

EXPLORING THE IMMUNOGENICITY OF DCs PULSED WITH HLA-A2 RESTRICTED TUMOR ANTIGENS IN COLOMBIAN INDIVIDUALS

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ABSTRACT: Despite vaccination with DCs pulsed with Tumor Associated Antigens HLA-A2 restricted (TAAs-A2) has been thoroughly evaluated in Caucasian individuals as alternative for cancer immunotherapy, the coverage and immunogenicity of this type of vaccine in Colombian population is yet to be established. In this study the frequency of HLA-A2 alleles suitable for immunotherapy with TAAs-A2 and the immuno-phenotyping of monocyte derived DCs and TAAs-A2 specific CD8 T-cells were examined in Colombian individuals. **METHODS:** A statistically significant sample of individuals representative of the Colombian population (180 individuals in the city of Bogotá) was selected to establish the frequency of the allele HLA-A* 02011 and HLA-A* 02013 using PCR-SSP. DCs obtained from peripheral blood monocytes of 24 individuals were purified using Rosette-Sep (StemCell Technologies) and the phenotype of DCs in response to maturation cocktail (IL-6, IL-1 β , TNF- α [CellGenix] and PGE-2 [Sigma]) was analyzed. To evaluate the immunogenicity of DC pulsed with TAA-A2 from HER-2/neu, NY-ESO1, Melan-A and

Telomerase, CD8 T cells from normal individuals purified using magnetic beads (Miltenyi Biotec) were co-cultured 10 days in IL-2 and IL-7 and re-stimulated 2 days with peptide pulsed DCs. Production of Interferon Gamma (IFN- γ), cytotoxicity and tetramer positive CD8 T cells specific for the TAA-A2 was assessed using Flow Cytometry. **RESULTS:** HLA typing evidenced an allelic distribution of HLA-A* 02011 and A*02013 of 28%. Whereas up-regulation of CD80, CD83 and CCR7 and down-modulation of CD209 was evidenced in response to maturation stimulus, no change in the expression of CD86 and HLA-DR in response to maturation was evidenced in DCs of the 24 individuals examined. Finally, the micro-culture system used allowed the detection and analysis of the response of CD8 T-cell precursors specific to TAA-A2 of HER-2, NY-ESO1 and Melan-A but not against Telomerase. **CONCLUSION:** The proved immunogenicity of DCs pulsed with TAA-A2 and the frequency of HLA-A2 alleles in the population led us to predict that DCs pulsed with TAA-A2 may be an important alternative for cancer immunotherapy in Colombia.

INTRODUCTION

The allele HLA-A*0201 is one of the most frequent in individuals of Caucasian and Hispanic origin, hence, numerous clinical studies of antitumor immunotherapy using HLA-A*0201 restricted antigens as vaccines have been made. The presence of CD8+ T cell with anti-tumor activity against tumor antigens that in response to tumor antigens produce INF-, and cytotoxic molecules such as perforin and granzymes has been widely described in healthy individuals, cancer patients [1, 2] and in HLA-A*02 volunteers participating in clinical cancer vaccine trials [3, 4, 5]. Because the high number of well characterized HLA-A*0201 epitopes highly immunogenic shared for a wide variety of tumor antigens, very often, one of the criteria for inclusion of patients in these studies is the presence of the allele HLA-A*0201. The population of Bogotá, is composed of a polyethnic mixture and is considered representative of the Colombian population. So far it is not known the distribution of allele HLA-A*0201 required for planning studies of immunotherapy in this population. In order to determine the frequency of the allele HLA-A*0201 in this population, typing HLA-A*0201 in a sample of the population in Bogotá city was conducted using a combination of flow cytometry and PCR-SSOP method described by Gatz [6]. The high frequency of this allele and the presence of CD8+ T cell precursors specific TAAs-A2, HLA-A*0201 evidenced in healthy individuals evidenced in our work, suggests that the use in this population of immunotherapy based on HLA-A*0201 restricted peptides would have a high coverage.

OBJECTIVES

- To establish the frequency of expression of HLA-A*0201 in Colombian individuals.
- To explore the immune-phenotype and functional proficiency of Fast-DCs in Colombian individuals.
- To evidence the presence and immunological status of CD8+ T lymphocytes precursors specific for TAAs-A2 tumor antigens in peripheral blood of healthy Colombian individuals.

METHODS

Volunteers and blood samples: A total of 146 healthy adult individuals residing in the city of Bogotá (Colombia), taken at random, agreed to participate in the study after firming the informed consent form. This study was approved by the Ethics Committee of the National University of Colombia, Medical School. Using a sample of 3 ml peripheral blood in a tube with sodium citrate, after red blood cells lysis a screening by flow cytometry using the antibody anti-HLA-A*02 (BB7.1 clone from BD Biosciences, CA, USA) was done. The genomic DNA was obtained with the kit Wizard™ Genomic DNA Purification Kit (Promega Corporation, Madison, WI).

PCR-SSOP: Only positive samples by flow cytometry for HLA-A*02, were subjected to PCR-SSOP according to previously published protocol [6]. Briefly, 25 μ g of genomic DNA were used for the first reactions out for PCR reactions (Step 1), which confirms or rules out the presence of an allele HLA-A*02 by the presence or absence of an expected amplicon that was co-amplified with 2-microglobulin. Samples that amplify the allele HLA-A*02 is subjected to a Step 2 (12 PCR reactions of dilutions of the product HLA-A*02 obtained in Step 1) which establishes the presence of an allele HLA-A*0201. The allele HLA-A*0201 was confirmed in a Step 3 (composed of 16 PCR reactions). The samples were analyzed in an agarose gel 2.5%.

Isolation and cell culture: Monocytes from peripheral blood of healthy donors were isolated using RosetteSep (StemCell Technologies) and frozen in RPMI, FBS and DMSO. Cells were cultured 1-2x10⁶ cells/ml in AIM-V supplemented with IL-4 (750U/ml) and GM-CSF 1000U/ml (CellGenix) during 24 hours (37°C/5% CO₂) then cultured with proinflammatory cytokines (CellGenix) during 24 hours more (TNF- α 10ng/ml, IL-1 β 10ng/ml, IL-6 1000U/ml and PGE2 1 μ g/ml). CD4+ and CD8+ T cell were purified by positive selection from PBMCs by MACS column (Miltenyi Biotec).

DCs phenotype characterization: DCs were collected in PBS+1% FBS and stained with mAbs: CD209 (DC-SIGN), HLA-DR, CD14, CD80, CD86, CD83 (Becton Dickinson) and CCR7 (R&D Systems) with standard cell staining protocol. Cells were analyzed in CyAn ADP (Beckman Coulter) and FACS Canto II (BD Biosciences, San José). Data were analyzed in Summit 4.3 (Beckman Coulter) and FlowJo 7.2.5 (Tristar).

Peptide specific CD8+ T cell line generation: Mature Fast-DCs, pulsed with each peptide 10M: Melan-A (ELAGIGILTV), NY-ESO-1 (SLLMWITQA), Her-2/neu (KIFGSLAFL), Telomerase (ILAKFLHWL) or irrelevant peptide were co-cultured with CD8+ T cells from HLA-A*0201 donor (1:5 ratio DCs:CD8 T cells). After 48 hours IL-2 (30U/ml) and IL-7 (5ng/ml) were added. Cells were collected after 10 days of culture (adding cytokines every 72 hours) counted and restimulated with peptide pulsed Fast-DCs (1:10 ratio). After 72 hours, cells were stained with mAbs: CD8 (Caltag) and specific tetramers (Dako Cytomation) and analyzed by flow cytometry.

Cytotoxicity assays: Co-cultures of T CD8+ lymphocytes restimulated with peptide pulsed Fast-DCs, ratio 1:2 Fast-DCs:CD8+, were incubated in 200 μ l of AIM-V + 1 μ l of Monensin (Becton Dickinson GolgiStop™) + 1 μ l of anti-CD107a and 1 μ l of anti-CD107b (Becton Dickinson). As a negative control an irrelevant peptide was used (Leishmania spp. DSLTNLRAL). After five hours of incubation, the cells were collected and washed with PBS + 0.5mM EDTA. Finally, cells were stained with CD8 and analyzed by FC.

IFN- γ measurement and CFSE proliferation assay: IFN- γ production was measured in supernatants from 40 hours of culture (ELISA, OptEIA – BD) and the CD4+ T cells proliferation was determined by flow cytometry evaluating the CFSE dilution after 112 hours (CFSE – Molecular Probes) in the following assays: (i) Mixed Lymphocyte Reaction (MLR) with Fast-DCs co-cultured with autologous or heterologous CD4+ T cells and (ii) Tetanus toxoid (TT 10 μ g/ml) pulsed Fast-DCs co-cultured with autologous CD4+ T cells. The ratio employed in both assays was 1:5 Fast-DCs:CD4+ T cells.

Statistical analysis: Statistical analysis (Mann-Whitney Test) and graphs were made in Prism 5.0 (GraphPad). p value <0.05 was considered significant.

RESULTS

Bogotá is a cosmopolitan city with about 8 million inhabitants that has a genetic component Indigenous, Caucasian, Hispanic and African descent in constant migration. The sample of 146 individuals chosen in this study was calculated assuming an estimated frequency of 15% expression of HLA-A* 0201 in the population (confidence value of 95% and an acceptable error of 4%). By screening with the antibody BB.7, 58 individuals carrying the allele HLA-A*02 57/146 (39%) confirmed in Step 1 PCR SSOP were identified (Fig 1). This frequency is lower than that reported for Caucasian and Hispanic American (46.9% and 50% respectively) and lower than that reported for African descent (34.6%) [7], and similar to that of the mestizo population in Cali, Colombia [8]. For the identification of carriers of HLA-A*0201 (HLA-A*02011/2/3) and non-HLA-A*0201, the products of PCR fragment of DNA HLA-A*02 of the 57 carriers were subjected to Step 2 PCR-SSOP [6]. The HLA-A*0201 were confirmed in a Step 3 SSOP-PCR. Table in Fig 1C shows that 28% (41/146) of HLA-A*02 carriers was identified the presence of the allele HLA-A* 0201.

Phenotypic frequency of 28% means that 1 out of 4 individuals in the population is carrying the phenotype HLA-A* 0201. The allelic frequency in a population is influenced by the presence of individuals heterozygous and homozygous for allele, taking into account 41/146 individuals carrying HLA-A* 0201, that 9 showed a pattern consistent with homozygous and 32 heterozygous for a total of 50 HLA-A* 0201 of the 292 alleles of the sample, the estimated frequency HLA-A* 0201 was 17%.

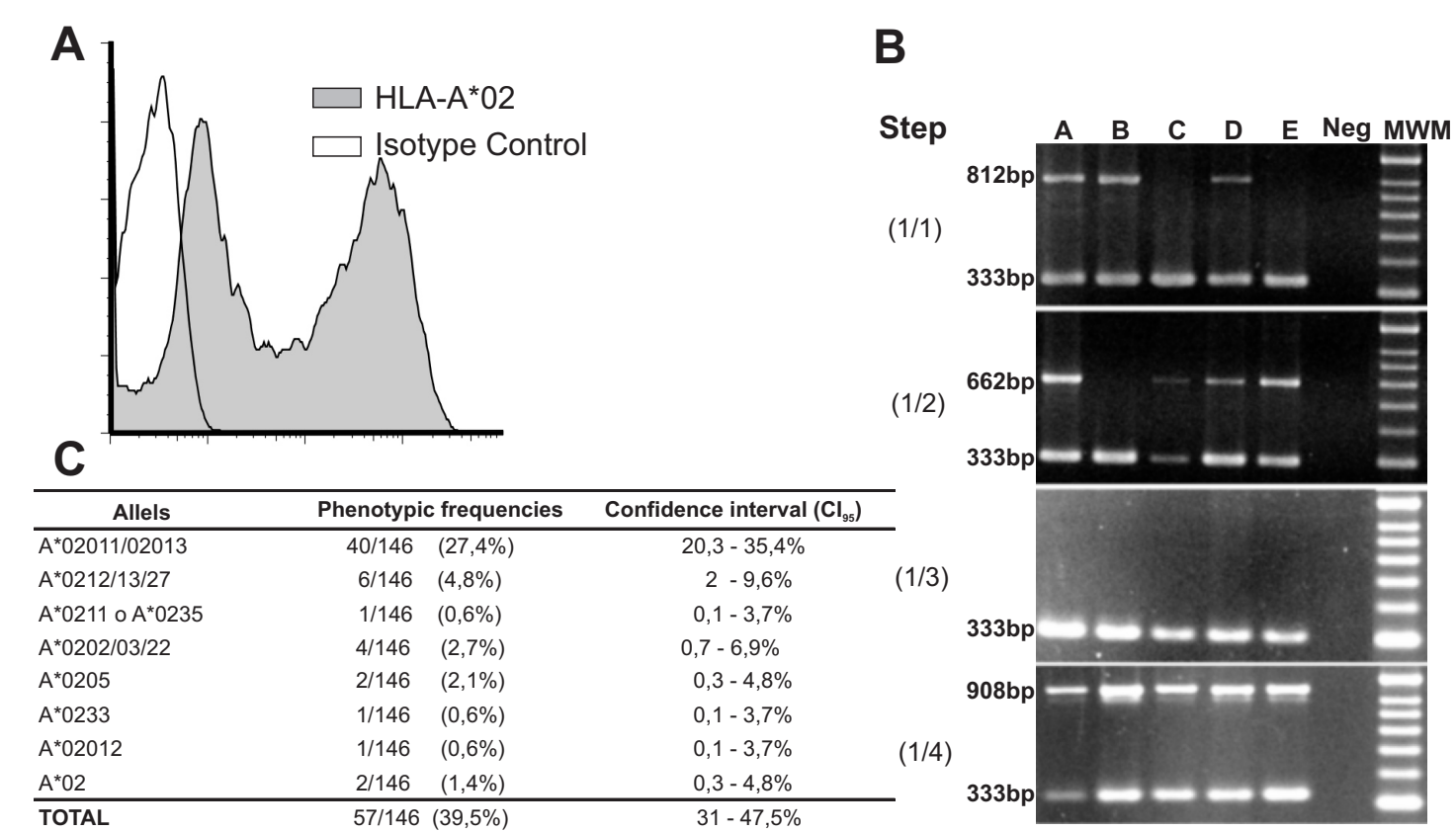


Figure 1. Frequency of HLA-A*0201 in the Colombian population. A. Histogram of white blood cells stained with isotype control antibody BB7.1 (anti-HLA-A*02) and analyzed by flow cytometry. B. A PCR-SSP based method described by Gatz et al., (Tissue Antigens 2000; 55: 532-547) was used to specifically select HLA-A*0201 individuals. Agarose gel that shows amplicons of different sizes that are typical for the four reactions of step 1 (reactions 1 / 1 to 1 / 4); ac, patterns 1, 2 and 5 obtained in the samples analyzed, d, cells SK MEL 23 - HLA-A*02 positive; e, U937 cells HLA-A* 02 negative, and f, PCR negative control. In all reactions are evidenced the co-amplification of 333 bp fragment from β 2-m amplified as control in each reaction. C. Table summarizing the frequency of HLA-A*0201 in the sampled population.

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) involved in the induction of T-cell-mediated immune responses and have appeared as important candidates for cellular-based therapies [8]. Because development of mature DCs from monocytes is labor intense efforts to reduce time of production of DCs is desirable. Dauer and co-workers have developed a protocol for the generation of DCs from human monocytes within 48 hours (Fast-DC) aimed to reduce processing time for generation of DCs important for large-scale clinical production of DCs [9]. In this study we compared Fast-DCs (2-day culture) with monocyte-derived DCs generated by a standard 7-day protocol (Standard DCs) for the expression of DC activation markers, sensitivity to proinflammatory and lymph node-directing chemokines, and their ability to induce proliferation and Ag-specific IFN-production in autologous T cells as well as to prime autologous naive T lymphocytes. Figure 2 shows that in Colombian individuals Standard and Fast-DCs exhibit similar immune-phenotype. Furthermore the functional capacity to elicit in CD4+ T-lymphocytes potent cellular responses such as proliferation (Figs 3A and 3B) and cytokine responses (Fig 4) in response to different stimuli.

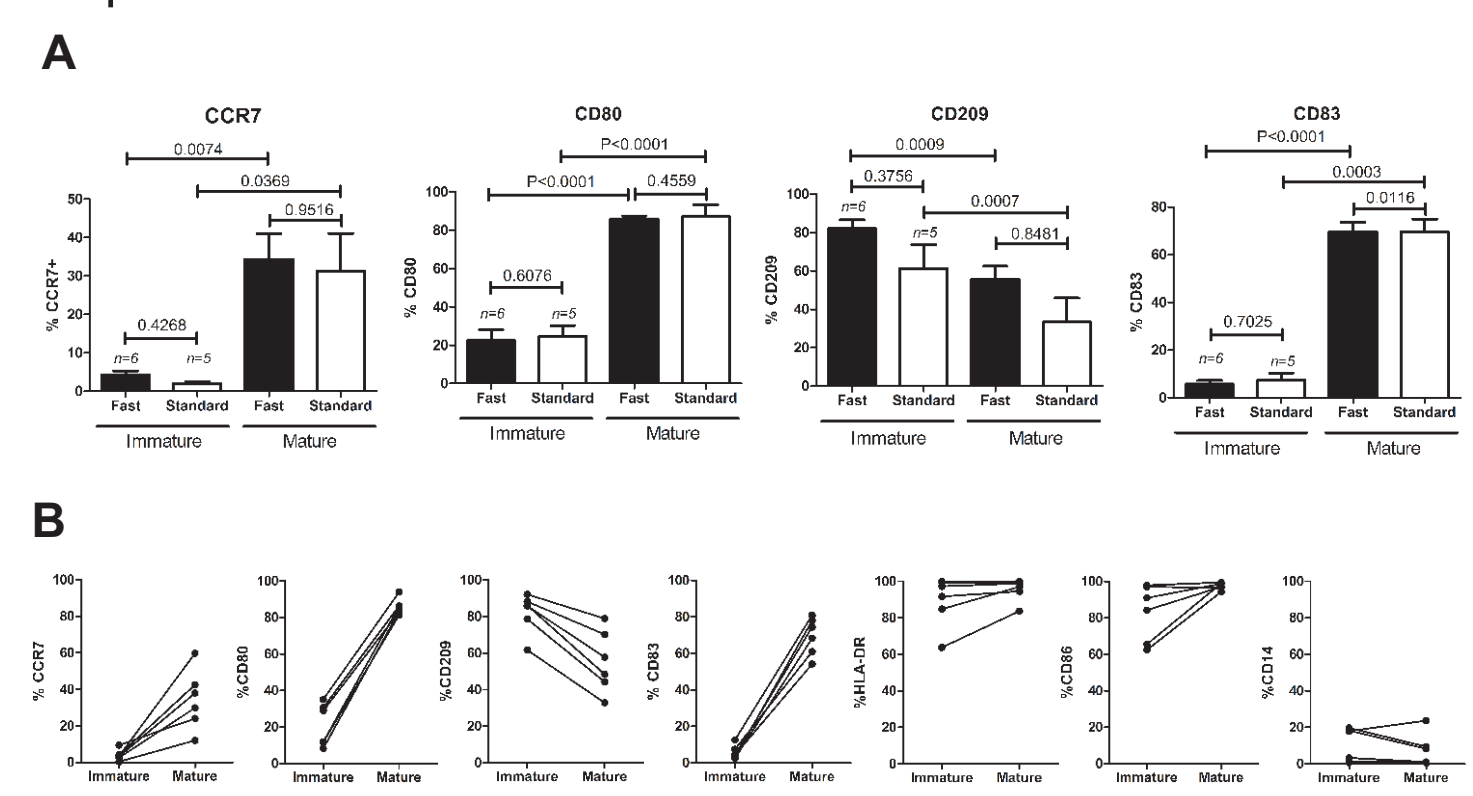


Figure 2. Phenotypic analysis by flow cytometry of Standard and Fast-DCs. A. Standard and Fast-DCs were cultured for 24 hours and 5 days respectively with IL-4 and GM-CSF (immature), and then matured with IL1 β , IL6, TNF α and PGE2 by 24 and 48 additional hours, respectively. The cells obtained in each case were stained with antibodies to CCR7, CD83, CD209 and CD80. B. Percentages of expression of surface markers in Standard and Fast-DCs isolated from six healthy individuals, prior (immature) and after (matured) treatment with pro-inflammatory cytokines.

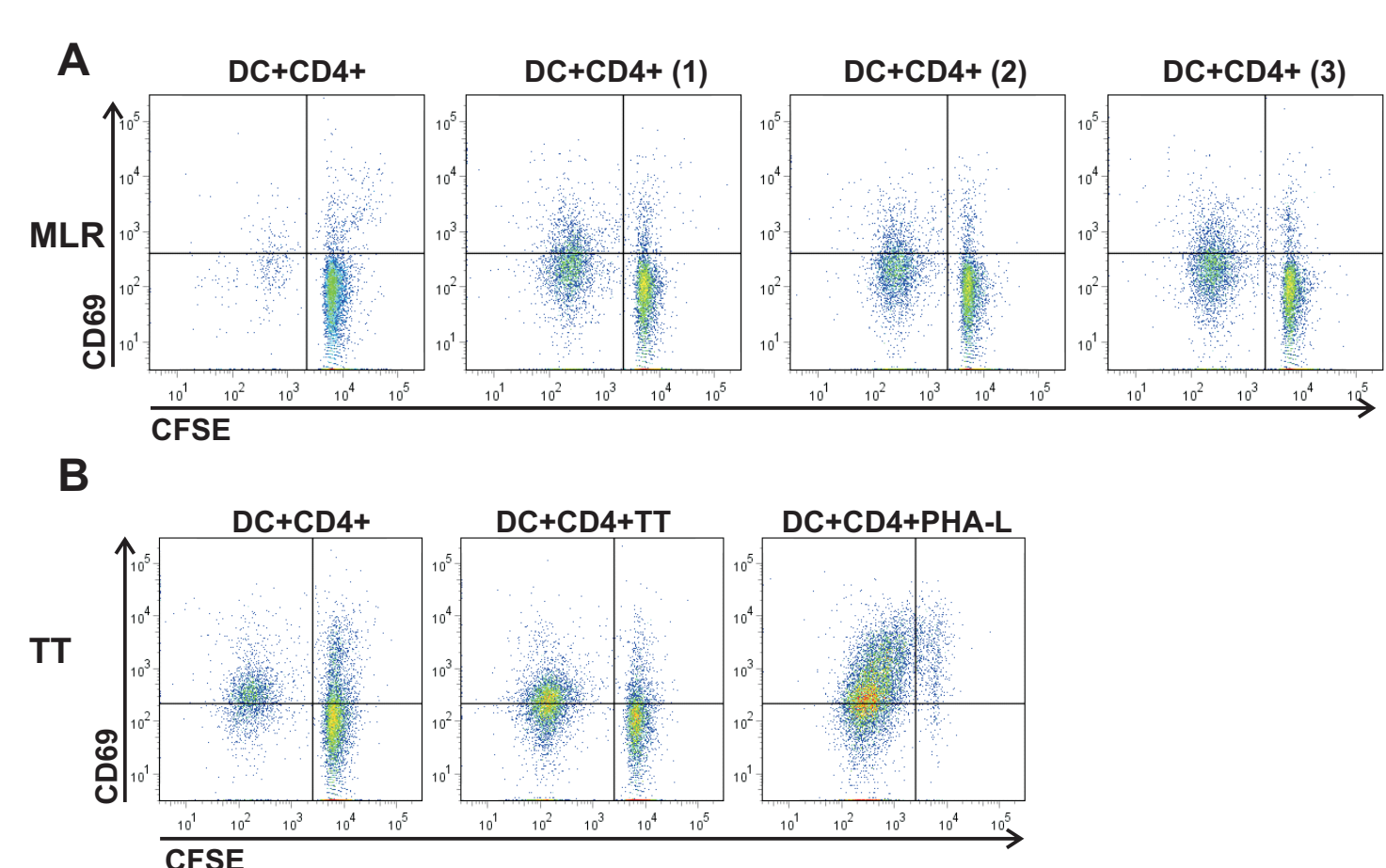


Figure 3. Proliferation and activation of CD4+ T-lymphocytes induced by Fast-DCs. CD4+ T-lymphocytes positively selected were labeled with CFSE 2 μ M, co-cultured (ratio 5:1) for 112 hours with Fast-DCs matured with the cocktail of pro-inflammatory cytokines and then stained with anti-CD69 antibody. A. Mixed Lymphocyte Reaction (MLR), plots profile of Fast-DCs cultured with autologous CD4+ T lymphocytes and from individuals 1, 2 and 3, respectively. B. Plots of CD4+ T lymphocytes co-cultured with Fast-DCs pulsed with 10 μ g/ml TT and Fast-DCs stimulated with PHA-M 2% v/v, respectively.

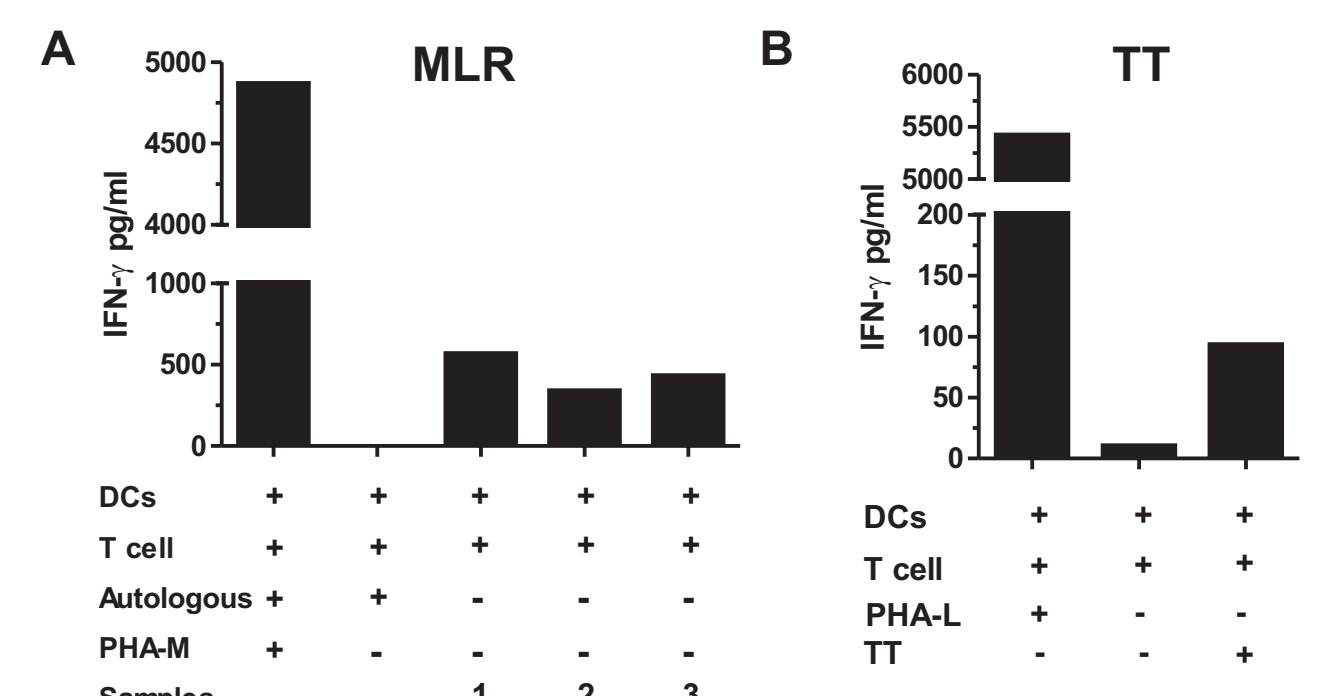


Figure 4. IFN- γ production by CD4+ T-lymphocytes after stimulation with Fast-DCs. The production of IFN- γ was determined by ELISA in culture supernatants of CD4+ T-lymphocytes co-cultured with (A) autologous and heterologous Fast-DCs in a typical MLR reaction and (B) Fast-DCs pulsed with or without TT.

Studies of anti-tumor immunotherapy would require the presence of specific CD8+ T-lymphocyte precursors against tumor antigens that vigorously expand in response to tumor antigens suitable to attack the tumor. We monitored by tetramer staining the capacity of Fast-DCs to expand CD8+ T-cell precursors specific for four TAAs-A2 tumor antigens in several HLA-A*0201 individuals. Figures 5 shows that vigorous expansion of CD8+ T-lymphocytes specific for TAA-A2 tumor antigens can be detected in normal Colombian individuals (one individual representative of five examined). Furthermore, Figure 6 and data not shown suggest that these cells elicit cytolytic responses against target cells expressing TAAs-A2 tumor antigens.

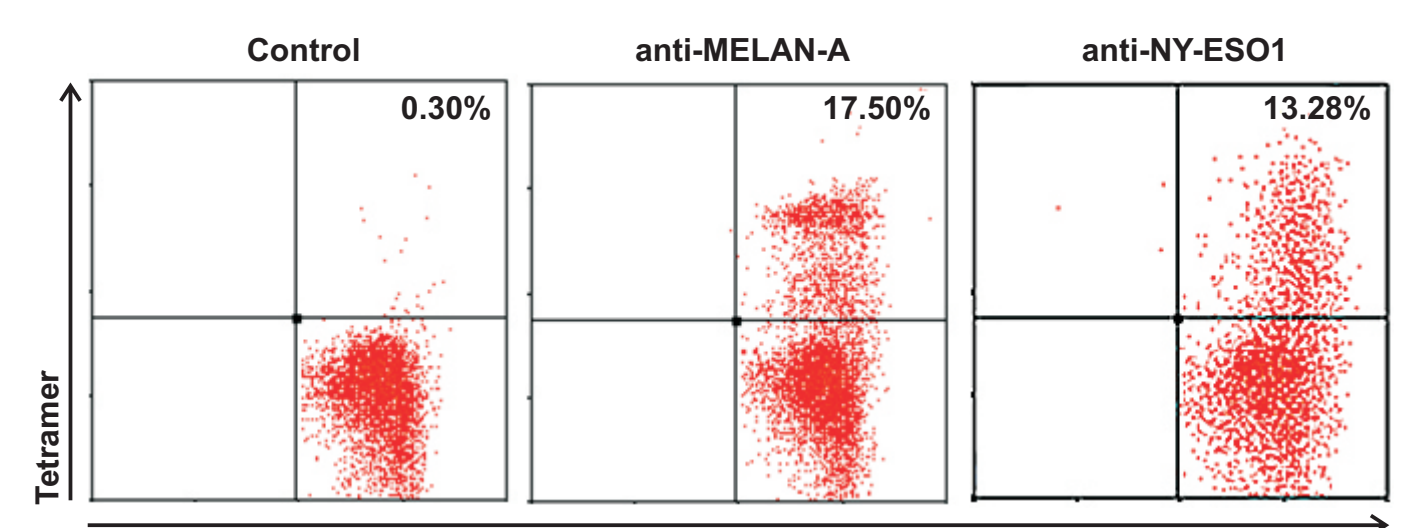


Figure 5. Peptide pulsed Fast-DCs efficiently amplify CD8+ T-lymphocytes specific for TAAs present in peripheral blood of normal individuals. CD8+ T-lymphocytes purified by positive selection were co-cultured with Fast-mature DCs pulsed with peptide Melan-A (ELAGIGILTV) or NY-ESO1 (SLLMWITQA) HLA-A*02 epitopes for 10 days in the presence of IL-2 and IL-7 and then re-stimulated and stained with HLA-A*0201 tetramers specific for each peptide.

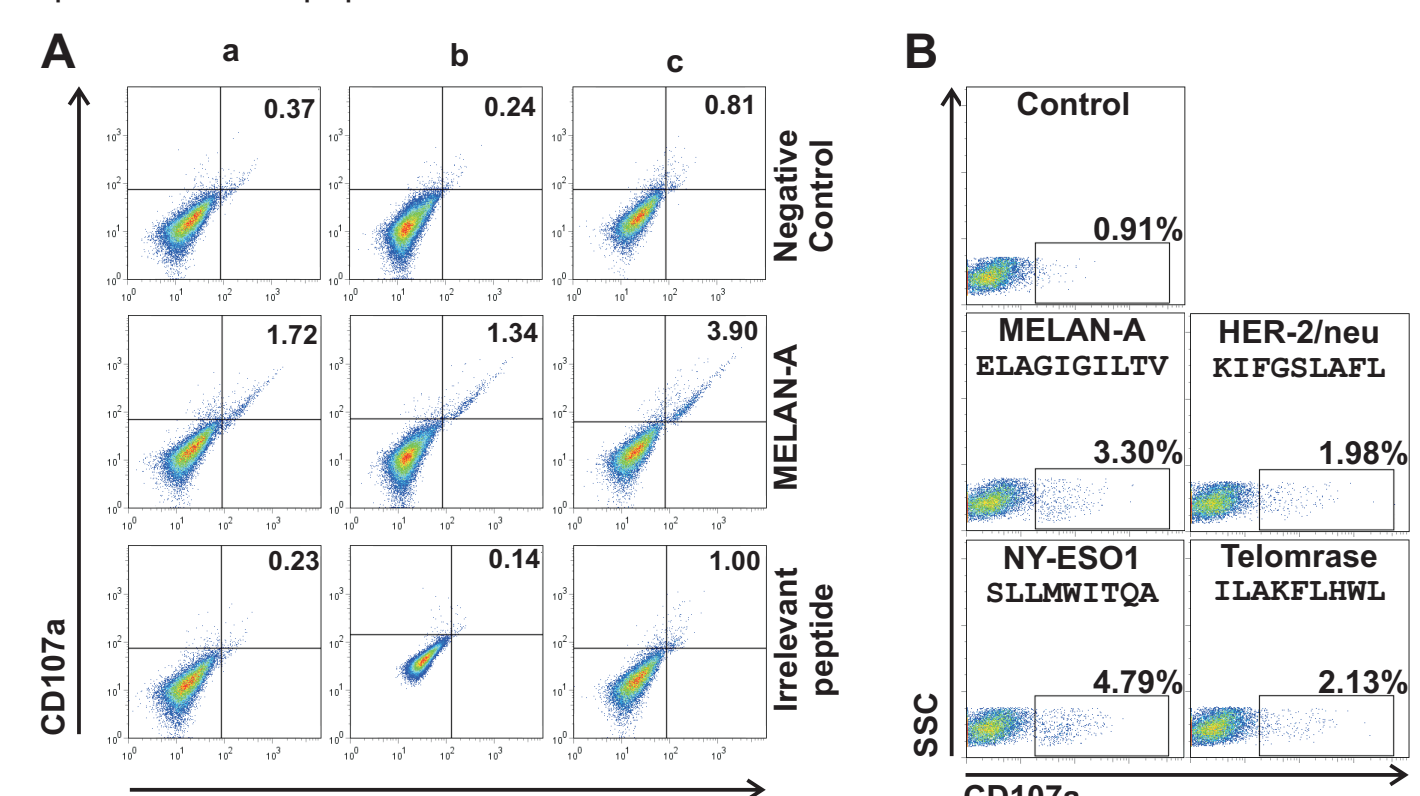


Figure 6. Test of CD107a and CD107b mobilization. CD8+ T-lymphocyte peptide specific were re-stimulated for 5 hours with Fast-DCs pulsed with peptide (ratio 2:1) in the presence of anti-CD107a/b and Monensin before being stained with anti-CD8 and analyzed by flow cytometry.

CONCLUSIONS

Our results suggest that one important reduction in processing time for generation of Fast-DCs suitable for large-scale clinical production is possible. On the other hand, the proved immunogenicity of Fast-DCs pulsed with TAAs-A2 tumor antigens and the frequency of the HLA-A*02011 and HLA-A*02013 alleles in the population led us to predict that Fast-DCs pulsed with TAAs-A2 tumor antigens may be an important alternative for cancer immunotherapy in Colombia.

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