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(54) Title: BEAD-ASSISTED VIRAL TRANSDUCTION

(57) Abstract: The present invention is directed to a method of viral transduction of a cell, wherein a bead is coated with a virus and the virus coated bead is contacted with the cell to transfer a nucleic acid to the cell. Moreover, the invention refers to a virus coated bead and composition comprising such bead and their use for the preparation of a medicament for preventing and/or treating a disease.

**Title: Bead-assisted** viral **transduction**

The present invention is directed to a method of viral transduction of nucleic acid to a cell via virus coated beads resulting in an increased efficiency of the transduction, in particular in case of a high cell density. Moreover, the invention refers to a virus coated bead and a composition comprising such bead and a cell, which are suitable for the present method of viral transduction.

Since the early 1990s genetic modification of human hematopoietic cells have been used for example for treating inherited genetic disorders, acquired immunodeficiency syndrome (AIDS), or cancer (Hwu P. J Immunol 1993; Bordignon C Science 1995; Woffendin, PNAS 1996). In order to get the gene of interest expressed by the target cell, these cells need to be transduced with a viral vector that integrates into the host DNA. Retroviruses are to date most commonly used for this process as they can integrate into dividing cells and large scale high-titer, stable supernatants can be produced. The process of retroviral transduction has been extensively studied in order to get optimal transduction efficiencies.

In early work, retroviral supernatant was added to the cells and medium replaced after 6-24 hours. However, transduction efficiencies were very low. A subsequent improvement of this process was obtained when cells and virus were transduced in the presence of polycations such as polybrene or protamine sulfate, presumably by increasing virus adsorption and/or penetration through a charge-mediated mechanism (Olsen JC, Nucl Acid Res 1993). An additional centrifugation step at for example 1000 x *g* has been reported to further increase transduction efficiencies in the presence of polycations (Bunnel, PNAS 1995); however, these agents were also toxic for the cells at high concentrations. A novel method was described that was highly effective in increasing transduction efficiencies of hematopoietic cells, including primary T cells of human and murine origin, by co-localization of the retrovirus and target cells on specific

fibronectin adhesion domains (Hanenberg H, Nat Med 1996; Pollok J Virol 1998), in particular the recombinant fibronectin fragment CH-296. This recombinant fibronectin fragment contains at least three distinct cell adhesion domains that interact with very late antigen (VLA-4) and VLA-5 integrins and proteoglycans that are expressed on T cells. Furthermore, the recombinant fibronectin fragments also contain a heparin-binding domain for adherence of retroviral particles (Moritz, Blood 1996).

Retroviral transduction is commonly used for gene-modification of cells to express a gene of interest. In order to efficiently transduce mammalian cells, fibronectin or a recombinant fibronectin molecule, for example Retronectin® (CH296 of Takara), has become widely utilized to coat the surface of a carrier such as a cell plate to facilitate virus binding as well as cell binding and thereby enhance viral uptake. In general, fibronectin is coated onto the wells of a non-tissue culture plate, and the viral suspension alone or together with the cells is added to the wells. This step is preferably followed by centrifugation of either the viral supernatant, or of cells together with the viral supernatant resulting in enhanced transduction, a procedure termed spin-transduction, which has become a standard transduction protocol (Zhou P, Hum Gen Ther, 2001).

In EP 1 561 819 Al a method for transducing a gene into activated T cells via paramyxovirus vector is described, and in WO 94/29438 Al a retroviral packaging system suitable for transduction of primary human cells. WO 95/26200 Al refers to a method of retroviral transduction of cells using fibronectin and WO 2004/097025 A2 discloses a method of transduction of neural stem cells using a fibronectin fragment.

While the strategy of viral transduction has become widely adopted, in particular for the genetic modification of hematopoietic cells, there are some limitations of this technique in particular with regard to clinical use, for example: it requires the use of a culture system that allows viral coating, something that is not possible for many of the closed cell culture systems that are utilized within

clinical trials; efficient transduction is only obtained by spinning the samples comprising either the virus or the virus plus the cells, thus, the culture systems must be suitable to be spun, and even under those optimal conditions, transduction efficiency is reduced at higher cell densities (Lamers CHJ, Cane Gene Ther, 2002).

It is therefore an object of the present invention to provide a method of viral transduction having increased transduction efficiency, which is independent of the viral coating of the culture system, the spinning of the culture system, and the density of the cells within the working range of hematopoietic cell culture where the nucleic acid has to be transferred to.

The object is achieved according to the present invention by the method of claim 1, a virus coated bead of claim 9, a composition comprising a virus coated bead and a cell according to claim 10, and the use of the virus coated bead or the composition according to claim 11.

Summary

The novel and potent strategy for the transduction of cells, for example cells of the hematopoietic system such as T cells, of the present invention is particularly appropriate in clinical scale. The virus, viral particle, or a fragment thereof is coated onto a bead, preferably an epoxy-coated paramagnetic bead (for example Dynabeads ® Epoxy of Invitrogen), the bead having a diameter of 2 to 20 micrometer. The virus, viral particle, or fragment thereof is either directly coated onto the bead or the bead has been precoated with a protein such as fibronectin, a derivative of fragment thereof such as Retronectin ® (RN). For the coating of the bead, the bead is added to viral supernatant, which can render such supernatant almost completely devoid of functional viral particles. Addition of the virus-loaded bead to a cell for example an activated T cell results in efficient viral infection.

An efficient transduction according to the present invention, which is higher than that obtained for example with conventional Retronectin-mediated transduction, does neither depend on the use of a culture system that is compatible with protein coating nor on centrifugation of the viral supernatant or the cells. The present invention also leads to high transduction efficiency, if cells at a high cell density are transfected. After transduction, the virus coated beads are removable from the transfected cells, and cell growth of regular-transduced versus bead-transduced cells is comparable.

The present invention facilitates the handling of viral transduction of nucleic acid to a cell, in particular in case of high cell density of the recipient cells, and leads to an increased number of the virally-modified cells. The present invention results in an improvement of viral transduction, which is particularly suitable for use in clinical trials.

**Figure 1: Microbead mediated retroviral transduction.** Epoxy-beads were coated with a fibronectin fragment, here Retronectin, and retrovirus. Retroviral beads were co-incubated with activated T cells at 0, 3, 10 or 30 beads per cell. Four days after transduction, the expression of the MART-I T cell receptor —that was encoded by the viral vector - was analyzed by staining with MART-I MHC tetramers and analysis by flow cytometry.

**Figure 2: Efficient** depletion of viral particles from retroviral **supernatants by** epoxy-coated microbeads. Bead-transductions of $IxIO^6$ cells with 10 beads per cell were compared with regular spin-transductions of $5xlO^5$ cells per well. For spin-transductions, plates were centrifuged at 2000 rpm for 90 minutes. In order to determine the binding capacity of the beads, Retronectin-coated epoxy-beads were incubated with retroviral supernatant for 2 hours, after which the beads and viral supernatant were separated. Coated beads alone were subsequently added to activated T cells, and the bead-depleted viral supernatant was used for spin-transduction of activated T cells. Four days after transduction, the expression of the MART-I T cell receptor - that was

encoded by the viral vector - was analyzed by staining with MART-I MHC tetramers and analysis by flow cytometry. Bar 1: Spin transduction of $5xlO^5$ cells per well with viral supernatant; Bar 2: Spin transduction of $5xlO^5$ cells per well with bead depleted viral supernatant; Bar 3: Bead transduction of $IxIO^6$ cells per well with virus coated beads alone; Bar 4: Bead transduction of $IxIO^6$ cells per well with viral supernatant comprising virus coated beads.

**Figure 3: Effect of centrifugal forces** on bead-transductions. To determine whether centrifugation has any additional impact on bead-transduction, Retronectin- and virus-coated beads were added to activated T cells ($IxIO^6$ cells / well) and placed directly at 37° C (Bar 1), or centrifuged for either 10 minutes at 1500 rpm (Bar 2) or for 90 minutes at 2000 rpm (identical to spin-transductions; Bar 3). Four days after transduction, the expression of the MART-I T cell receptor - that was encoded by the viral vector - was analyzed by staining with MART-I MHC tetramers and analysis by flow cytometry.

**Figure 4: Efficiency** of bead-transduction **in** preclinical **and** clinical transductions **in absence** of centrifugation. (A) Activated T cells ($IxIO^6$ cells / well) were transduced in plates that were coated with Retronectin (without (Bar 1) or with centrifugation (Bar 2)) according to a commonly used method and compared to cells that were transduced with 10 virus coated beads per cell according to the present invention (Bar 3). (B) At clinical scale, activated T cells were transduced in bags, one bag (Cell Expansion bag, Miltenyi) coated with a fibronectin fragment such as Retronectin for spin transduction (Bar 1), and another bag (Lifecell Bag, Baxter) without fibronectin coating, wherein the T cells were transduced with 10 virus coated beads per cell (Bar 2). Four days after transduction, the expression of the MART-I T cell receptor - that was encoded by the viral vector - was analyzed by staining with MART-I MHC tetramers and analysis by flow cytometry.

**Figure 5: Efficiency** of **bead** transduction **at high** cell **densities.** Spin-transductions according to a commonly used method and microbead-assisted

transductions (at 10 virus coated beads per cell) according to the present invention were performed with $5xlO^5$, $IxIO^6$ and $2xlO^6$ cells per ml (and per well). Four days after transduction, the expression of the MART-I T cell receptor —that was encoded by the viral vector - was analyzed by staining with MART-I MHC tetramers and analysis by flow cytometry. Bar Ia: Spin transduction of $5xlO^5$ cells per well, Bar Ib: bead transduction of $5xlO^5$ cells per well; Bar 2a: spin transduction of $IxIO^6$ cells per well, Bar 2b; bead transduction of $IxIO^6$ cells per well; Bar 3a: spin transduction of $2xlO^6$ cells per well, Bar 3b: bead transduction of $2xlO^6$ cells per well.

**Figure 6: Growth curves of spin transduced and bead transduced T cells.** T cells ($IxIO^6$ cells per well) were transduced either via spin transduction (square) or via bead-assisted transduction (diamond). 6, 10 and 13 days after transduction, cells were enumerated and split to a density of $0.25xl0^6$/ml. Fold expansion was calculated relative to day 2 (the day on which transduction is initiated) and is depicted.

Figure 7: Microbead mediated retroviral transduction comparing different bead surfaces and different bead sizes.

## Detailed description

Transfer of nucleic acid to a cell for gene-modification of the cell in order for the cell to express a gene of interest is widely performed via viral transduction. A common method for such viral transduction is spin transduction, wherein a (free) virus comprising a gene of interest is added to the activated cell, which is conjugated to a fibronectin coated surface. The virus and the activated cell are commonly centrifuged such that the gene of interest is transferred from the virus to the activated cell. The efficiency of commonly used transduction methods such as spin transduction decreases with an increase of the cell density of the activated cells.

To improve the transduction efficiency, which is for example reached with conventional spin-transduction, the present invention is directed to a method of viral transduction of a cell *in vitro*, wherein a bead is coated with the virus and the virus coated bead is contacted with the cell to transfer a nucleic acid to the cell via the virus coating the bead. The virus coated bead is any type of bead, preferably a hydrophobic bead, which is suitable to be coated by a virus, such as a magnetic bead, a paramagnetic bead, a glass bead, a polylactic bead, a latex bead, a polystyrene bead, a sugar polymer bead, optionally comprising active groups, preferably on the surface of the bead, for example a glycidyl ether (epoxy) reactive group (epoxy group), a maleimide group, an active ester such as N-hydroxysuccinimide esters, or a cyanogen bromide-activated group. In an alternative embodiment, the bead is precoated with a protein before the virus coating, wherein the protein is for example fibronectin, a derivative thereof, or a fragment thereof, such as Retronectin, Streptavidin, avidin, monocolonal antibody, or a receptor. Before the bead is coated with the virus, the bead is preferably blocked for example with a protein such as BSA. For coating the bead with the virus, the bead is added to a viral suspension and incubated for a certain time, preferably 30 min to 5 hours, 1 hour to 3 hours, 1.5 hours to 3 hours, or 1.5 to 2 hours.

In a preferred embodiment, the bead is coated with the virus at a temperature of 15°C to 30$^0$C, 15°C to 25°C, or 15°C to 20$^0$C, preferably at 15°C, 16°C, 17°C, 18°C, 19°C, 20$^0$C, 21°C, 22°C, 23°C, 24°C, or 25°C, and at a preferred pH of 5 to 14, 6 to 12, or 7 to 10, more preferred at a pH of 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 10.5, 11, 11.5, 12, 12.5, 13, 13.5, or 14. In a further embodiment the viral transduction of the present invention using the virus coated beads of the invention is preferably performed at a temperature of 20$^0$C to 40$^0$C, 25°C to 40$^0$C, or 30$^0$C to 40$^0$C, more preferably at a temperature of 25°C, 27°C, 30$^0$C, 32°C, 35°C, or 37°C and pH as described for the virus coating of the bead.

The size of the virus coatable beads varies, preferably the bead has a size of 1 nm to 10 μm, 1 nm to 7.5 μm, 1 nm to 5 μm, 1 nm to 2.5 μm, 1 nm to 1 μm, 1 to 750 nm, 1 nm to 500 nm, 1 to 250 nm, 1 to 100 nm, 1 to 50 nm, 1 to 40 nm, 1 to 30 nm, 1 to 20 nm, 1 to 10 nm, 1 to 5 nm, more preferably 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 nm or 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 μm.

In a most preferred embodiment, the bead has a size of 2 μm to 20 μm, more preferred 2 μm to 10 μm, such as 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 μm.

The virus conjugated to the bead is any virus suitable for viral transduction such as a retrovirus, a lentivirus, an adeno-associated virus, or an adenovirus. The number of viruses coated on the bead varies, preferably a bead is coated with 1 to IxIO$^{12}$ viruses per bead, 1 to IxIO$^{11}$ viruses per bead, 1 to IxIO$^{10}$ viruses per bead, 1 to IxIO$^9$ viruses per bead, 1 to IxIO$^8$ viruses per bead, 1 to IxIO$^7$ viruses per bead, 1 to IxIO$^6$ viruses per bead, 1 to IxIO$^5$ viruses per bead, 1 to IxIO$^4$ viruses per bead, 1 to IxIO$^3$ viruses per bead, 1 to IxIO$^2$ viruses per bead, 1 to 90 viruses per bead, 1 to 80 viruses per bead, 1 to 70 viruses per bead, 1 to 60

viruses per bead, 1 to 50 viruses per bead, 1 to 40 viruses per bead, 1 to 30 viruses per bead, 1 to 20 viruses per bead, or 1 to 10 viruses per bead.

The viral transducable cell is preferably a hematopoietic stem cell, or a cell of the hematopoietic stem cell system such as a T cell, a thymocyte, a B cell, an NK cell, a hematopoietic progenitor cell, or a fibroblast. The cell is either freely floating in suspension, or is conjugated to a surface, e.g., a bead, a cell plate, or a bag such as a cell expansion bag. This surface consists of any material suitable for cell growth and/or cell proliferation. In one embodiment the surface is coated with a protein such as fibronectin, a derivative or fragment thereof, for example Retronectin.

In a preferred embodiment a virus coated bead isolated from the viral supernatant, or a viral supernatant comprising virus coated beads is added to cells in suspension or to cells conjugated to a surface, such as a hematopoietic stem cell, or a cell of the hematopoietic stem cell system, e.g., a T cell, comprising $IxIO^3$ cells to $IxIO^{10}$ cells, $IxIO^3$ cells to lxl $\theta^9$cells, $IxIO^3$ cells to $IxIO^8$ cells, $IxIO^3$ cells to $IxIO^7$ cells, $IxIO^3$ cells to $IxIO^6$ cells, $IxIO^3$ cells to $IxIO^5$ cells, or $IxIO^3$ cells to $IxIO^4$ cells, preferably 1, 2, 3, 4, or 5 $xlO^3$ cells, 1, 2, 3, 4, or 5 $xlO^4$ cells, 1, 2, 3, 4, or 5 $xlO^5$ cells, 1, 2, 3, 4, or 5 $xlO^6$ cells, 1, 2, 3, 4, or 5 $xlO^7$ cells, 1, 2, 3, 4, or 5 $xlO^8$ cells, 1, 2, 3, 4, or 5 $xlO^9$ cells, or 1, 2, 3, 4, or 5 $xlO^{10}$ cells.

For the viral transduction according to the present invention the virus coated bead is added to a cell, which is optionally activated by ligands that induce proliferation, for T cells for example by antigen-MHC containing beads or cells, anti-CD3/anti-CD28 beads, anti-CD3 antibody (in suspension or coated), lectins, hematopoietic growth factors, costimulatory ligands, IL-2, IL-7, IL-15, IL-21 cytokines or combinations thereof. In a preferred embodiment, the amount of virus coated bead per cell is 1 per cell, 2 per cell, 3 per cell, 4 per cell, 5 per cell, 6 per cell, 7 per cell, 8 per cell, 9 per cell, 10 per cell, 15 per cell, 20 per cell, 25 per cell, 30 per cell, 35 per cell, 40 per cell, 45 per cell, 50 per cell, 100 per cell, 150 per cell, 200 per cell, 250 per cell, 300 per cell, 350 per cell, 400 per cell, 450

per cell, 500 per cell, 550 per cell, 600 per cell, 650 per cell, 700 per cell, 750 per cell, 800 per cell, 850 per cell, 900 per cell, 950 per cell, or 1000 per cell. Preferably, the number of the virus coated beads per cell increases with the decrease of the bead size. For example taking a virus coated bead having a size of 1 to 5 nm, the amount of virus coated bead per cell is preferably 500 to 1000 per cell, more preferably 600 to 1000 per cell, 700 to 1000 per cell, 800 to 1000 per cell, or 900 to 1000 per cell, or a virus coated bead having a size of 1 to 5 μm, the amount of virus coated bead per cell is preferably 10 to 500 per cell, more preferably 10 to 450 per cell, 10 to 400 per cell, 10 to 350 per cell, 10 to 300 per cell, 10 to 250 per cell, 10 to 200 per cell, 10 to 150 per cell, 10 to 100 per cell, or 10 to 50 per cell. The cells of these examples are preferably in suspension.

The composition comprising the virus coated bead and the cell is incubated preferably at a temperature of 25°C, 27°C, 30°C, 32°C, 35°C, or 37°C overnight, preferably without centrifugation; alternatively, the composition is incubated 30 min to 12 hours, 1 hour to 10 hours, 2 hours to 8 hours, or 3 hours to 5 hours, preferably without centrifugation. The temperature might vary during incubation, for example the virus coated bead and the cell are incubated at 37°C overnight and further incubated at a different temperature for some hours, e.g., 5 h at 25°C. Most preferably, the virus coated bead and the cell are incubated at 37°C for several days, in particular 1 to 7 days, 1 to 6 days, 1 to 5 days, 1 to 4 days, 1 to 3 days, or 1 to 2 days. In the most preferred embodiment, the viral transduction is independent of centrifugal force, but based on gravity. After transduction of the cells, the cells are harvested according to common methods.

In one embodiment, the supernatant comprising the virus coated beads is added to the cells for viral transduction; in another embodiment the virus coated beads are isolated from the supernatant and the isolated virus coated beads are added to the cells. When the virus coated beads and the cells are combined and incubated for viral transduction, the beads are optionally removed during incubation time or before harvesting the transduced cells, for instance in case patient administration of beads is undesirable.

In case the cells are conjugated to a surface, the cells have to be removed from the surface before harvesting the cells or the cells are harvested conjugated to the surface. In a preferred alternative, the transduced cells are conjugated to a bead and the cells are separated from the bead before or after harvesting the cells. The cells are preferably used for the preparation of a medicament for preventing and/or treating a disease which is responsive to the administration of a gene modified cell, wherein the cells are separated from the bead or conjugated to the bead. Dependent on the nature and size of the beads, in a preferred embodiment cells are infused conjugated to the beads. Alternatively, the cells are separated from the beads by placing the beads plus cells against a magnetic force. In another alternative, beads and cells are separated by centrifugation, preferably using a Ficoll separation step based on gradient centrifugation.

The virus coated bead, and the composition comprising a virus coated bead and a cell, respectively, are used for the preparation of a medicament for preventing and/or treating a disease which is responsive to the administration of a gene modified cell, preferably such disease is a genetic disease. A genetic disease is a disease that is treatable by provision of genetically modified autologous or allogeneic cells. A genetic disease treatable by the present invention is for example selected from the group consisting of cancer, infection, immunodeficiency, storage disease, and autoimmune disease. The gene modified cell is preferably administered via infusion.

In a preferred embodiment, a paramagnetic epoxy bead that covalently couples to free amino and sulfhydryl groups is incubated with a fibronectin fragment, in particular a recombinant fibronectin fragment, which is shown to contain domains that enable co-localization of viral particles and target cells. Following a blocking step, beads are incubated with a viral supernatant for example a retroviral or lentiviral supernatant, or a supernatant of an adeno-associated virus containing a gene of interest such as an pMP71-lD3 vector encoding for example a MART-I reactive T cell receptor, or other T cell receptors against

cancer, or chimeric receptors which consist of an antigen recognition domain linked to a cell signaling domain for example, or other genes such as enzymes used for (ADA)-SCID patients. Beads plus viral supernatant are then added for example to T cells at different bead to cell ratios (B/C ratios), preferably 3:1, 10:1, or 30:1 to determine whether these coated beads are able to transduce T cells using different bead-to-cell ratios. The preferred size of the virus coated bead is 1, 2, 3, 4, 4.5, 5, 5.5, 6, 7, or 8 μm, more preferred 2, 3, 4, 4.5, 5, 5.5, 6, 7, or 8 μm. An increase in the beads per cell ratio results in an increased transduction efficiency, whereas only a low level transduction is achieved with virus alone in the absence of beads. Fig. 1 shows results of retroviral coated microbeads.

To directly compare the efficiency of bead-assisted, preferably microbead-assisted, viral transduction, such as retroviral or lentiviral transduction, or transduction using adeno-associated virus, with conventional transduction, regular spin transductions are performed in parallel at a cell concentration of $5xlO^5$ cells per well in a further embodiment. The B/C ratio is preferably 10:1. Transduction efficiencies between the viral transduction of the present invention and the regular spin transduction are comparable. The bead transduction results in higher frequencies of transduced cells, e.g., of MART-I tetramer positive cells (mean ± SEM = 1.16 ± 0.08 fold higher transduction after bead transduction as compared to spin transduction from n=7 experiments). The results shown in Fig. 2 refer to a microbead assisted retroviral transduction of $IxIO^6$ cells per well.

To determine the efficiency with which viral particles are captured from the viral supernatants, for example retroviral or lentiviral supernatants, or supernatants of adeno-associated viruses, by beads, in particular microbeads, coated with epoxy groups, maleimide groups, active esters such as N-hydroxysuccinimide esters, or cyan bromide-activated beads, virus-coated beads were incubated with the viral supernatant and separated from the viral supernatant after incubation, for example after two hours of incubation. In a preferred embodiment, T cells were transduced with either the virus-coated beads alone, or were spin-transduced on Retronectin-coated plates with either untreated viral supernatant,

or the viral supernatant that had been exposed to the beads, preferably microbeads. Preferably, the B/C ratio is 10:1. Figure 2 shows that as compared to the efficiency of retroviral modification with unmanipulated retroviral supernatant, a prior exposure to epoxy coated microbeads leads to a loss in transduction efficiency of approximately 90%. As viral transduction with microbeads obtained from the pre-exposure of coated beads with the viral supernatant is highly efficient, the virus of the supernatant is almost completely conjugated to the bead, rendering the supernatant almost devoid of functional virus.

The above embodiments indicate that at least part of the mode of action of the claimed procedure occurs through binding of virus from viral supernatants by the added microbeads.

In a further embodiment, bead-transduction with or without centrifugation were compared, wherein a bead coated with a retrovirus, a lentivirus or an adeno-associated virus was added to a cell such as a T cell. The B/C ratio was preferably 10:1. Figure 3 shows that a short spin of 10 minutes at 1500 rpm, a 90 minute spin at 2000 rpm (identical to conventional spin-transduction), or no spin at all of the composition comprising a retrovirus coated bead and a T cell resulted in very similar transduction efficiencies. Co-localization of cells and beads due to gravity forces is thus sufficient to obtain maximal transduction, independent of centrifugal forces.

In an alternative embodiment, magnetic forces were used to bring cells and beads in contact, which showed transduction efficiency comparable to the efficiency of the present transduction method based on gravity forces.

In a further embodiment, a cell, preferably a T cell, was transfected with a virus coated bead for example a retroviral or lentiviral bead, or a bead coated with an adeno-associated virus. The transduction using the virus coated beads of the present invention resulted in an approximately three-fold higher transduction

rate as compared to transductions performed with viruses not conjugated to a bead in the absence of centrifugation in preclinical experiments. Fig. 4A shows this effect for retrovirus transduction of T cells, wherein the B/C ratio is 10:1.

In another preferred embodiment, viral transduction, in particular retroviral or lentiviral transduction, or transduction using an adeno-associated virus is performed with cells cultured in Life Cell bags. For transduction, a Cell Differentiation Bag is coated, e.g., with fibronectin, or a derivative of a fragment thereof, such as Retronectin, a recombinant fibronectin fragment. These Cell Differentiation Bags represent a closed culture system, for which centrifugation, and thus, spin transduction is precluded. Fig. 4B shows the results of a closed culture system, wherein retroviral transduction led to a transduction efficiency of close to 30% after precoating the bag with a fibronectin fragment, e.g., Retronectin. Transduction with the same retroviral supernatant reached an efficiency of close to 70%, when beads were added to the supernatant for binding the viruses to the beads, and the transduction was performed with virus coated beads according to the present invention.

In a most preferred embodiment, viral transduction, for example retroviral or lentiviral transduction, or transduction using adeno-associated viruses, is performed in clinical scale processes, where gene modification is preferably performed at high cell density, to allow the production of the cell numbers required for adoptive transfer. With conventional spin transduction, the efficiency of retroviral modification is significantly reduced at higher cell densities. Fig. 5 shows results of a four-fold increase in cell number (for example from $5xlO^5$ to $2xlO^6$ T cells) resulting in a four-fold reduction in the frequency of transduced T cells in the case of spin transduction. In fact the number of gene-modified cells is unaltered with only an increase in the number of non-transduced cells in the spin-transduction method. In contrast, efficiency of retroviral transduction is almost not affected at high cell densities in case bead-assisted transduction is performed in this case with a B/C ratio of 10:1. Using the present method of bead-assisted transduction, less viral supernatant is used to

transduce higher cell numbers, which makes bead-transductions very cost-effective.

To determine whether the presence of virus-coated beads, in particular microbeads, in the transduction process influenced their capacity to expand, the growth curve of T cells transduced by classical spin transduction and bead-assisted transduction was compared. In one embodiment, bead-transduced cells expanded to similar numbers as spin-transduced T cells indicating that the growth potential of the cells is not impacted by the different transduction method (Fig. 6).

The present invention using virus coated beads is an improvement over spin-transduction, preferably in clinical scale closed systems, where protein coating in combination with high centrifugal forces is difficult or even impossible to achieve, and a high cell density reduces the transduction efficiency.

The invention further provides the use of a bead with a size of 2 to 20 micrometer for viral transduction of a cell *in vitro*. More preferred is a bead with a size of 2 to 10 micrometer, such as 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, or 10 μm. Most preferred is a bead with a size of 4.5 μm. The bead is preferably a hydrophobic bead, which is suitable to be coated by a virus, such as a magnetic bead, a paramagnetic bead, a glass bead, a polylactic bead, a latex bead, a polystyrene bead, or a sugar polymer bead. The bead optionally comprises active groups, preferably on the surface of the bead, for example a glycidyl ether (epoxy) reactive group (epoxy group), a maleimide group, an active ester such as N-hydroxysuccinimide esters, or a cyanogen bromide-activated group. A most preferred bead is a polystyrene bead or a hydrophobic bead with glycidyl ether (epoxy) reactive groups.

Virus transduction is mediated by conjugation of a virus to the bead. Said virus is any virus suitable for viral transduction such as a retrovirus including lentivirus, an adeno-associated virus, or an adenovirus such as, for example, Ad5

or Ad51-based adenoviruses. A preferred virus is a retrovirus such as an ecotrophic retrovirus, amphotrophic retrovirus, VSVG pseudotype retrovirus, or a lentivirus. Said retrovirus, for example, is a Moloney murine leukaemia virus (MMLV)-based retrovirus, a human immunodeficiency virus (HIV)-based retrovirus or a chimeric vector such as, for example, a MP71-based retrovirus which is based on the myeloproliferative sarcoma virus (MPSV) and the murine embryonic stem cell virus (MESV). Said virus preferably is produced in packaging cell that expresses viral gag and pol genes. A suitable packaging cell line is, for example, an ecotropic HEK 293-based packaging cell line such as Phoenix-E (Orbigen, San Diego), an amphotrophic HEK 293-based cell line such as Phoenix-A (Orbigen, San Diego), a T-lymphoma-based cell line such as Jurkatt cell line (Reuszlig et al., 2007. Gene Therapy 14, 595-603), a NIH/3T3-based packaging cell line, or a pantropic cell line that expresses pVSV-Glycoprotein. Further preferred are packaging cell lines that express Moloney murine leukemia virus gag-pol proteins and a further protein such as, for example, measles virus glycoprotein (Buchholz et al. 2009. Trends Biotechnol 27 :259-65), feline endogenous virus envelope protein RD114 (Ward et al. 2003. MoI Ther 8: 804-12) or the gibbon ape leukemia virus envelope protein such as PG13 (Miller et al. 1991. J Virol 65: 2220).

The bead is preferably precoated with a protein, for example with streptavidin, avidin, a monoclonal antibody, a receptor, or fibronectin, a derivative of fibronectin, or a fragment of fibronectin, such as Retronectin. A most preferred bead is a polystyrene bead or a hydrophobic bead with glycidyl ether (epoxy) reactive groups that is coated with Retronectin.

Examples

The following examples show aspects of the present invention in more detail, however, the present invention is not limited to these examples.

Example 1

*Stimulation of PBMC*

Peripheral blood mononuclear cells (PBMC) were obtained from healthy blood bank donors and isolated using a Ficoll gradient. PBMC were stimulated and enriched for T cells with αCD3/αCD28 magnetic beads (e.g., ClinExVivo, Invitrogen) at a 3 beads per T cell ratio. Following enrichment, T cells were cultured in 24 well plates at ~5xlO$^5$/ml in X-Vivol5 medium (e.g., Lonza) supplemented with penicillin and streptomycin, 20 mM Hepes (e.g., Invitrogen), 2 mM L-Glutamine (e.g., Invitrogen) and 200 IU/ml interleukin-2 (IL-2). For clinical scale transduction, cells were enriched with αCD3/αCD28 magnetic beads and cultured in bags (e.g., Baxter Lifecell). Activated T cells were transduced after 2 days of culture as described below. Cells were cultured for a total of 12-14 days and were counted and split every ~3 day of culture to a final density of 0.25xl0 $^6$/ml.

Example 2

*Coating of RN and virus on microbeads, plates and bags*

Microbeads that allow protein binding (e.g., Epoxy beads, Invitrogen) were washed once with 0.1 M sodium phosphate buffer by magnetic separation and incubated with Retronectin (RN, 50 μg/ml, e.g., Takara) at IxIO$^8$ beads per ml RN overnight at 4°C at slow speed rotation. Unbound retronectin was removed by magnetic separation of beads, and beads were blocked in PBS with 2% bovine serum albumin (BSA) for 30 minutes, followed by a wash with PBS. Subsequently, beads were slowly rotated for 1.5-2 hours at room temperature (RT) with retroviral supernatant containing the MP71-1D3 (MART-I-reactive T cell receptor (TCR)) retrovirus at 2xlO$^7$ beads/ml viral supernatant. Where indicated, virus-coated beads were separated from the viral supernatant by

magnetic separation to determine the efficiency of virus capture. For comparison with spin-transduction in plates, 24-well non-tissue culture plates were coated with 500 µl/well RN (50 µg/ml) for 3 h at RT, followed by a blocking step with PBS/2% BSA for 30 minutes and a wash with PBS. For clinical scale experiments, bags (e.g., Cell Differentiation Bags, Miltenyi) were coated with RN at 3 µg/cm$^3$ overnight at 4°C at slow speed rotation. Bags were subsequently blocked with PBS/2%BSA, washed with PBS and incubated with 1 ml virus per IxIO$^6$ cells.

## Example 3

*Retroviral transduction of PBMC*

For transduction in tissue culture plates, αCD3/αCD28 activated T cells were incubated with retrovirus-coated beads at a bead per cell ratio of 0, 3:1, 10:1, or 30:1 in 500 µl/well at a final concentration of 5xlO$^5$ or IxIO$^6$ cells/ml. T cells were transduced at 37°C with 5% CO2 overnight. Where indicated, plates were centrifuged to determine the impact of centrifugal force on transduction efficiency. Results in Fig. 1 to 5 show that centrifugal force has no or only marginal effect on the results of bead transduction.

As a control, regular spin-transductions (Jorritsma et al, Blood 2007) were performed in parallel. Viral supernatant (500 µl) and activated T cells (final concentration of 5xlO$^5$ in 500 µl) were added per well. Where indicated, plates were centrifuged at 2000 rpm (= 430 x g) for 90 minutes (no brake) after which cells were cultured at 37°C and 5% CO2. One day after bead transduction or standard transduction, 1 ml/well of X-vivo medium containing 400 IU/ml IL-2 was added and cells were analyzed at the indicated time point. Results in Fig. 2, 4, or 5 show high efficiency of bead transduction.

Example 4

*Retroviral transduction in clinical scale experiments*

For clinical scale experiments, beads were coated with retrovirus as described in Example 2 and were added at a 10 bead per cell ratio to a bag (e.g., Baxter Lifecell bags) comprising T cells; medium was added to a final concentration of

$5xlO^5$/ml. In comparison, T cells were transduced in alternative bags (for example Cell Differentiation bag, Miltenyi, coated with a fibronectin fragment) and medium was added to a final concentration of $5xlO^5$/ml. One day after bead transduction in the Baxter Lifecell bags and transduction in the Cell Differentiation bag, one volume of medium with 400 IU IL-2/ml was added to each bag to obtain a final concentration of 200 IU IL-2/ml. T cells were cultured in bags and split every 2-3 days to a density of $2.5x10^5$/ml and were analyzed at the indicated time points. Results in Fig. 4B show high efficiency of bead transduction.

## Example 5

*Flow cytometry analysis*

Transduction efficiencies were determined by flow cytometry. On day 6 after stimulation (day 4 after transduction), cells were stained with anti-CD3-PE and anti-CD8-FITC conjugated antibodies in conjunction with APC-labeled tetramers containing the MART-I27L peptide. These MHC tetramers detect T cells expressing the MART-I reactive TCR encoded by the MP71-1D3 vector. After a 20-30 minute staining in FACS buffer (PBS with 0.5% BSA and azide), cells were washed and analyzed by acquisition on a Becton Dickinson FACS Calibur I and data were analyzed using Flowjo software. Results are shown in Fig. 1 to 5.

Example 6

Beads, here epoxy-beads (Invitrogen) and polystyrene beads (Polybeads Polystyrene Microsphere, PolyScience) were coated with a fibronectin fragment, here Retronectin, and retrovirus. Epoxy-beads were coated as described above. Particle numbers of the polystyrene beads were calculated from the percentage and diameter of the beads in the suspension. Polystyrene beads were washed with ethanol, recovered by centrifugation, and coated with Retronectin and retrovirus in an identical manner as the epoxy-beads. Epoxy-coated beads and polystyrene beads of 4.5 μm diameter were compared to polystyrene beads with a diameter of 1 μm. Retrovirus coated beads were co-incubated with activated T cells at 10 or 30 beads per cell for beads of 4.5 μm and at 10, 30 and 150 beads

per cell for 1 μm size beads. Four days after transduction, the expression of the MART-I T cell receptor - that was encoded by the viral vector - was analyzed by staining with MART-I MHC tetramers and analysis by flow cytometry as indicated in Example 5.

Results in Figure 7 show enhanced transduction efficiency mediated by beads with a diameter of 4.5 μm compared to beads with a diameter of 1 μm.

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## Claims

1. Method of viral transduction of a cell *in vitro*, wherein a bead is coated with a virus and the virus coated bead is contacted with the cell to transfer a nucleic acid to the cell via the virus coating the bead, whereby the size of the bead is 2 to 20 micrometer.

2. Method according to claim 1, wherein the cell is freely floating in suspension.

3. Method according to claim 1 or 2, wherein the bead is precoated with a protein.

4. Method according to claim 3 wherein the bead is precoated with fibronectin, fragments or derivatives thereof.

5. Method according to any of claims 1 to 4, wherein the bead comprises an epoxy group, an maleimide group, an activated ester, or cyanogen bromide-activated group.

6. Method according to claim 5, wherein the activated ester is a N-hydroxysuccinimide ester.

7. Method according to any of claims 1 to 6, wherein the cell is a cell of the hematopoietic system.

8. Method according to any of claims 1 to 7, wherein the virus is a retrovirus, a lentivirus, an adenovirus, or an adeno-associated virus.

9. Virus coated bead for viral transduction of a cell according to the method of any of claims 1 to 8, whereby the size of the bead prior to coating is 2 to 20 micrometer.

10. Composition comprising the virus coated bead according to claim 9 and a cell, wherein the cell is freely floating in suspension.

11. Use of the virus coated bead according to claim 9, or the composition according to claim 10 for the preparation of a medicament for preventing and/or treating a disease which is responsive to the administration of a gene-modified cell.

12. Use according to claim 11, wherein the disease responsive to administration of a gene-modified cell is cancer, infection, immunodeficiency, storage disease, or autoimmunity.

13. Use of a bead with a size of 2 to 20 micrometer for viral transduction of a cell *in vitro.*

14. Use according to claim 13, wherein the size of the bead is 4.5 micrometer.

15. Use according to claim 13 or claim 14, wherein the bead is a polystyrene bead or a hydrophobic bead with glycidyl ether (epoxy) reactive groups.

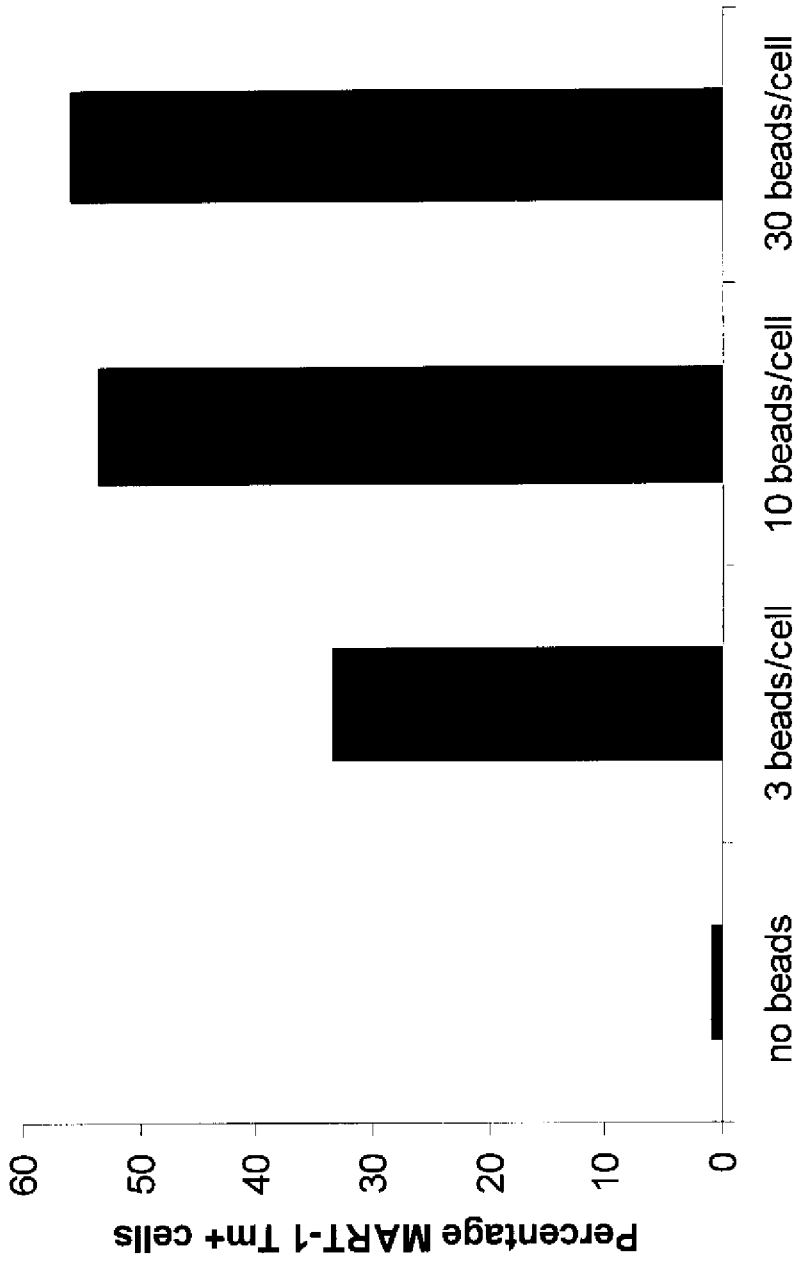

Percentage MART-1 Tm+ cells
0
10
20
30
40
50
60
no beads
3 beads/cell
10 beads/cell
30 beads/cell

Figure 1

Figure 2

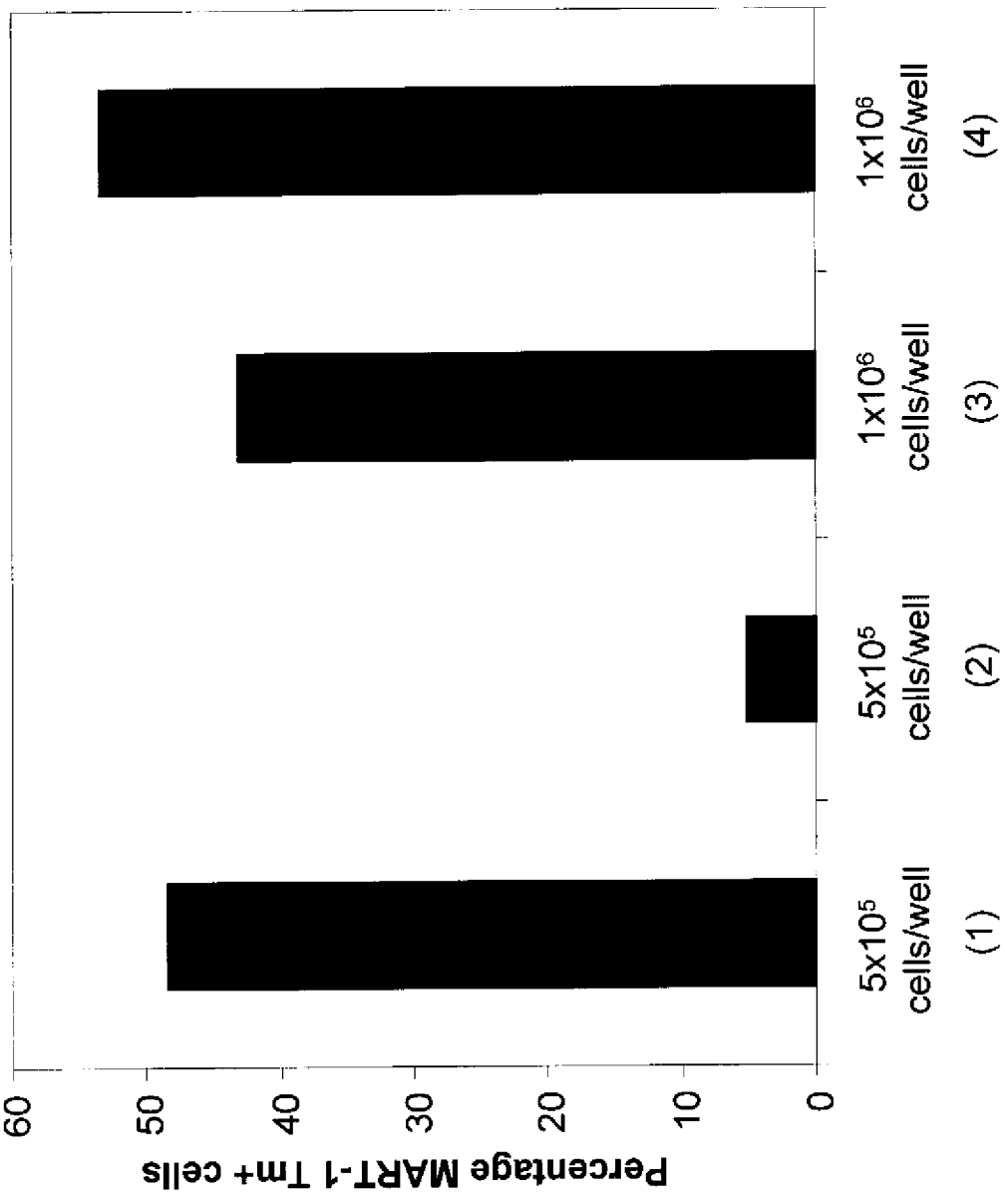

Percentage MART-1 Tm+ cells
0
10
20
30
40
50
60
5x10⁵ cells/well (1)
5x10⁵ cells/well (2)
1x10⁶ cells/well (3)
1x10⁶ cells/well (4)

Figure 3

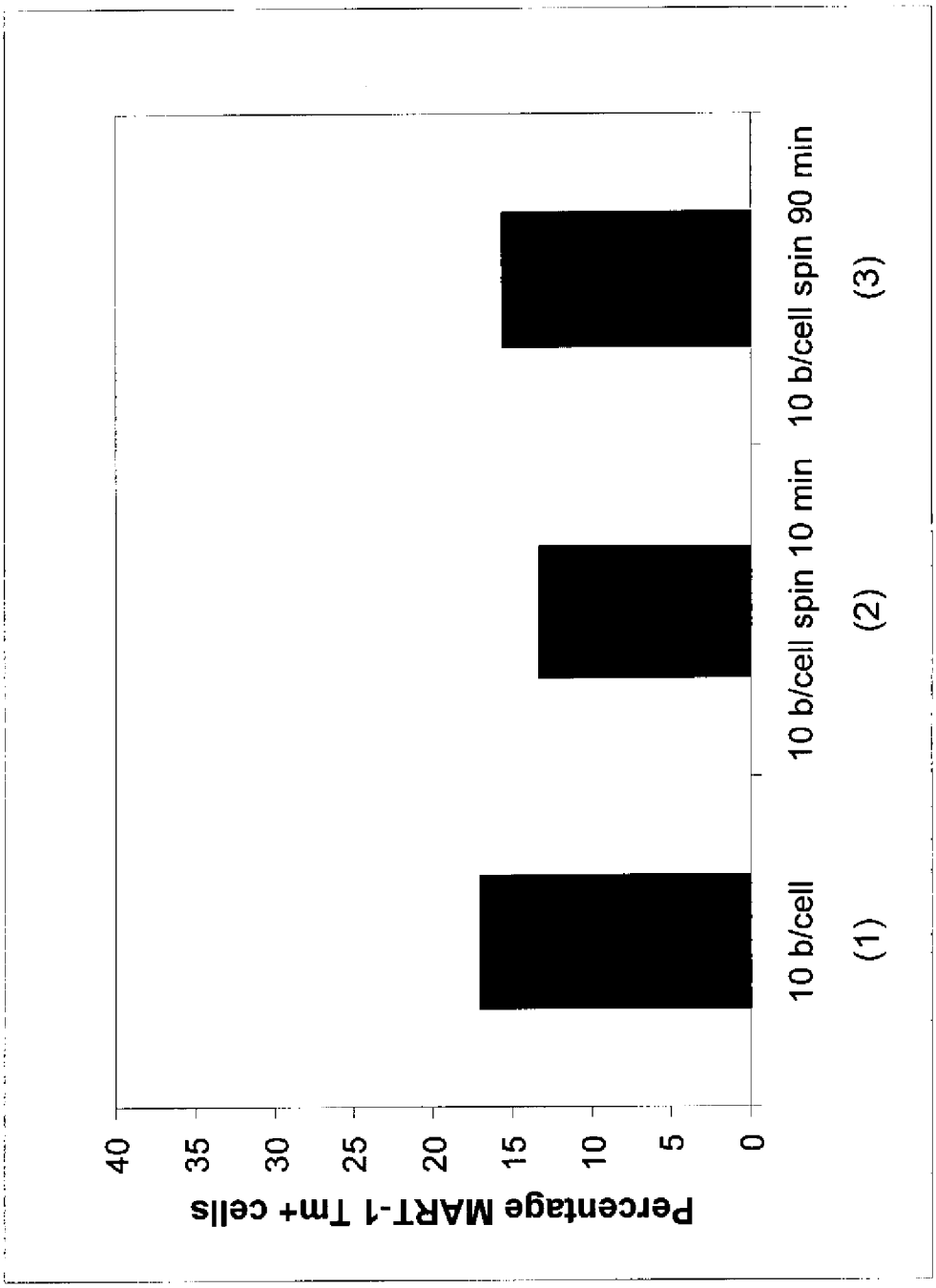
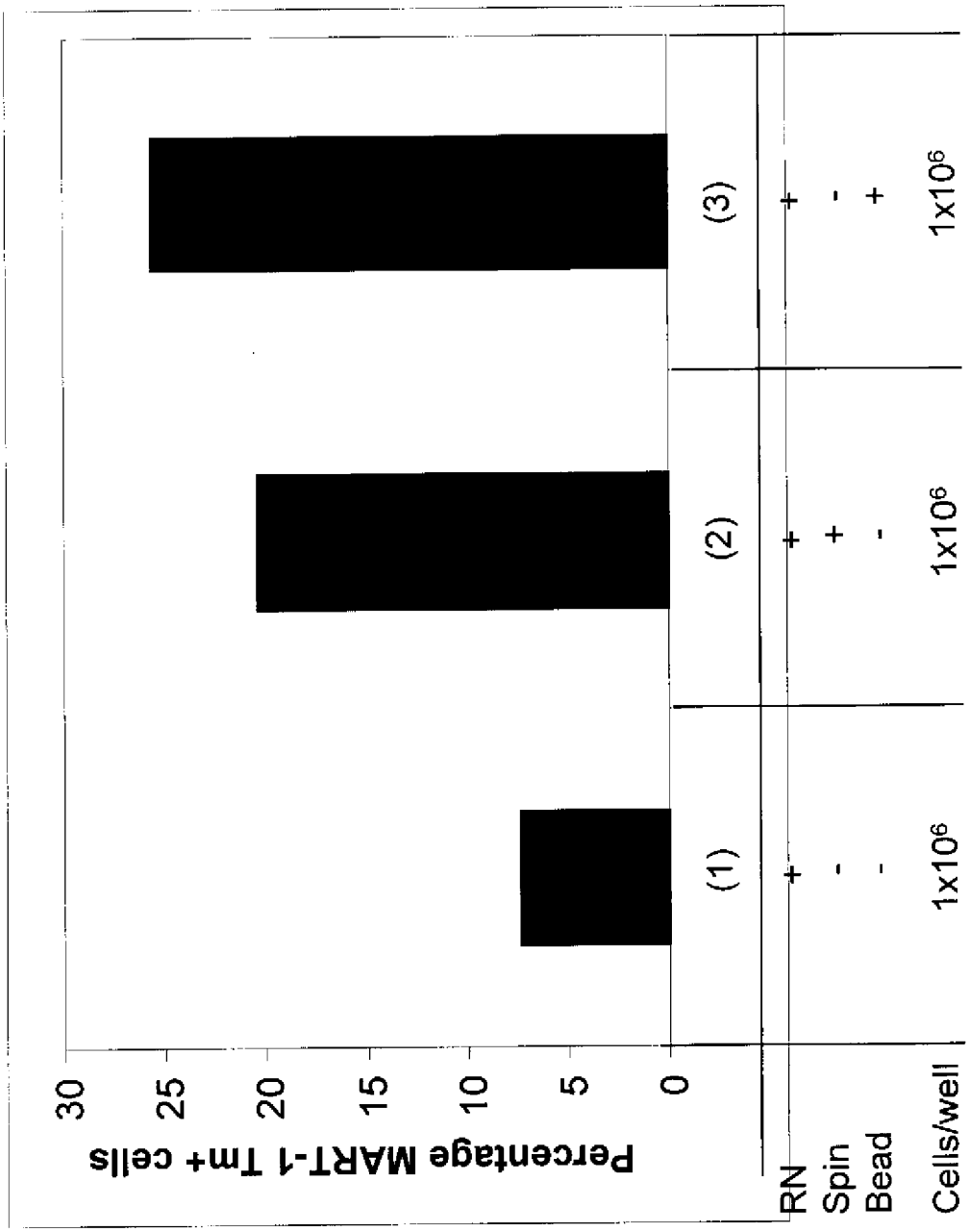

Percentage MART-1 Tm+ cells
0
5
10
15
20
25
30
35
40
10 b/cell
10 b/cell spin 10 min
10 b/cell spin 90 min
(1)
(2)
(3)

Figure 4A


Percentage MART-1 Tm+ cells
30
25
20
15
10
5
0
(1)
(2)
(3)
RN
Spin
Bead
Cells/well
+
-
-
1x10^6
+
+
-
1x10^6
+
-
+
1x10^6

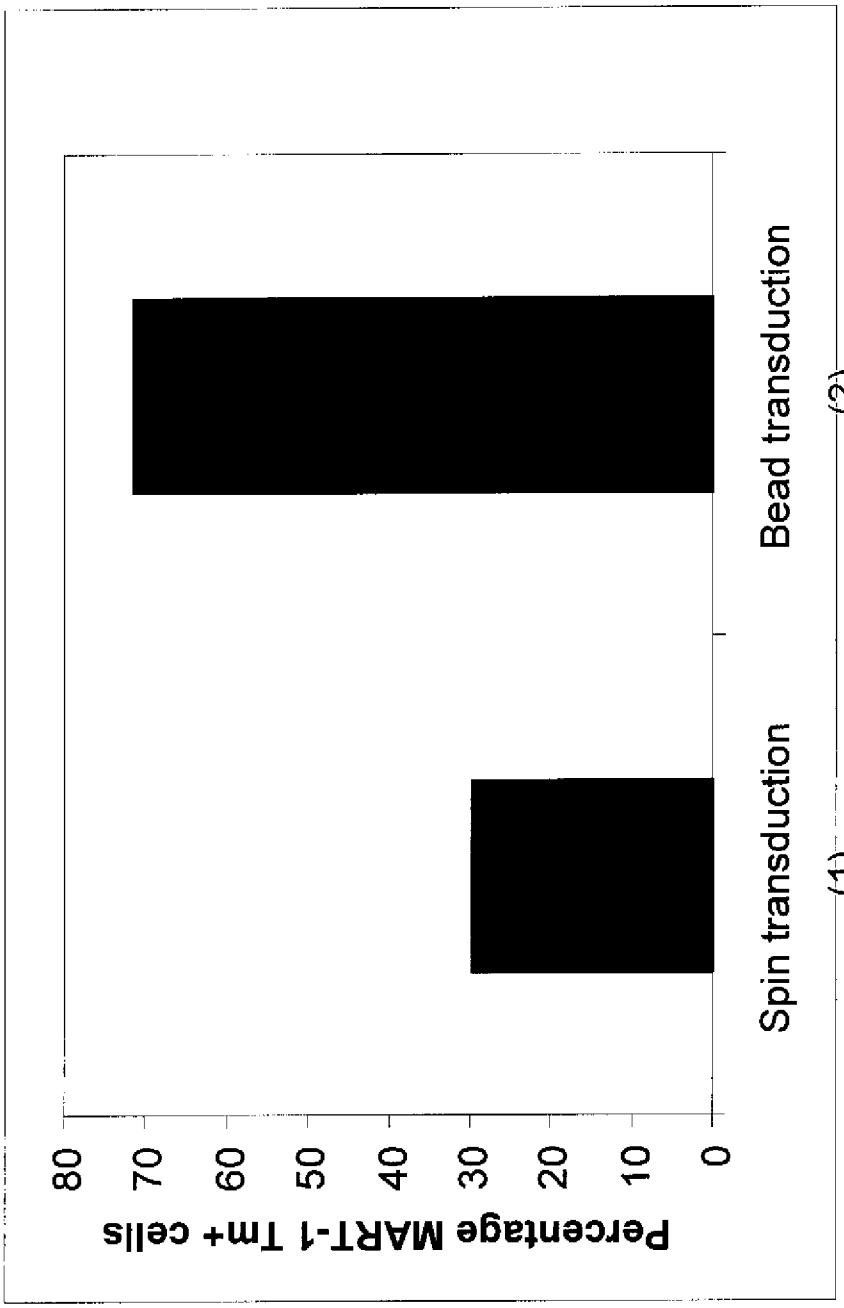

Percentage MART-1 Tm+ cells
0
10
20
30
40
50
60
70
80
Spin transduction
Bead transduction
(1)
(2)

Figure 4B

Figure 5

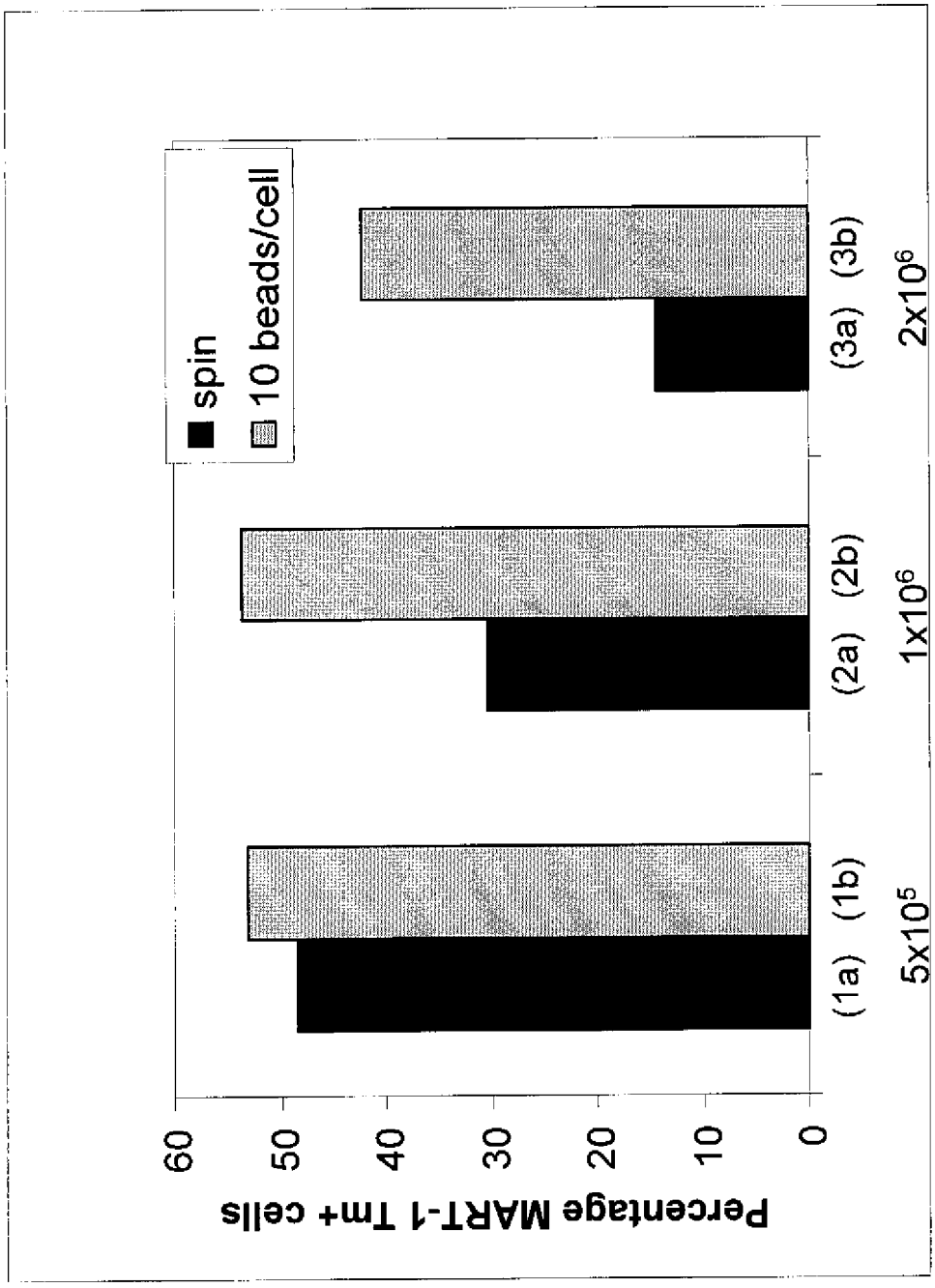

Percentage MART-1 Tm+ cells
0
10
20
30
40
50
60
spin
10 beads/cell
(1a) (1b)
(2a) (2b)
(3a) (3b)
5x10^5 cells/well
1x10^6 cells/well
2x10^6 cells/well

Figure 6

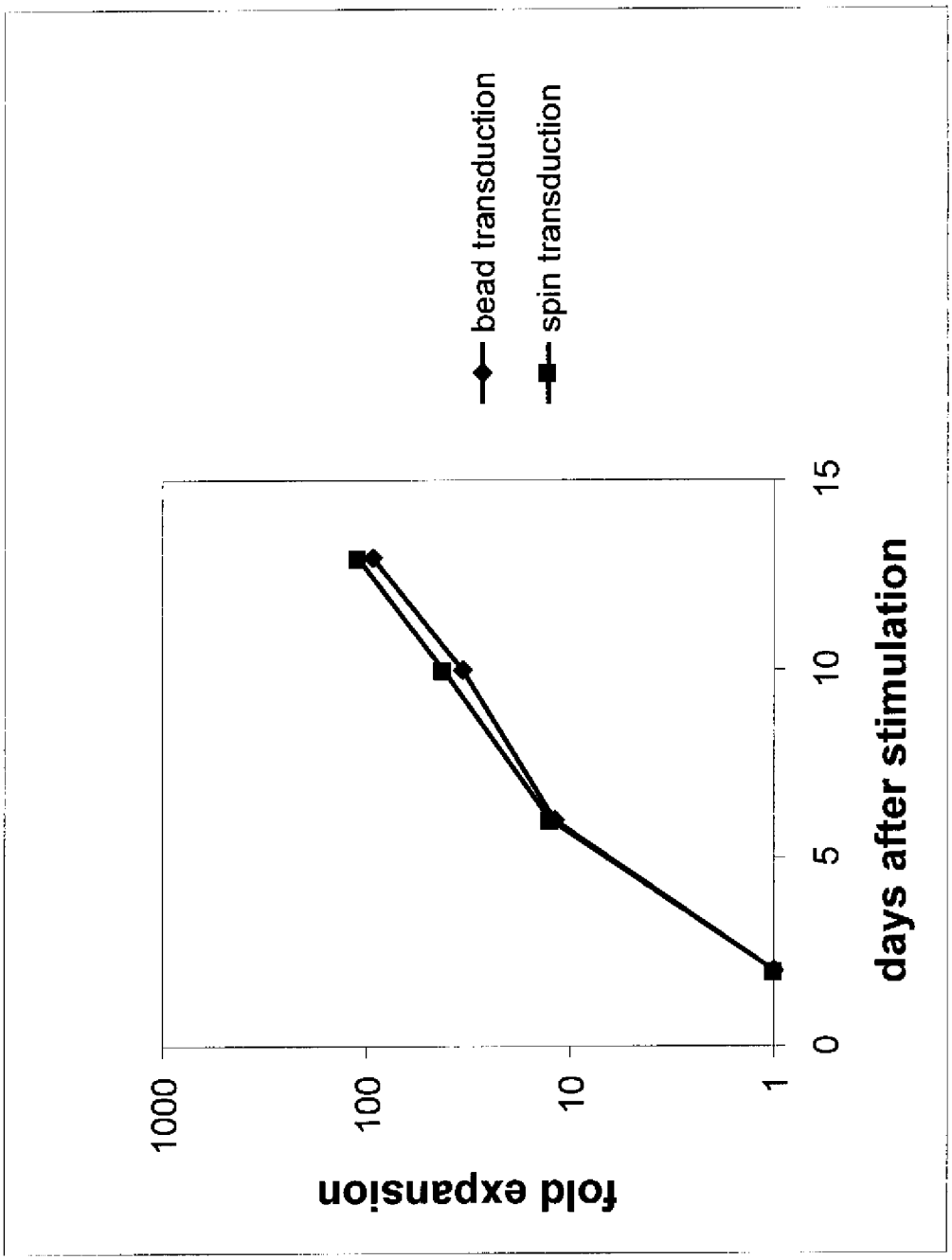
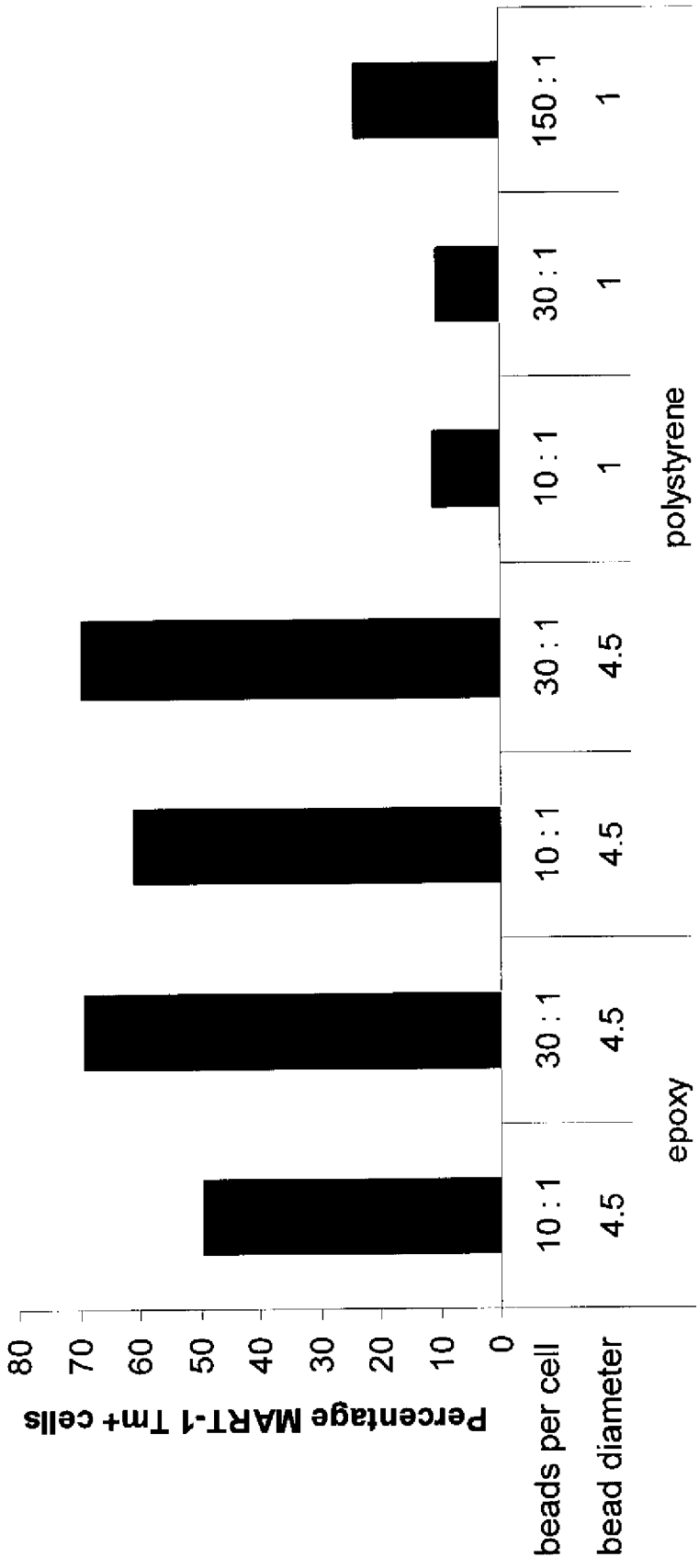

bead transduction
spin transduction
fold expansion
1000
100
10
1
0
5
10
15
days after stimulation

Figure 7


Percentage MART-1 Tm+ cells
0
10
20
30
40
50
60
70
80
beads per cell
bead diameter
10 : 1
4.5
30 : 1
4.5
10 : 1
4.5
30 : 1
4.5
10 : 1
1
30 : 1
1
150 : 1
1
epoxy
polystyrene