dose IL-2 plus IL-15. Cell counts were assessed using the trypan blue dye exclusion test on days 7 and 14 after stimulation. Results are representative of three independent experiments with cells from different donors. Error bars indicate the standard deviation of the mean (*: $p \leq 0.001$). (B) Maturation status defined by CD45RA versus CD62L expression 14 days after stimulation at a T cell/aAPC ratio increased by one or two log. (C) Estimation of the amount of aAPC and the culture volumes required to expand 10^9 cells (target T-cell number) using different aAPC/T-cell ratio.**

cells for anticancer immunotherapy, since they make it feasible to expand low avidity tumor-specific T cells, even when tumor-associated antigens are unknown.

Compared to microbeads or anti-CD3-stimulation, a single administration of our artificial APC to polyclonal CD3$^+$ T cells cultured with high-dose IL-2 plus IL-15 resulted in the greatest expansion of viable cells. In addition, after 14 days, APC cultured-polyclonal CD3$^+$ T cells showed only a low amount of apoptotic and necrotic cells, a finding of great relevance for the practical development of artificial APC-based adoptive immunotherapy protocols.

A growing awareness of lymphocyte characteristics that can affect lymphocyte behavior *in vivo* is influencing the practice of adoptive cell transfer therapy. For instance, the discovery that the maturation status of CD8$^+$ T lymphocytes determines their *in vivo* migration and persistence during an immune response[42-46] opens up new avenues for manipulating T-cell activity. These might include modification of *in vitro* T-cell cultures to avoid terminal differentiation of these cells.[43] We found that associating artificial APC, as a source of T-cell receptor stimulation, to high-dose IL-2 plus IL-15 produced the best expansion efficiency while generating T cells with high expression of both CD69, a marker associated with T-cell receptor engagement, and CD62L/CCR7, secondary lymphoid organ homing molecules. Moreover, our system was able to expand T cells with granzyme B, with limited expression of perforin. Collectively, these findings suggest that some of the expanded cells were acutely activated and could exert immediate effector functions upon *in vivo* infusion, but other cells retaining the ability to traffic through lymph-nodes, showing a naïve or effector phenotype, could be stimulated *in vivo* and then persist in the memory compartment.[44] The presence of these two dominant phenotypes (CD45RA$^+$CD62L$^+$ and CD45RA$^+$CD62L$^-$) in the APC-expanded T cells suggests that our approach may boost the expansion of naïve T cells (supported by T-cell receptor triggering) while inducing central memory T-cell differentiation (promoted by T-cell receptor triggering and γc cytokines) towards the effector stage of maturation.[47,48]

In addition, recent reports describing a requirement of CD4$^+$ T cells for persistence of CD8$^+$ T cells after an immune response underline a potential role of CD4$^+$ T cells in T-cell cultures for adoptive cell transfer therapy.[49-51] Our artificial APC, while preferentially sustaining the expansion of CD8$^+$ T cells, which are crucial for a productive antitumor response, maintained a sizable fraction of CD4$^+$ T cells. Contrariwise, the demonstration that CD4$^+$CD25$^+$ regulatory T cells suppress autoimmunity and might be potent inhibitors of antitumor effects in mice indicates a rationale for additional investigations on lymphodepleting conditioning for adoptive cell transfer therapy.[52,53] For these reasons, we evaluated the frequencies of CD4$^+$ and CD8$^+$ regulatory T cells defined as FoxP3$^+$ and CD25$^+$ in expanded cultures, and found that they were kept at the lowest level when artificial APC were used. In contrast, the amount of regulatory T cells was increased using the other two systems (anti-CD3/-CD28 microbeads or immobilized anti-CD3).

When the effect of the artificial APC on the stimulation of pre-enriched anti-MART-1 CD8$^+$ T cells was assessed, it was seen that they efficiently expanded the T-cell population which retained antigen specificity close to the levels seen when these T cells were maintained in the presence of the cognate antigen. Moreover, similarly to the expanded polyclonal T-cell population, MART-1-specific CD8$^+$ T cells cultured with our artificial APC still exhibited a phenotype consistent with their lymph node homing associated with a high expression of CD69 and no increase in the frequencies of regulatory T cells. Thus, artificial APC can be used to boost the expansion of previously enriched antigen-specific CD8$^+$ T cells without any limitations due to HLA compatibility, suggesting their potential application in adoptive immunotherapy

programs even when tumor-associated antigens are unknown.

Finally, the results of this study indicated that efficient T-cell expansion with artificial APC can be achieved starting with a larger amount of T cells in a reduced volume, compared to standard conditions, laying the basis for scaling up our approach for clinical applications. In particular, using artificial APC to expand aspecifically the complete T-cell population of cancer patients, we envisage the possibility of increasing the frequency of tumor-associated antigen specific lymphocytes, which are usually represented at low level in the immune repertoire even after their initial amplification by *in vivo* boosting with specific vaccination.[3,54] Moreover, in the case of highly immunogenic tumors, for which tumor-associated peptides are known and the related peptides available, it is possible to perform aspecific expansion with artificial APC after *ex vivo* enrichment of antitumor specific cytotoxic T-lymphocytes using *in vitro* co-culture with autologous tumor cells.[55]

Authorship and Disclosures

RZ co-designed the study, collected patients' samples, performed the T-cell expansion experiments and FACScan analysis, and wrote the manuscript; MDN co-designed the study, analyzed the results and wrote the manuscript; CCS, RM analyzed results and wrote the manuscript; AM collected the cell samples, generated human TAL cell lines; CV collected the cell samples, generated human TAL cell lines and performed cytotoxicity assays; SA supplied the artificial antigen presenting cell reagents and co-designed the study; AA co-designed the study, analyzed the results and wrote the manuscript; AMG co-designed the study, analyzed the results and wrote the manuscript. All authors approved the final version of the manuscript. The authors reported no potential conflicts of interest.

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