Retroviral gene transfer, rapid selection, and maintenance of the immature phenotype in mouse dendritic cells

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Abstract: We used the retroviral vector PINCO [which expresses the green fluorescent protein (GFP) as a selectable marker], to infect growth factor-dependent immature D1 dendritic cells (DC). The efficiency of infection in different experiments was between 5 and 30%, but subsequent cell sorting led to a virtually homogeneous population of GFP-positive cells. Retroviral infection did not modify the immature DC phenotype, as shown by the low expression of major histocompatibility complex and co-stimulatory molecules. Furthermore, the GFP-positive D1 cells underwent full maturation after lipopolysaccharide treatment, as indicated by a high expression of cell-surface MHC and co-stimulatory molecules, and also by strong stimulatory activity in allogeneic mixed lymphocyte reaction. The high efficiency of this retroviral system, the rapidity of the technique, and the possibility to overcome *in vitro* selection make this method very attractive for the stable introduction of heterologous genes into proliferating immature mouse D1 cells. Furthermore, this approach is suitable for functional studies of new DC-specific genes involved in DC maturation and survival. *J. Leukoc. Biol.* 66: 263–267; 1999.

Key Words: *green fluorescent protein · retroviral vector*

INTRODUCTION

Dendritic cells (DC) are highly potent antigen-presenting cells capable of inducing a primary T lymphocyte response [1]. These bone marrow-derived leukocytes are widely distributed in tissues, especially in those with an environmental interface (skin and mucosal membranes) and in lymphoid organs. They express a unique repertoire of surface molecules involved in the uptake of microorganisms or their products. Because pattern recognition receptors, such as the Toll-like molecules [2], have recently been described as lipopolysaccharide (LPS) receptors [3, 4] these molecules are likely to be involved in microbial uptake. Once activated by inflammatory stimuli and infectious agents, DC migrate to the lymphoid organs to interact with antigen-specific T cells. During migration, the immature DC acquire professional antigen-presenting capacity, translocate major histocompatibility complex (MHC) molecules to the cell surface, and up-regulate co-stimulatory molecules [4]. As mature DC, they direct both the quality and the quantity of the immune response, providing all the signals for the activation of unprimed T cells [1].

Several groups have generated large numbers of functional DC/Langerhans cells from the precursors in either bone marrow, cord blood, or peripheral blood. DC precursors have been isolated with granulocyte-macrophage colony-stimulating factor (GM-CSF) alone or in combination with other cytokines [6–12]. However, such DC could only be propagated for limited periods because *in vitro* these cells undergo spontaneous maturation and cell death.

We have previously described a DC culture system that allows the propagation of homogeneous immature mouse DC that are growth factor-dependent and that remain immature in the presence of the conditioned medium DC-GM [5, 13]. This long-term, growth factor-dependent immature D1 cell line can be fully matured in response to inflammatory signals or bacteria, mimicking the *in vivo* DC maturation process. Using this unique differentiation system, the coordinated molecular events and the kinetics of DC maturation have been studied [14, 15, F. Granucci et al. unpublished results, and M. Foti et al. unpublished results].

We report the use of the recently described retroviral vector PINCO [16] to genetically modify the immature D1 cells without altering their morphological and functional properties. The characteristics of the PINCO vector (modified from the original plasmid LZRSpBMN-Z [17]) are the presence of the Epstein Barr virus origin of replication, and the EBNA1 gene outside the long terminal repeats (LTRs). This confers stable episomal maintenance of the retroviral constructs within the packaging cell line. In addition, the vector contains the gene encoding the green fluorescent protein (GFP) driven by a cytomegalovirus promoter and a cloning site for heterologous gene insertion driven by the 5′ LTR. Transient transfection of

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Abbreviations: GFP, green fluorescent protein; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; IMDM, Iscove's modified Dulbecco's medium; PBS, phosphate-buffered saline.

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the PINCO vector in the amphotropic packaging cell line, Phoenix, results in high-titer production of retroviral particles. Furthermore, it has been shown that this group of vectors is not prone to rearrangements, when maintained episomally in the packaging cells, and that viral stocks are helper-virus free [17].

Using this system, we have obtained a virtually homogeneous population of GFP-positive mouse DC that preserve their immature phenotype after retroviral infection. Moreover, the transduced cells are still able to undergo both functional and phenotypical maturation in response to lipopolysaccharide (LPS) or bacteria. The ability to insert and express heterologous genes with high efficiency in immature DC is full of promise for future studies of new DC-specific genes involved in DC maturation and survival.

MATERIALS AND METHODS

Cells and reagents

The D1 cells were derived from murine splenic DC and maintained *in vitro* as growth factor-dependent DC [5]. D1 cells were grown in complete Iscove's modified Dulbecco's medium (IMDM) supplemented with 30% of DC-GM, as described [13].

LPS (*Escherichia coli* serotype 026:B6) was purchased from Sigma Chemicals.

Retroviral infection of DC

The PINCO vector used in this study encodes the enhanced GFP under the transcriptional control of a cytomegalovirus promoter. The generation of retroviral vector and the production of high-titer vectors have been previously described [16]. To infect D1 cells, the cells were cultured in the presence of viral supernatant (filtered with a 0.45-μm filter) supplemented with 4 μg/mL of polybrene (Sigma) for 3 h. Three infection cycles were performed. After infection, the D1 cells were plated in complete IMDM supplemented with 30% of DC-GM for 24 h. The efficiency of transduction was then evaluated by FACS analysis (see below) and by fluorescence microscopy. Infected D1 cells were grown for 1 day on glass cover slips, fixed with 4% paraformaldehyde, and observed with a fluorescence microscope.

Sorting of GFP-positive cells

Infected cells were sorted by a FACScan (FACS-Vantage; Becton-Dickinson, Mountain View, CA) following the standard procedure with a standard excitation wavelength of 488 nm.

Phenotypical maturation

Twenty-four hours after LPS (10 μg/mL) activation, the D1 cells were detached with 2 mM EDTA in phosphate-buffered saline (PBS) and incubated with one of the following mAb: PE-conjugated anti-MHC II (I-Ad/I-Ed), biotinylated anti-CD86 (B7.2), PE-conjugated anti-CD40 (PharMingen). Appropriately diluted PE-streptavidin was used to detect the binding of biotinylated mAb.

To block Fc receptor binding, staining was carried out in the presence of 2-4G2 (anti-CD32) antibody. The cells were washed and analyzed using the FACScan (Becton-Dickinson).

Mixed lymphocyte reaction (MLR) assay

Primary allogeneic MLR was set up with sorted GFP-positive DC as stimulators. Stimulator cells were treated with mitomycin C (50 μg/mL, 20 min at 37°C; Sigma) and co-cultured with 2×10^5 BALB/c lymph node cells/well in 200 μL of complete IMDM. Cultures were pulsed on day 3 with 1 μCi/well of [^{3}H]thymidine (specific activity 85 Ci/mmol); Amersham, Amersham Place, UK). Incorporation of [^{3}H]thymidine was measured 8 h later with a liquid scintillation counter (MicroBeta Plus; Wallac, Turku, Finland). Each point on the graph represents the mean count per minute from triplicate cultures.

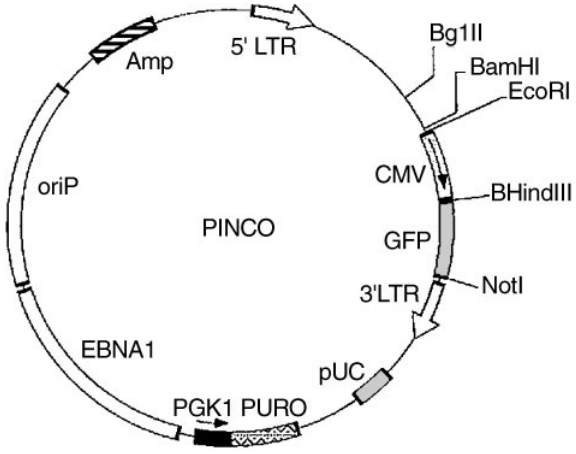

Fig. 1. PINCO vector. This plasmid contains the full-length Moloney LTRs and the extended ψ sequence and carries the GFP cDNA under the control of the cytomegalovirus (CMV) promoter. Outside the LTRs, it contains the PGK-1 promoter that drives puromycin resistance, the EBV origin of replication, and the EBNA-1 gene. The last two endow the plasmid with the capacity to replicate as an episome and develop 5–20 copies/mammalian cell. In 5′ to the CMV promoter there are cloning sites for the insertion of the cDNAs.

RESULTS

Infection of D1 cells

The packaging cell line Phoenix was transfected with the PINCO vector (**Fig. 1**) by the calcium-phosphate chloroquine method. Culture supernatants containing viral particles were collected 48 h after transfection. Infection was performed by culturing D1 cells with viral supernatant for 3 h. Three infection cycles were performed. The percentage of transduced cells was evaluated by FACS analysis 24 h after the last infection. The efficiency of transduction in different experiments was between 5 and 30%. The infected population was viable and dividing actively. Two days later the infected cells were sorted on the basis of GFP expression. A population of 97% GFP-positive cells was thus obtained (**Fig. 2**).

Phenotypical maturation of D1/GFP

The GFP-positive DC population (D1/GFP) was studied for its phenotypical and differentiative properties and compared with the non-transduced D1 cells.

The D1/GFP cells had a morphology comparable to the noninfected cells. The GFP expression resulted in a diffuse cytoplasmic fluorescent pattern in both fixed and living cells (**Fig. 3**).

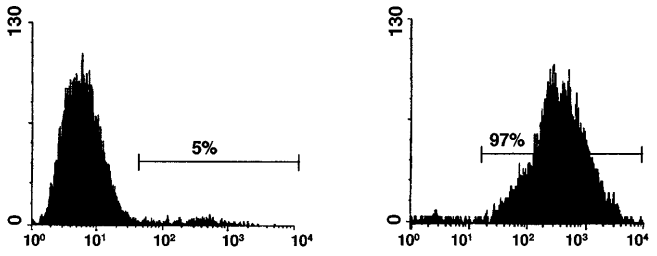

Fig. 2. D1 cells were infected with the PINCO vector. GFP expression before (left panel) and after (right panel) FACS sorting.

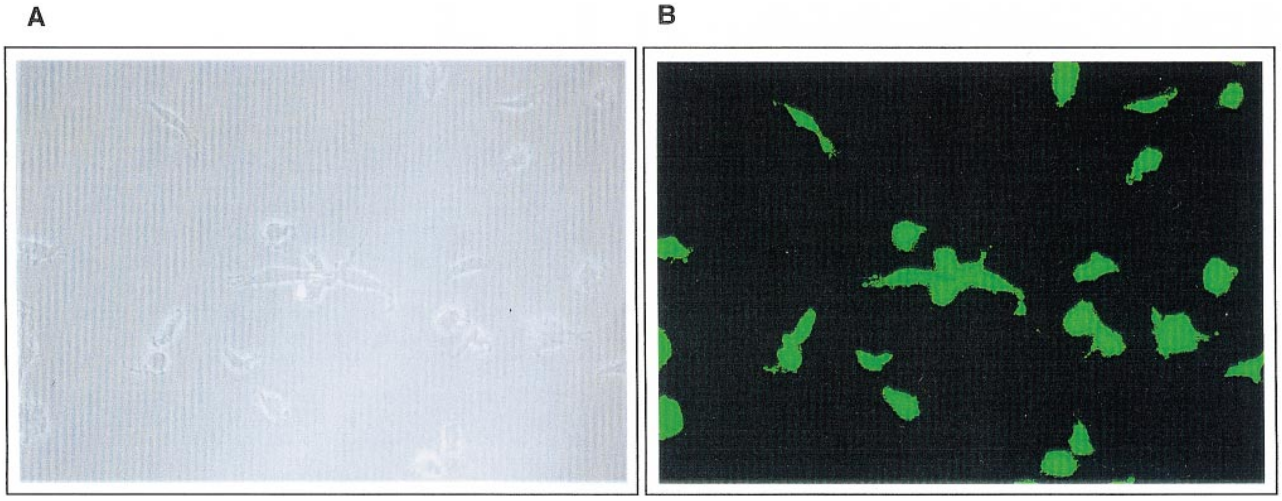

Fig. 3. To detect the expression of GFP, the infected cells were grown directly on coverslip, fixed with 3% paraformaldehyde, and observed using a fluorescence microscope (A, phase contrast; B, GFP fluorescence).

The D1 cells are maintained in an immature state in the presence of the conditioned medium (DC-GM). Various activating signals such as living bacteria or LPS promote their full maturation. During this process MHC class II molecules are translocated to the cell surface, adhesion/costimulation molecules are up-regulated, the actin-based cytoskeleton is rearranged, and cell motility is increased. After cell sorting, the pattern of the expression of surface molecules in the GFP-positive population was investigated by FACS analysis. The expression of MHC class II, B7.2, and CD40 was comparable in the infected and in the non-infected population.

To test whether retroviral infection altered the capacity of DC to mature, we induced the maturation of the sorted D1 cells. Cells were cultured for 24 h in the presence of LPS (10 µg/mL) and then the expression of the surface molecules was analyzed by FACS. The shift in the expression patterns of MHC class II molecules, B7.2 and CD40, was comparable in both the GFP/D1 and in the noninfected D1 cells (**Fig. 4**). In conclusion, retroviruses do not induce DC maturation.

Functional maturation of D1/GFP

To investigate whether retroviral infection altered the ability of mature DC to stimulate unprimed T cells, the sorted GFP-positive D1 population was activated with LPS and used as a stimulator of unprimed BALB/c lymph node T lymphocytes. Consistent with a well-known property of DC, the GFP-D1 cells developed strong stimulatory activity after maturation induced by LPS (**Fig. 5**). This result further indicates that the infected D1/GFP maintain their immature phenotype and are still susceptible to fully functional maturation.

DISCUSSION

Several transfection methods to introduce heterologous genes into DC have been described, including DNA liposome complexes, electroporation, and $CaPO_4$ precipitation [18]. Nevertheless, the efficiency of these methods, when applied to DC, has always been very low. In contrast, retroviral vectors have been successfully used to introduce genetic information into the chromosomal DNA of DC progenitors. The limitation of this system is the requirement of proliferating DC or DC progenitors for stable integration of the retroviral constructs. For this reason, the current approach is to infect the actively proliferating hematopoietic progenitor cells ($CD34^+$) and then to differentiate the infected cells *in vitro* to mature DC [19–24]. However, these cells can survive for only a few days due to their *in vitro* spontaneous maturation.

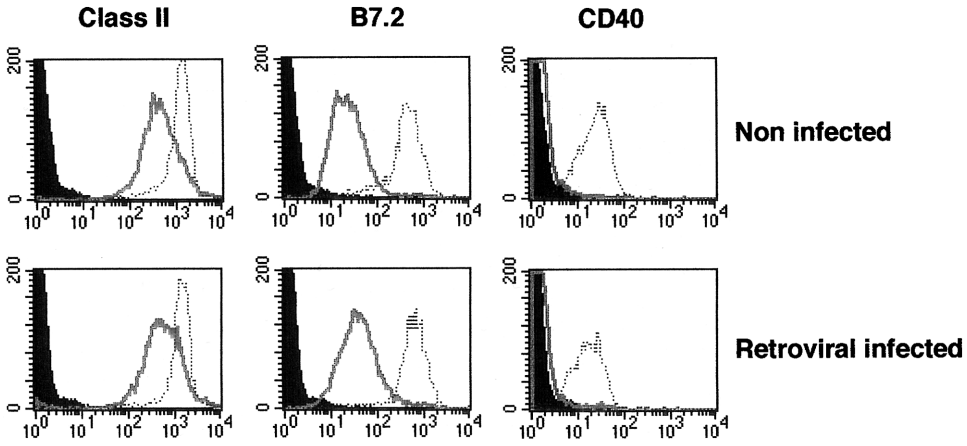

Fig. 4. Surface marker expression on D1 cells. Open histograms represent the expression in the immature D1 population, dotted histograms represent the expression in the mature population after LPS treatment, filled histograms represent the isotype control.

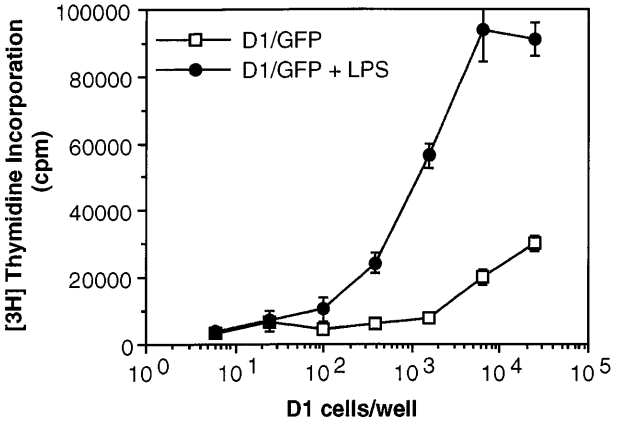

Fig. 5. Mixed lymphocyte reaction using sorted GFP-D1 cells as antigen-presenting cells. The D1 GFP-positive cells develop a strong stimulatory activity after the LPS maturation (10 μg/mL for 24 h). This agrees with the behavior observed with freshly isolated DC.

In contrast, in the mouse system, we could take advantage of a method that allows the production of growth factor-dependent, immature DC (named D1 cells) that indefinitely proliferate *in vitro*.

Using the PINCO vector carrying GFP as a selectable marker, the D1 cells were easily infected and selected. Genes for drug resistance have been extensively used to detect transduced cells, but positive selection of the modified cells is only possible after several weeks of culture. Alternative strategies are based on the use of heterologous genes coding for proteins that can be further detected by incubation with the appropriate fluorescin-labeled antibodies. However, the detection of such gene products is time-consuming and is prone to cellular toxicity or DC maturation [25]. In contrast, GFP-based selection has a number of advantages: the GFP expression does not influence the immature DC phenotype and it can be detected after a short time, thus allowing rapid flow cytometry sorting and quantification of the transduced cells. The infection and selection of D1 cells can be carried out in 2 days.

The high efficiency of this retroviral system, the rapidity of the technique, and the possibility of overcoming *in vitro* selection render this method very attractive for the stable introduction of heterologous genes into proliferating immature mouse D1 cells. Furthermore, this approach is suitable for functional studies of new DC-specific genes involved in DC maturation and survival.

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