Artificial Antigen Presenting Cells With Preclustered anti-CD28/-CD3/-LFA-1 Monoclonal Antibodies Are Highly Effective To Induce The Ex-Vivo Expansion Of Functional Human Antitumor T Cells.

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Abstract

Effective adoptive T cell therapy requires the ex-vivo generation of functional T lymphocytes with a long lifespan in-vivo. We evaluated in-vitro T cell expansion by artificial antigen presenting cells (aAPC) generated with activating (human anti-CD3), co-stimulating (human anti-CD28) and adhesion (human anti-LFA-1) monoclonal antibodies pre-clustered in microdomains (MDs) held by a liposome scaffold. The co-localization of T cell ligands in MDs and the targeting of an adhesion protein, increasing the efficiency of immunological synapse formations, represent the novelties of our system. These aAPCs allowed increased expansion of polyclonal CD4+ and CD8+ T cells and of tumor antigen-specific CD8+ T cells compared to anti-CD28- and anti-CD3-coated microbeads and to immobilized anti-CD3. These aAPCs allowed the generation of T cells displaying an immunophenotype consistent with long-term in-vivo persistence, without increasing the frequency of regulatory T cells. Finally, our aAPCs proved to be suitable for large scale T cell expansion required in immunotherapy trials.

Introduction

The in vitro activation and expansion of antigen-specific T cells for therapeutic purposes has yielded impressive results in the context of prophylaxis and treatment of virus-associated infection and disease post-transplant[1, 2]. These successes prompted the extension of this method for the treatment of tumors in immunocompetent individuals[3, 4], given the finding that antitumor specific T lymphocytes exist in cancer patients[5-7], even if they are not able to overcome tumor immune escape mechanisms and to react efficiently against the tumor.

For clinical purposes, the use of natural antigen presenting cells (nAPCs), usually dendritic cells (DCs) or virally infected B cells, to activate and expand T cells for adoptive immunotherapy has been studied[8], but still poses a lot of challenges. In particular, they require several months to produce sufficient numbers of T cells that may be a limiting factor in the setting of malignancy. Moreover, after a long-term ex-vivo expansion, T cells are expected to have limited replicative capacity and persistence upon infusion into patients. Finally, the use of nAPC is limited by the difficulties in standardize protocols for their in-vitro generation, which, being highly sensitive to the culture conditions, is hardly reproducible [9], and by the prerequisite for their application only in an autologous setting.

These evidences 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 modifying the composition of the expressed molecules. Although several artificial systems have been produced[10-21], only the following systems are suitable for clinical use being generated under "Good Manufacturing Practice" (GMP): immunomagnetic beads coated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs)[22, 23] and anti-CD3 mAb immobilized on well bottom of culture plates. Even if these technologies are able to efficiently support the expansion of $CD4^+$ T cells[24], the

long-term growth of purified $CD8^+$ T cells [25] is hampered. More likely, the reason consists in a not efficient interaction of these systems with T cells[26, 27], since they are not supplied with a fluid membrane, which makes it impossible for ligands to cluster and efficiently activate T cells.

The present work has been aimed at engineering an aAPC-based system with the properties of a fluid cellular membrane and the flexibility derived from an artificial structure, which can be properly tailored at the purpose. A stable interaction with APC for T cell activation is dependent not only on the absolute affinity between the T cell receptor (TCR) and its ligand but also on the relative density of relevant molecules at the interaction site. Furthermore, it has been recently shown that preclustering of MHC-peptide complexes in membrane MDs on the APC surface affects the efficiency of immune synapse formation and the related T cell activation[28, 29]. For these reasons, starting from aAPCs manufactured by Albani in which a liposomal formulation is loaded with class II HLA molecule-peptide complexes associated with costimulatory molecules, we modified aAPC by adding anti-CD3, instead of HLA molecule, and anti-LFA-1 adhesion molecule on MDs in lipidic bilayer to allow an efficient aAPC-T cell interaction.

We demonstrated that these aAPCs are able to expand more efficiently, compared to the other commercial artificial techniques, both viable and functional polyclonal T cells and antigen specific cytotoxic T lymphocytes (CTLs) with immunophenotypic characteristics that suggest a potential long-term persistence in vivo.

Materials and Methods

Generation of aAPC

For the preparation of aAPCs, the materials used by Albani et al. for the identification of rare antigen-specific T cells in autoimmune disorders[30-32] was made suitable for ex-vivo T cell activation and expansion with appropriate modifications. The resulting aAPCs consisted in a scaffold made of GM1 enriched-liposomes, which anchor microdomains (MDs) where mouse antihuman CD3, CD28 (BD PharMingen, San Diego, California) and LFA1 (Biodesign, Saco, Maine) triggering mAbs have been preclustered (Figure1A). At this end, biotinylated anti-CD3, anti-CD28 and anti-LFA1 mAbs were combined with biotinylated cholera toxin B (CTB; Sigma-Aldrich Inc.) in a 3:1 molar ratio for 5' at room temperature (RT), as previously described[30]. Therefore, neutravidin (NA; Pierce Biotechnology, Rockford, Illinois) was added at a ratio of 1 mol of NA per 4 biotinylated moieties. After 15' of incubation at RT, the ganglioside GM1 enriched liposomes (gently 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). After 90' of incubation while mixing, the aAPC solution was washed in PBS at 14000 rpm for 10'. The pellet with aAPCs was resuspended in culture medium and used for T cell expansion. As controls, only the liposomial formulation (liposome alone) or the biotinylated mAbs on neutravidin rafts without liposomes (MD alone) were used in T cell cultures in the same conditions of complete aAPCs (Figure 2). In order to corroborate the choice of the association of those three mAbs on aAPCs to achieve the best T cell expansion, initial experiments were performed using aAPC whose MDs 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 biotinylatd mAbs in comparison. Briefly, CFSE (CFDA, SE; Molecular Probe Inc. Eugene, Oregon) stained PBMCs from healthy donors were stimulated with anti-CD3/-CD28/-LFA1 aAPCs, or anti-CD3/-CD28 aAPCs or anti-CD3/LFA1 aAPCs or anti-CD28/-LFA1 or anti-CD3 aAPCs or anti-CD28 aAPCs or

anti-LFA1 aAPCs where mAbs were added at serial dilutions (1-1/64). CFSE dilution was assayed after 4, 7 and 10 days by FACSCalibur device and results were analyzed with CellQuest software (Becton Dickinson).

Human cells and culture conditions

Two sources of T cells were tested for the expansion: i. $CD3^+$ lymphocytes, negatively isolated from peripheral blood mononuclear cells (PBMCs) obtained from the heparinized blood of healthy donors after Ficoll-Hypaque density gradient separation, using Pan T cell isolation kit with a MiniMACS device (Miltenyi Biotech, Gladbach, Germany), according to the manufacturer's protocol; ii**.** Lymphocytes from melanoma associated lymph nodes were cultured for 2 weeks with HLA-A*0201^+ T2 loaded with 10 μg/ml of Melan-A/MART-1 $_{27\text{-}35}$ (modified sequence 27–35: ELAGIGILTV; PRIMM s.r.l., San Raffaele Biomedical Park, Milan Italy)[33, 34] as described[35]. Frequency of $tetramer^+$ T cells in the $CD8^+$ fraction was analyzed before and at the end of culture. T cells were cultured at a concentration of 0.2 x 10^6 cells/ml in a 96 flat-bottom well plate with 250 μl/well of RPMI 1640 (Cambrex, Verviers, Belgium) containing 10% human serum (HS), 1% PenStrep (Cambrex, Verviers, Belgium), 1% Glutamine (Cambrex, Verviers, Belgium) and 1% Hepes buffer (Cambrex, Verviers, Belgium). T cell expansions were performed with a single administration of aAPCs or immunomagnetic beads (Figure 1B) coated with mouse antihuman CD3 and CD28 mAbs (Dynabeads CD3/CD28 T cell Expander, Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's instruction, or mouse antihuman CD3 mAb (Functional grade purified anti-hCD3, OKT3 clone, eBioscience, San Diego, California) immobilized on well bottom. Anti-CD3 was cross-linked (Figure 1C) to the well bottom of 96 flat bottom well plates precoated with anti-mouse IgG (whole molecule, SIGMA). Briefly, the plates were incubated with anti-mouse IgG at10 μg/ml in PBS (Cambrex, Verviers, Belgium) at 4°C, over night. The day after, anti-mouse IgG was removed from the plates and the wells have been washed three times with PBS. Then, anti-CD3 was added at 0.5 μg/ml of RPMI 1640 (50 μl/well) and the plates were incubated for 45' at

4°C. The incubation was stopped by blocking the plates with 20% HS supplemented RPMI 1640 (50 µl/well).

These cultures were carried on with the following combination of γ-chain cytokines accordingly to previous results[36]: i. low dose (LD) rhIL-2 (300U/ml, Proleukin, Chiron, Emeryville, California), ii. rhIL-15 (Peprotech Inc., Rocky Hill, New Jersey) 10 ng/ml, iii. high dose (HD) rhIL-2 (3000 U/ml) plus rhIL-15 10 ng/ml. Complete culture medium with cytokines was changed every 2 days during the 14 day-expansion with the artificial systems.

The aAPC stimulation: T cell ratio was reduced by diminishing mAb dose for the generation of aAPCs or by increasing the starting T cell amount in culture with aAPCs produced as previously described. In the first case, the biotinylated mAbs were added at 1/10 or 1/100 of the standard dose, resulting with biotinylated cholera toxin B and NA in 3/10 or 3/100: 1 molar ratio respectively. These aAPCs were used for 14 day-expansion of 0.05 x 10^6 $CD3^+$ purified T cells in association with HD IL-2 and IL-15 and they were compared to the standard ones in the same culture condition. In addition, standard aAPCs were tested for the expansion of 10 or 100 fold increase number of starting T cell. Briefly, 0.5 x 10^6 or 5 x 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 aAPCs in the presence of HD IL-2 plus IL-15. Culture medium with cytokines was changed every 2 days during the 14 day-expansions.

Flow cytometric analysis

Flow cytometric 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 status and activation phenotype were evaluated by multiparametric analyses using the following mouse-antihuman mAbs and MHC tetramers in different combinations: FITC-labeled anti-CD62L (BD BioSciences, San Jose, California), FITC-labeled anti-CD3 (Miltenyi Biotech, Gladbach, Germany), PE-labeled anti-CD45RA (BD Pharmingen, San Diego, California), PE-

labeled anti-CD4 (BD Biosciences, San Jose, California), PE-labeled anti-CD25 (Miltenyi Biotech, Gladbach, Germany) mAbs, PE-labeled tetramers of HLA-A 0201 containing Melan-A/MART-1 peptide (Beckman Coulter Inc., Fullerton, California), PerCP-labeled anti-CD4, anti-CD69 and anti-CD3, APC-labeled anti-CD8 and anti-CD4 (BD Biosciences, San Jose, California). Surface stainings were performed by incubating mAbs at 4°C for 30'.

Regulatory T cell expansion was assayed by intracellular hFOXP3 staining using FITC-labeled anti-human FOXP3 staining kit (eBioscience, San Diego, California) in association with PE-labeled anti-CD25, PerCP-labeled anti-CD8 and APC-labeled anti-CD4 surface mAbs, according to the manufacturer's protocol.

Apoptosis assay was performed 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 after 14 days from artificial stimulations, expanded T cells were permeabilized with Cytofix/Cytoperm (BD Biosciences, San Jose, California) and then stained with mouse-antihuman FITC–labeled anti-Perforin (BD Biosciences, San Jose, California), or PE–labeled anti-Granzyme B (CLB, Amsterdam, The Netherlands) in the presence of Perm/Wash solution (BD Biosciences, San Jose, California). Multiparametric analyses of perforin or granzyme B expression in T cell subsets, defined by anti-CD-3, anti-CD8 and anti-CCR7 (mouse-antihuman APC-labeled CCR7 mAb, R&D systems, Minneapolis, Minnesota) mAb staining, were performed.

Cytotoxic assay

The cytotoxic activity of expanded specific MART-1 T cells was assessed through ^{51}Cr-release assay using as target Melan-A/MART-1_{27-35} (AAGIGILTV, PRIMM s.r.l., San Raffaele Biomedical Park, Milan Italy) pulsed lymphoblastoid cell line (LCL 9742). As negative controls, unpulsed-LCL or LCL pulsed with Influenza A (Flu) Matrix$_{58-66}$ (GILGFVFTL, PRIMM s.r.l., San Raffaele

Biomedical Park, Milan Italy) or HIV (ILKEPVHGV, PRIMM s.r.l., San Raffaele Biomedical Park, Milan Italy) were used. Sintetic peptides were ≥95% pure[35]. Results were expressed as follows:

% Lysis = (experimental release (cpm) - spontaneous release (cpm))/ (maximum release (cpm) - spontaneous release (cpm)) x 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

Analysis of statistical significance were performed by using the Student's t test at the two-sided 0.05 significance level on Prism software (*: P≤ 0.05;**: P≤ 0.02; ***: P≤ 0.01).

Results

aAPCs efficiently bind and expand human polyclonal T cells

As shown in Figure 1, aAPCs consists of a scaffold made of GM1 enriched-liposomes, which anchor microdomains (MDs) of preclustered activating biotinylated mAbs (anti-CD3, anti-CD28, and anti-LFA1) through biotinylated CTB subunit and NA. The highest proliferation rate of selected polyclonal $CD3^+$ T (mean purity 90±3%) cells was obtained combining all the three activating molecules on aAPCs (data not shown). In the following experiments aAPCs containing the mixture of all the three mAbs were tested in association with LD IL-2, or IL-15 or HDIL-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 MDs alone. After 7 days from the stimulation, the greatest efficiency was observed when complete aAPC were associated to any cytokine combinations. However, after 2 weeks, aAPCs combined with exogenous HD IL-2 plus IL-15 resulted in the greatest T cell fold increase that was statistically significant when compared to the other conditions (Figure 2; *: $P \leq 0.05$; ***: $P \leq 0.01$).

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

aAPC stimulation enhance survival of human polyclonal T cells

Our aAPCs were compared with GMP systems (anti-CD3 and anti-CD28 coated immunomagnetic microbeads or immobilized anti-CD3) for the efficiency in expanding purified polyclonal $CD3^+$ T cells. The cytokines reported above were added to T cell culture. Two weeks following the stimulation, the best methods resulted microbeads and aAPCs in combination with HD IL-2 plus IL-15 displaying comparable efficacy of polyclonal T cell expansion (Figure 3A). However, when apoptosis of expanded T cells was evaluated, aAPCs preserved the highest cell viability in culture

compared to the other artificial systems (Figure 3B). In the bead-stimulated culture, only the 46% of expanded T cells was viable (annexin V-/propidium iodide-), whereas 83% of T cells were still alive 14 days after the stimulation with aAPCs that in turn displayed the statistically highest expansion (Figure 3C).

Immunophenotype of expanded polyclonal T cells

Fourteen days after stimulation, T cells displayed a naïve/effector phenotype, with a still evident CD62L positivity and a high expression of the activation marker CD69, in all experimental conditions. In particular, bead and aAPC stimulated cells displayed an even higher expression of CD62L compared to immobilized anti-CD3 expanded lymphocytes. The expression of the homing adhesion molecule CD62L on the expanded T cells should confer to them the capability of trafficking through lymph nodes, where they could be involved in physiological responses and in the following memory development. On the other hand, the high expression of a marker associated with TCR engagement (CD69) suggests that such cells are acutely activated and could exert immediate effector functions upon in vivo re-infusion.

In addition, when our aAPCs were used, $CD8^+$ T cells were predominantly expanded, while $CD4^+$ T cells were preserved at low level. On the contrary, anti-CD3/-CD28 microbead and immobilized anti-CD3 stimulation preferentially gave rise to $CD4^+$ lymphocyte expansion (Figure 4A-B). Finally, the risk to expand also regulatory T cells was evaluated calculating their frequency in the culture of polyclonal T lymphocytes stimulated with the different artificial systems. Two weeks after the stimulation with aAPCs, the frequency of $CD4^+FoxP3^+$ T cells or of $CD8^+FoxP3^+$ T cells was statistically lower (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 independent of the CD25 one[37].

By comparing the mean fluorescence intensity (MFI) of granzyme B staining on the differently expanded $CD8^+$ or $CD4^+$ T cells, our APCs resulted in the lowest up-regulation of granzyme B

expression among artificial stimulations tested (Figure 4C, left panel). Moreover, when our aAPCs were used, perforin expression was kept at the lowest level and was confined to a minimal fraction of expanded $CD8^+$ or $CD4^+$ T cells (Figure 4C, right panel). The reduced differentiating properties of our aAPCs were confirmed even analyzing the expression of perforin and granzyme B in the $CCR7^+$ and $CCR7^-$ sub-populations in both $CD4^+$ and $CD8^+$ cell subsets (data not shown). These observations suggest that T cells expanded in the presence of our aAPCs are prone to kill, since they express granzyme B, but they require a further activation to do it, due to the limited induction of perforin expression following the in vitro stimulation. This phenotype is consistent with the high expression of CD62L and with the increased survival of T cells expanded with aAPC and further support the possibility that our method of T cell stimulation might generate T cells with prolonged lifespan in vivo and more likely able to persist in the memory compartment compared to the other artificial GMP systems.

Specific cytotoxic activity of efficiently expanded T cells by aAPCs is preserved

Since aAPCs rapidly activated and expanded polyclonal T lymphocytes, we have also tested aAPCs for the expansion of human anti-melanoma CTLs enriched for MART-1 specificity. MART-1 loaded HLA-A*0201^+ TAP-deficient T2 cell line was used as APCs for anti-MART-1 specific TAL expansion, isolated from melanoma involved lymph nodes. After two culture weeks with the cognate peptide, they were aspecifically stimulated with aAPCs or immobilized anti-CD3 or with anti-CD3/-CD28 microbeads and expanded for additional 14 days in the presence of HD IL-2 and IL-15. As shown in Figure 5A, the highest expansion was obtained using aAPCs. In addition, anti-MART-1 $CD8^+$T lymphocytes stimulated with aAPCs preserved the closest specific cytolytic activity to the anti-MART-1 $CD8^+$ enriched T cell grown with MART-1 pulsed-T2 cell line (Figure 5B). By contrast, while maintaining a highly pure anti-MART-1 $CD8^+$ T cell population, the stimulation with the cognate antigen exhibited a limited expansion efficacy (Figure 5A-B). The aAPC trend in preferentially supporting the expansion of $CD8^+$ T lymphocytes seen in polyclonal

setting could reflect aAPC capability to preserve the specific activity of expanded anti-MART-1 specific $CD8^+$ T cells that in turn might be responsible of the antitumor effect.

Moreover, the viability of aAPC expanded anti-MART-1 $CD8^+$ T cells was preserved even in this setting (Figure 5C).

Immunophenotype of expanded anti-MART-1 CTLs

The immunophenotype of $CD4^+$ and $CD8^+$ T cells generated during the expansion of anti-MART-1 CTLs with aAPCs is shown Figure 6 that reported a representative FACScan analysis. The high positivity for CD62L expression in both $CD8^+$ and the rare $CD4^+$ T cells is consistent with in-vivo long-term persistence (Figure 6). On the other hand, the widespread expression of CD69 suggested that the stimulation of aAPCs could activate specific CTLs as efficiently as polyclonal T cells (Figure 6). In addition, after 14 culture days with aAPCs plus HD IL-2 and IL-15, the frequency of $FoxP3^+$ regulatory T cells was not increased for both total $CD8^+$ and anti-MART-1 specific CD8+ T cells, as well for the less frequent $CD4^+$ T cells (Figure 6).

Optimization of aAPC T cell expansion culture

In order to allow the feasibility of the procedure on large–scale for clinical application, several experiments have been performed reducing the dose aAPC stimulation or increasing the concentration of T cells in cultures. Diminishing to 1/10 or 1/100 the dose of biotinylated mAbs added to the aAPC mixture did not saturate the MDs, present on the liposome scaffold in the same number, saturated of stimulating molecules. These systems were less efficient than standard aAPCs in expanding the same number of starting T cells (data not shown). The best results were achieved when starting T cell number was increased of 10 or 100 fold (Figure 7A). Moreover, the maturation status and the potential capability of in vivo persistence, on the basis of CD62L expression, are preserved even on large-scale (Figure 7B). In addition, at 1:10 aAPCs: T cell ratio, 10 billion of

antitumor T cells can be generated in a reduced volume with a limited cost, which makes this procedure suitable for clinical application (Figure 7C).

Discussion

In this report we demonstrate the advantages in T cell expansion conferred by a fully artificial system capable of mimicking nAPC membrane reorganization during immune synapse formation. Our aAPCs exhibit high efficiency (1) in generating large numbers of T cells starting from polyclonal $CD3^+$ T cells, (2) preserving the highest cell viability in culture compared to the other commercial artificial systems, (3) enriching the fraction of T cells expressing a central memory-naïve phenotype, (4) without increasing the frequency of $CD25^{high}$ $FoxP3^+$ regulatory T cells; (5) moreover they show a great activity in expanding functionally competent anti-MART-1 specific $CD8^+$ T cells and (6) finally prove to be suitable for clinical application on large scale.

To engineer our artificial system, we exploit the structure of the aAPCs developed by Albani S. et al.[30-32] By simply substituting the HLA-peptide complexes, which originally covered one site on MDs, with the ubiquitous TCR triggering signal conferred by anti-CD3 mAb and by adding the most suitable molecules for a productive T cell activation, we obtain aAPCs capable to efficiently stimulate and expand T cells irrespective to their antigen-specificity. These aAPCs are provided with triggering mAbs, which mimic the function of activating, co-stimulatory and adhesion molecules, preclustered in MDs within a liposome mixture. Our aAPCs differ from the other acellular artificial systems for the presence of a fluid membrane that allows the free movement of the MDs and their effective orientation after T cell contact for the productive formation of immunological synapses. Moreover, the possibility to create in advance a first level of clusterisation by grouping the triggering molecules for T cell activation on MDs and the presence of the anti-LFA-1 mAb among them, which acts as the most important adhesion molecule on nAPC (ICAM-1), represent the other advantages of our system. In fact, favoring a more stable interaction with T cells, these strategies increase the possibility of the immune synapse formation, the essential prerequisite for T cell activation. The aspecific but highly sensitive activity of our aAPCs could be useful for

anticancer immunotherapy, since it might be desirable to expand low avidity tumor specific T cells, in particular when tumor associated antigen are unknown.

A single administration of our aAPCs to polyclonal $CD3^+$ T cells cultured with HD IL-2 plus IL-15 gives rise to the highest expansion of viable cells when compared to the other artificial commercial systems (anti-CD3/-CD28 microbeads and immobilized anti-CD3). The evidence that, after 14 days, aAPC cultured-polyclonal $CD3^+$ T cells have a negligible amount of apoptotic and necrotic cells, further supporting the paraphysiologic stimulation performed by our aAPCs, is of great relevance in the practice of adoptive immunotherapy which include criopreservation and thawing of T cells, whose viability is strongly related with their capability to persist in vivo and with the possibility to generate a clinical response.

Concurrently, a growing appreciation regarding lymphocyte characteristics that can impact on lymphocyte behavior in vivo is influencing the practice of ACT therapy. For instance, the discovery that the maturation status of $CD8^+$ T lymphocytes determines their in vivo migration and persistence during an immune response[38-42] indicates new avenues for manipulating T cell activity. These might include the alteration of in vitro T cell cultures to maintain a central memory phenotype[39]. Our work demonstrates that by associating aAPCs to HD IL-2 plus IL-15 it was possible to obtain the best expansion while generating T cells with a high expression of CD69, a marker associated with TCR engagement, and CD62L, a secondary lymphoid organ homing molecule. Moreover, our system was able to extensively arm expanded T cells with granzyme B, while reserving the capability to kill the target to a limited fraction of $perforin^+$ T cells, suggesting that, 14 days after stimulation, T cells were not terminal differentiated. Together these evidences suggest that part of expanded cells are acutely activated and could exert immediate effector functions upon in vivo infusion, but other cells retaining the capability to traffic through lymph-

nodes, showing a naïve-memory phenotype, could be stimulated in vivo and then persist in the memory compartment[40].

In addition, recent reports describing a requirement of $CD4^+$ T cells for persistence of $CD8^+$ T cells after an immune response underlines a potential role of $CD4^+$ T cells in TIL cultures for ACT therapy [43-45]. Since cancers are for the most part weakly immunogenic and develop over long period of time, the presence of $CD4^+$ T cells after ex-vivo expansion may be crucial for the effectiveness of ACT. Our aAPCs, while preferentially sustain the expansion of $CD8^+$ T cells, exhibit a good activity even on $CD4^+$ T cells. By contrast, 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 investigation on lymphodepleting conditioning for ACT therapy[46, 47]. For these reasons, we evaluated the frequencies of $CD4^+$ and $CD8^+$ regulatory T cells defined as $FoxP3^+$ and $CD25^+$ in expanded cultures, and we found that they were kept at the lowest level when aAPCs were used. By contrast, using the other two systems, the amount of regulatory T cells was increased.

When aAPCs were assayed in pre-enriched MART-1 specific $CD8^+$ T cell culture as aspecific stimulation, mimicking a possible program of anti-cancer immunotherapy, they highly efficiently generate the T cell population with the closest specific cytolytic activity to the same lymphocytes maintained in culture with MART-1 pulsed-T2, compared with antitumor CTLs expanded with microbeads or OKT3. Moreover, similarly to the expanded polyclonal T cell population, MART-1 specific $CD8^+$ T cells cultured with our aAPCs still exhibit a central memory-naïve phenotype associated with a high expression of CD69, with no increase in regulatory T cell frequencies. Thus, previously enriched antigen specific $CD8^+$ T cells can be efficiently expanded with aAPCs without any limitations due to HLA compatibility.

Because T cell expansion with aAPCs can be achieved with high efficiency even starting from a larger amount of T cells in a reduced volume limiting the costs for large-scale applications, it is likely that this system could have therapeutic potential in clinical setting for patients with cancer and viral diseases. In particular, using aAPCs to expand aspecifically the full T cell population of cancer patients, we envisage the possibility to increase the frequency of TAA specific lymphocytes which are usually represented at low level in the immune repertoire even after their initial amplification by boosting in vivo with specific vaccination[48,40]. Moreover, in case of highly immunogenic tumors where TAAs are known and the related peptides available, it is possible to perform the aspecific expansion with aAPCs after the ex-vivo enrichment of antitumor specific CTLs using in vitro co-cultivation with autologous tumor cells[49].

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Figure Legends

Figure 1: **Structures of artificial systems compared for T cell expansion efficiency.** (A) Representation of MD structure loaded on aAPCs (adapted from Giannoni F. et al.[30]). GM1 ganglioside is part of the lipid bilayer and binds one molecule of biotinylated CTB. In each MD, one molecule of NA anchors the biotinylated molecules to the aAPC surface through one biotinylated CTB. The three free valences of NA have been saturated with anti-CD3 and anti-CD28 and anti-hLFA-1 triggering antibodies. (B) Schematic representation of CD3/CD28 T cell expander dynabeads. Anti-CD3 and anti-CD28 triggering mAbs on immunomagnetic beads can interact with the cognate molecules on T cells, activating them. The involved molecules at the site of interaction can cluster exclusively on T cells. (C) Schematic representation of mouse-antihuman CD3 mAb (OKT3 clone) cross-linking through anti-mouse IgG on the plate bottom. Anti-CD3 mAbs are linked through their Fc domains to the anti-mouse IgG mAbs. The exposed anti-CD3 mAb Fab domains can interact with the cognate molecules on T cells, activating them. The involved molecules at the site of interaction can cluster exclusively on T cells.

Figure 2: **Activity of complete aAPCs compared to their single components.** Expansion fold increase of purified polyclonal $CD3^+$ T cells 14 days after stimulation with aAPC or MDs or liposomes, starting from 0.05 x 10^6 T cells freshly isolated from healthy donor PB for each condition. LD IL-2, or IL-15 alone, or the combination of both with HD IL-2 was added in culture. Cellular fold increase was evaluated by Tripan Blue dye exclusion test on days 7 and 14 of expansion. Results are representative of four experiments with different donors. Error bars indicate standard deviation (s.d.) of the mean. Statistically significant differences, calculated using two-tailed T test, are reported (*: $P \leq 0.05$; ***: $P \leq 0.01$).

Figure 3: **Activity of aAPCs compared to commercial artificial systems.** (A) Expansion fold increase of polyclonal $CD3^+$ T cells, purified from healthy donors, stimulated with aAPCs or immobilized anti-CD3 or anti-CD3/-CD28 microbeads. Fifty thousand T cells freshly isolated from healthy donor PB were cultured with one of the three artificial systems and with LD IL-2, or IL-15 alone, or the combination of both with HD IL-2. Cellular fold increase was evaluated by Tripan Blue dye exclusion test on days 7 and 14 of expansion. Results are representative of four experiments with different donors. Error bars indicate s.d. of the mean. (B) Apoptosis assessment 14 days after stimulating purified polyclonal $CD3^+$ T cells with aAPCs or anti-CD3/-CD28 microbeads or immobilized anti-CD3 plus LD IL-2, IL-15 alone or HD IL-2 and IL-15. Cytograms represent flow cytometric analysis of cultured cells stained with FITC-labeled ANN-V (x-axis) and PI (y-axis). (C) Fold increase of $ANN\text{-}V^-/PI^-$ viable $CD3^+$ T cells 2 weeks after stimulation with aAPC or anti-CD3/-CD28 microbeads or immobilized anti-CD3. Error bars indicate s.d. of the mean. P values were calculated using two-tailed T test (*: $P \leq 0.05$;***: $P \leq 0.01$).

Figure 4: **Phenotypic characteristics of expanded polyclonal human T cells with aAPCs or other artificial systems plus HD IL2 and IL 15 for 14 days**. (A) Histogram representation of cell subset frequencies in cultures expanded with HD IL-2 plus IL-15 and aAPCs or OKT3 or anti-CD3/-CD28 microbeads. Results are representative of four experiments with different donors. Error bars indicate s.d. of the mean. Statistical analyses of aAPC versus microbeads results and of aAPC versus immobilized anti-CD3 ones were performed using two-tailed 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 HD IL-2 plus IL-15. Multiparametric FACS analysis were performed by gating on $CD4^+$ versus SSC and CD8 versus SSC populations (C) Histogram plot representation of intracellular expression of granzyme B (left panel) and perforin expression (right panel) in $CD8^+$ T cells (upper panel), identified by gating on $CD3^+CD8^+$ T cell subset, and in $CD4^+$T cells (lower panel), identified by gating on $CD3^+CD8^-$ T cell subset.

Figure 5: **Expansion of anti-MART-1 human specific T lymphocytes.** (A) Expansion fold increase of After 2 of pre-enriched anti-MART-1 specific human T lymphocytes 14 days after stimulation with anti-CD3/-CD28 microbeads or immobilized anti-CD3 or our aAPCs in the presence of HD IL-2 and IL-15. Cellular fold increases were evaluated by Tripan Blue dye exclusion test on days 7 and 14 of expansion with artificial systems. X-axis, days in culture; y-axis, fold increase. (B) Apoptosis assessment in cultures of anti-MART-1 specific human T cells 14 days after stimulation with aAPCs or anti-CD3/-CD28 microbeads plus HD IL-2 and IL-15. Cytograms represent flow cytometric analysis of cultured cells stained with FITC-labeled ANN-V (x-axis) and PI (y-axis). (C) Cytotoxicity of anti-MART-1 specific human T cells after 14-day expansion from the stimulation with aAPCs or anti-CD3/-CD28 microbeads or immobilized anti-CD3 plus HD IL-2 and IL-15, or with MART-1 loaded T2 cell line (T2-MART-1) was tested by ^{51}Cr release assay using LCL target cells pulsed with the specific MART-1 antigen. Specific activity was measured from the difference between the lysis of LCL presenting the cognate tumor antigen and the lysis of LCL alone or loaded with the unrelated Flu or HIV peptides. Results are representative of three experiments. Error bars indicate s.d. of the mean. Statistical analyses were performed using two-tailed T test (*: $P \leq 0.05$). (D) Representative example of anti-MART-1 T cell immunophenotype 2 weeks after aAPC stimulation. Cell suspensions were analyzed by FACS, gating on $CD4^+CD3^+$, $CD4^-CD3^+$ (CD8 cell subset) and MART-$1^+CD4^-CD3^+$ populations. Cells were analyzed for the expression of CD62L versus CD45RA, CD69 and of FOXP3 to define their maturation and activation level and regulatory T cell frequency in expanded cultures respectively.

Figure 6: **Effect on T cell expansion of the 1: 10 or 1: 100 aAPC dose: T cell ratios.**

Ten and 100 fold superior amount of purified polyclonal $CD3^+$ T cells isolated from healthy donor PB were cultured for two weeks with the same dose of aAPCs previously used, with HD IL-2 and IL-15. (A) Fold increase representation of polyclonal $CD3^+$ T cells expanded for 14 days with

aAPCs. Five millions or 0.5 x 10^6 T cells were cultured with the same dose of aAPCs used for 0.05 x 10^6 starting cells and HD IL-2 plus IL-15. Cellular fold increase was evaluated by Tripan Blue dye exclusion test on days 7 and 14 of expansion. Results are representative of three experiments with different donors. Error bars indicate s.d. of the mean. (B) Maturation status defined as CD45RA versus CD62L expression 14 days after stimulation with 1: 10 or 1: 100 aAPC = dose: T cell ratio. (C) Evaluation of aAPC amount and culture volumes required to hypothetically expand 10^9 cells for adoptive immunotherapy using different aAPC dose: T cell ratio. From the empirically found fold increase, it was possible to derive the starting cell number and the related amount of aAPCs required to obtain 10^9 T cells in the different conditions of aAPC dose: T cell ratio.

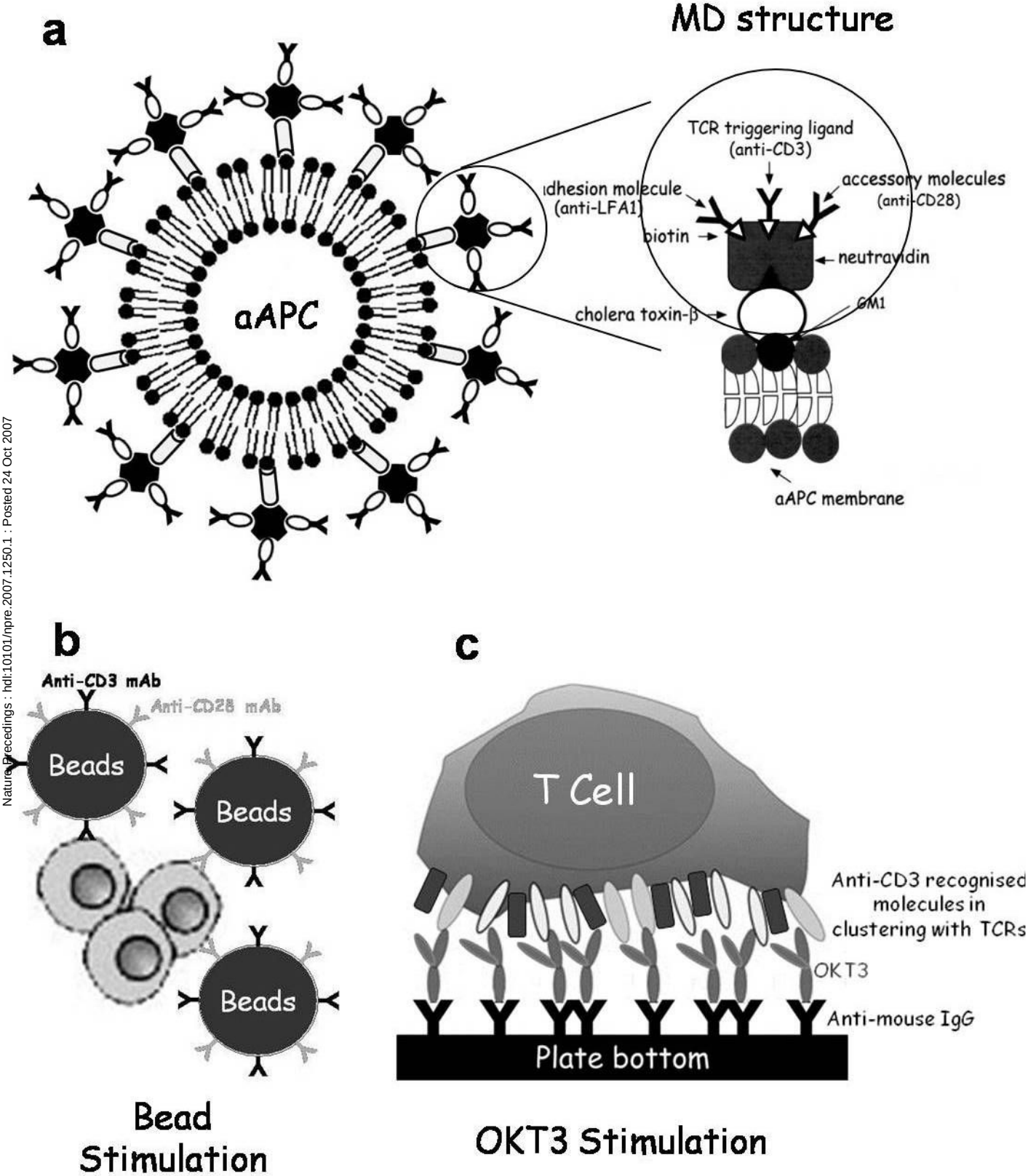


Figure 1

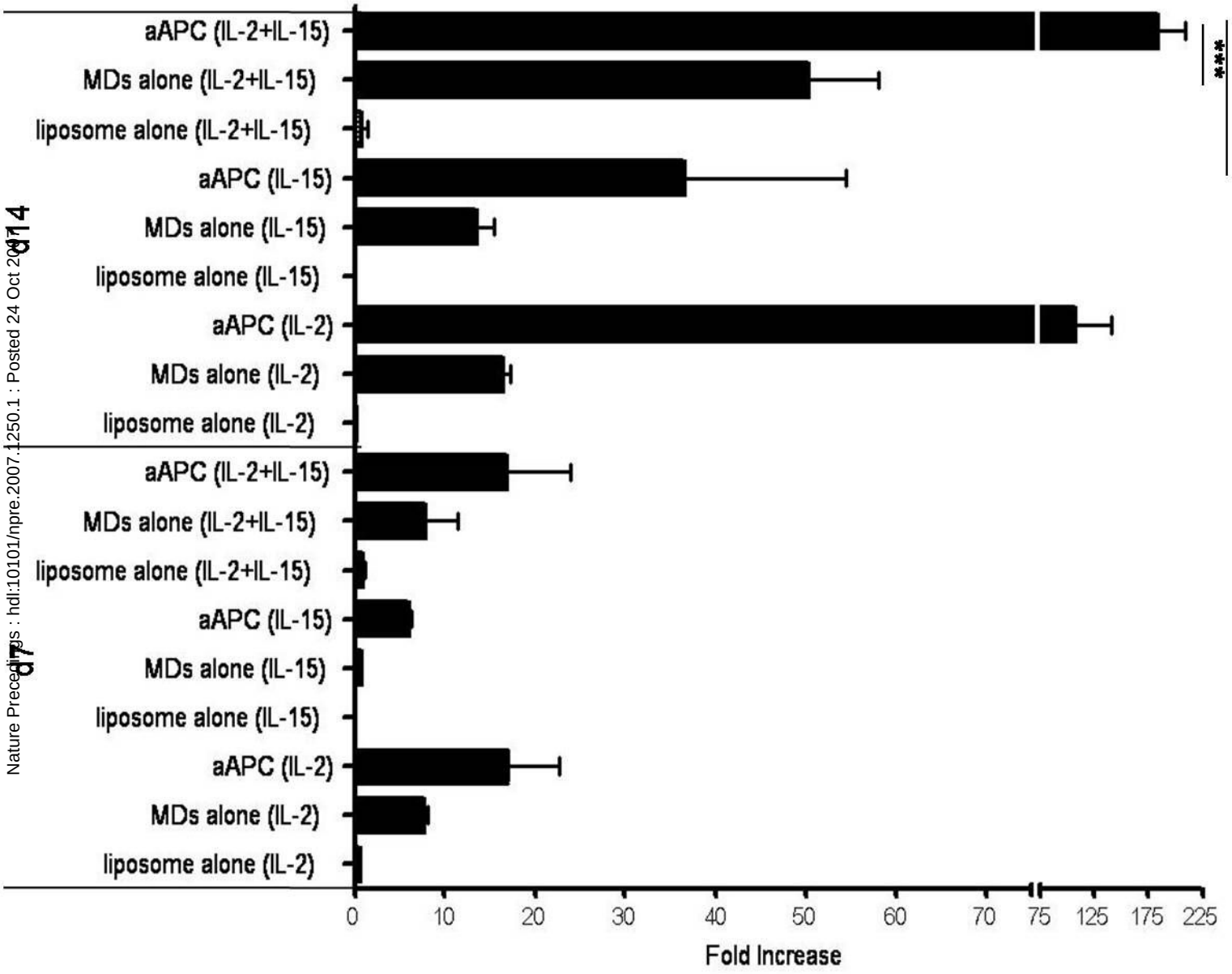
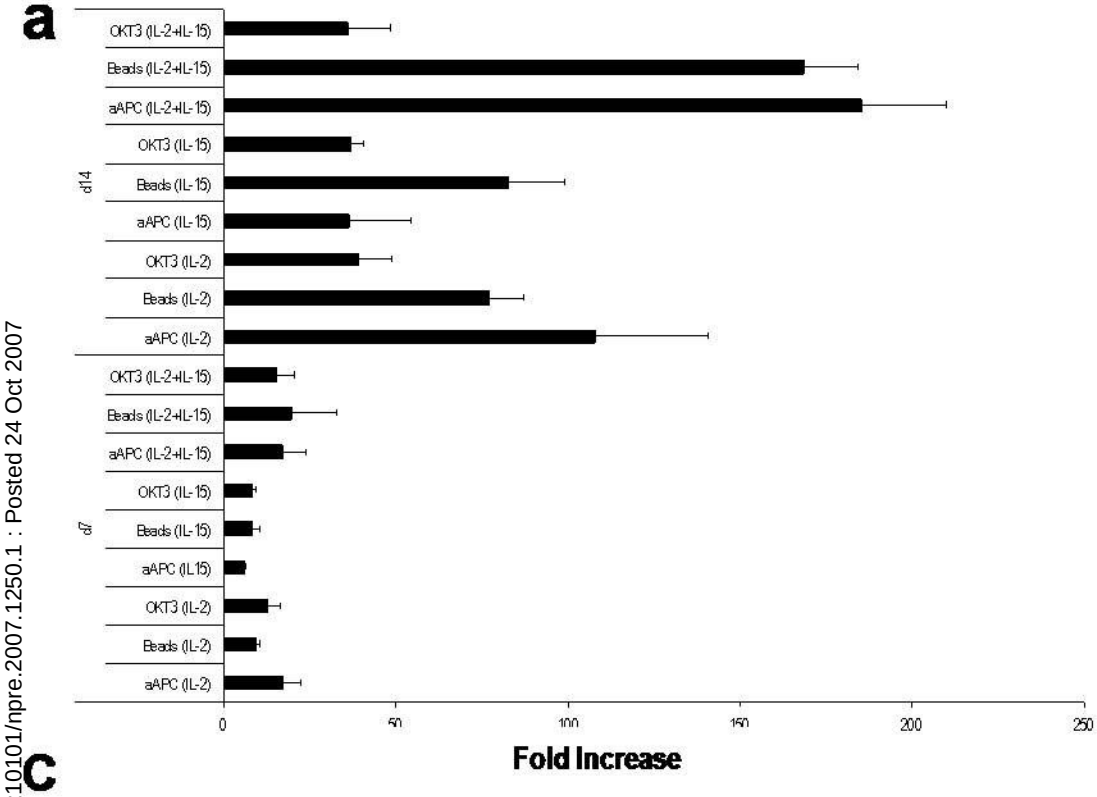
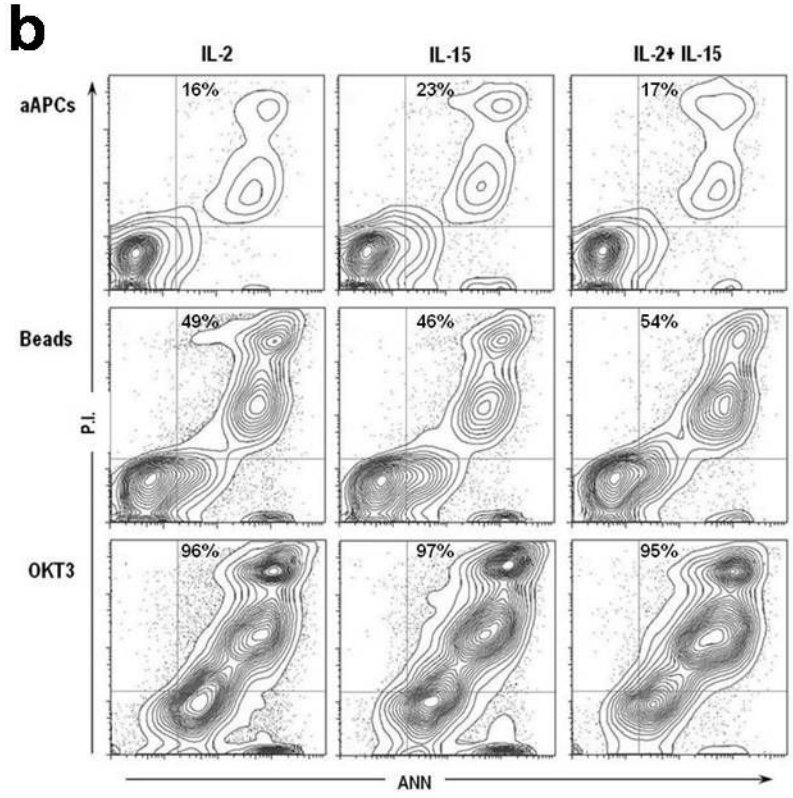


Figure 2





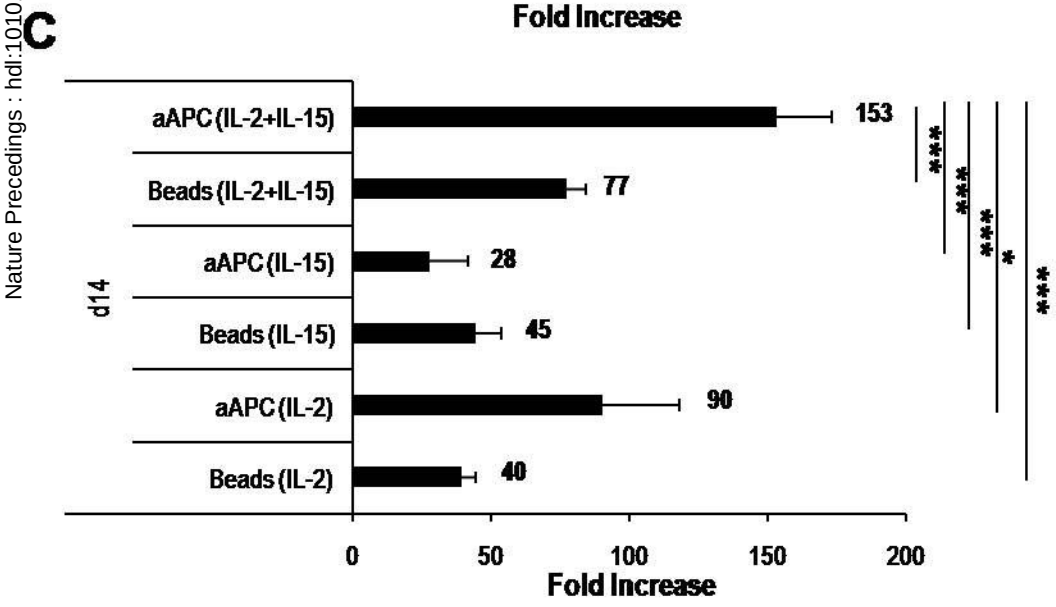
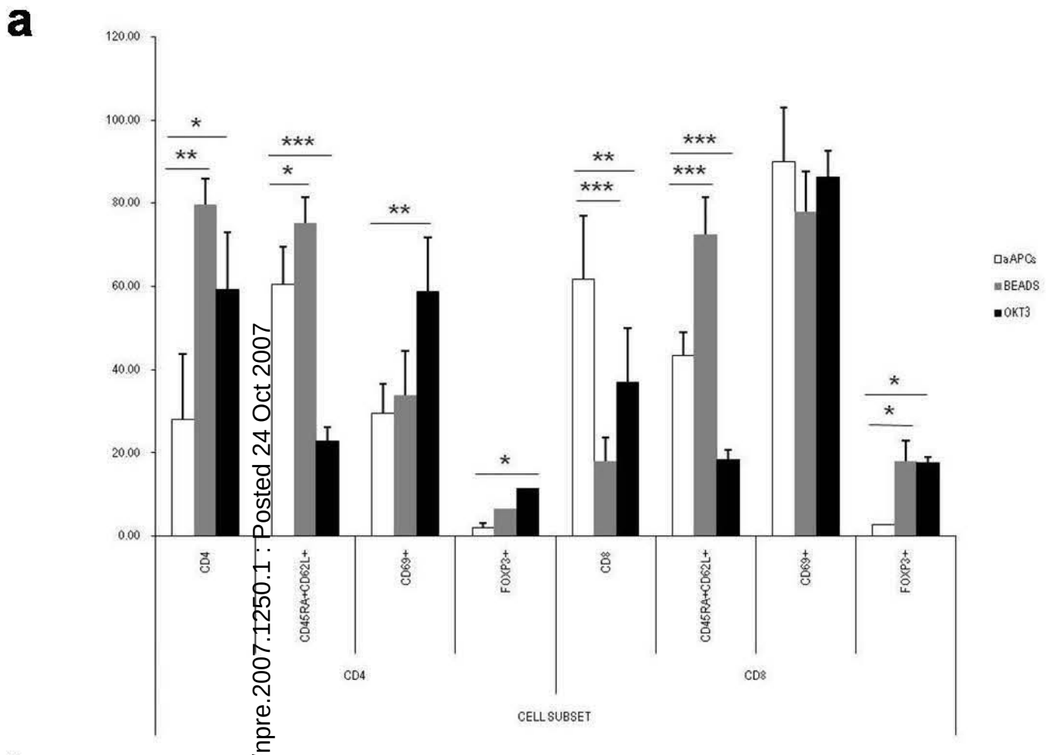
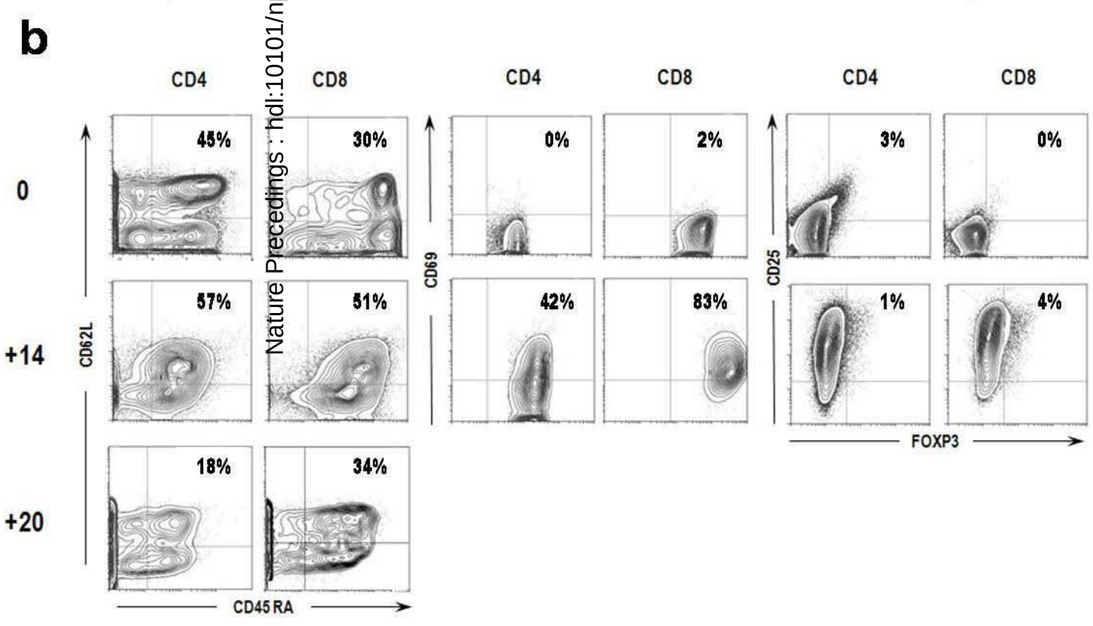


Figure 3





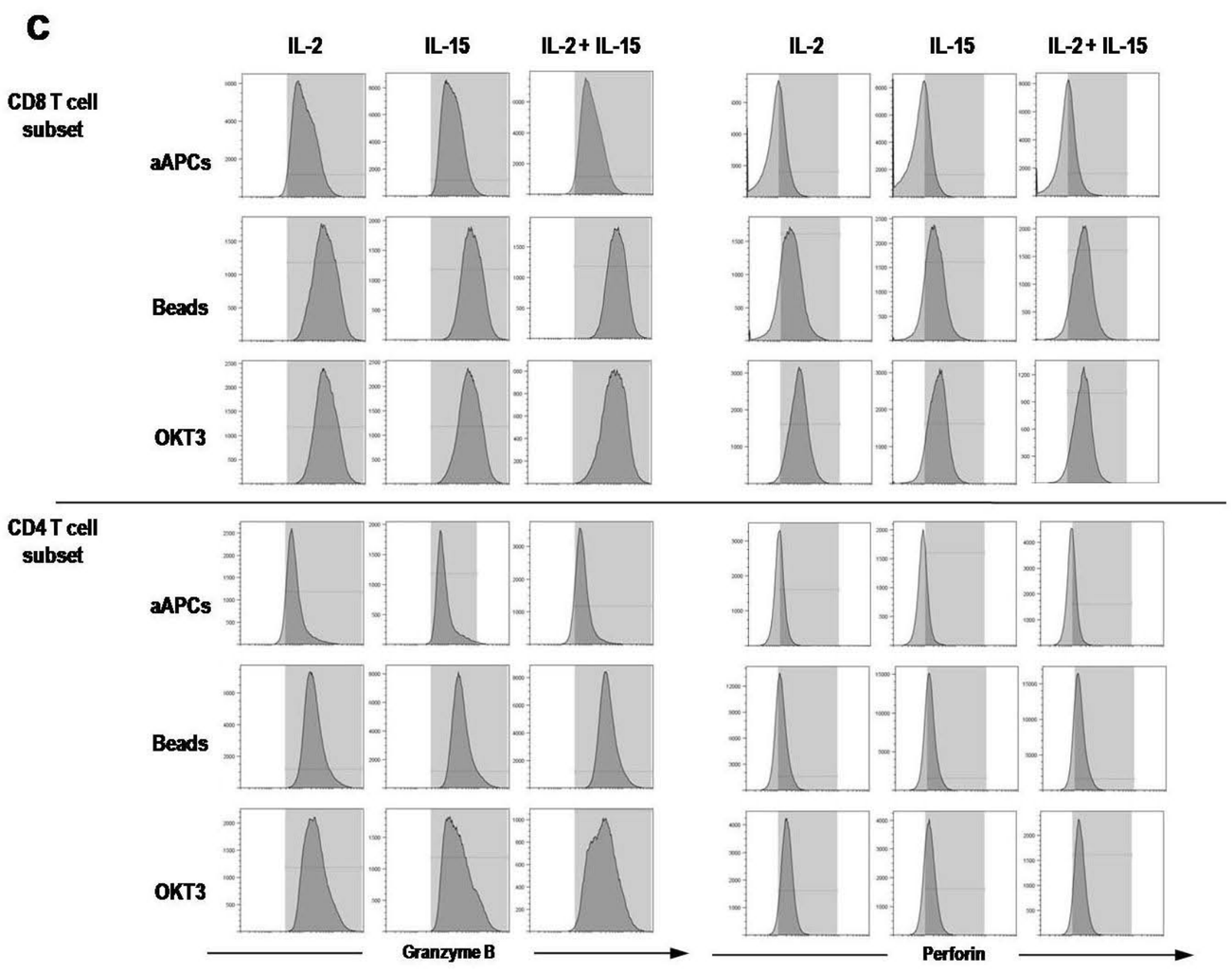


Figure 4

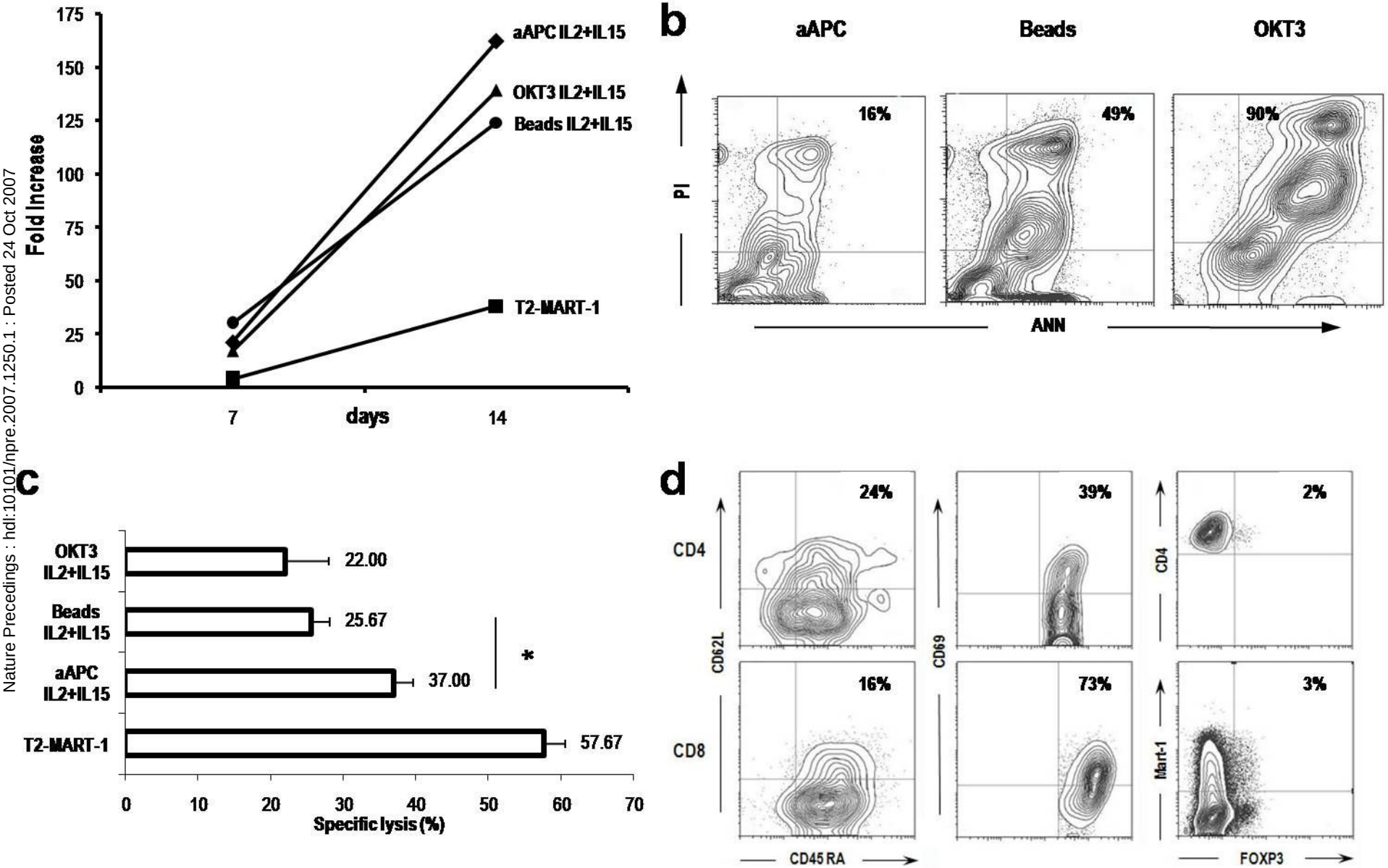
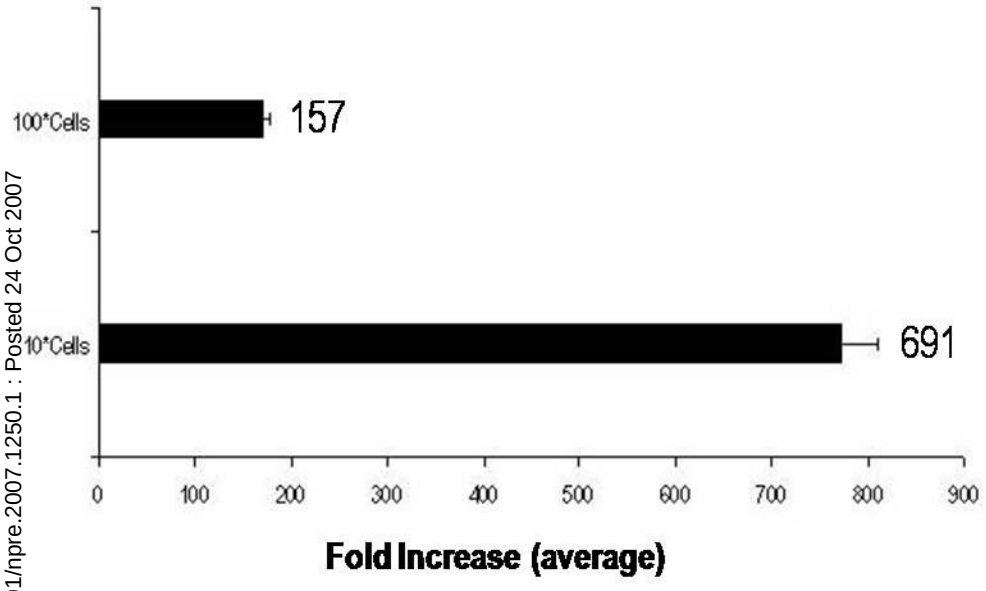


Figure 5

a



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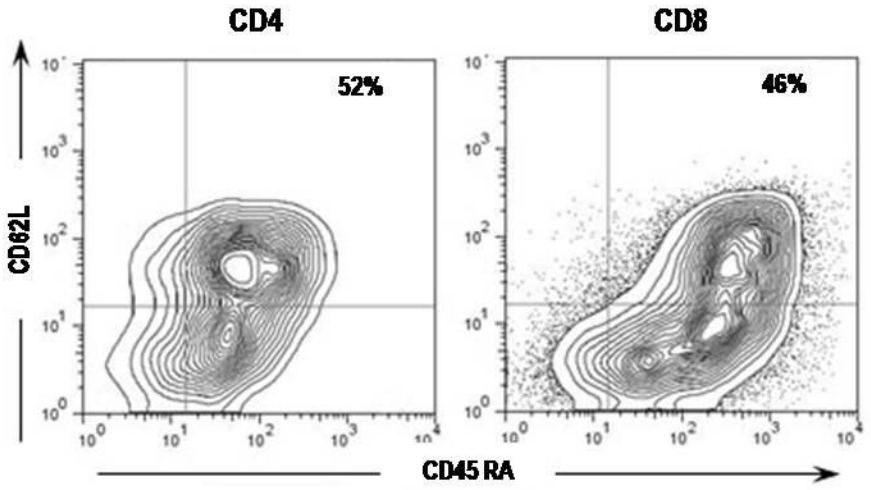

c

Ratio	aAPC dose: T cells	Target cell n°	Expansion fold increase	Starting T cell n°	Required aAPC doses	Starting Volume (L)	Final Volume (L)
Standard	1:0.05 x 10^6	10^9	153	65,4 x 10^6	1274	3,268	50
1: 10	1: 0.5 x 10^6	10^9	651	15,4 x 10^6	31	0,031	20
1:100	1:5 x 10^6	10^9	157	63,7 x 10^6	13	0,051	8

Figure 6