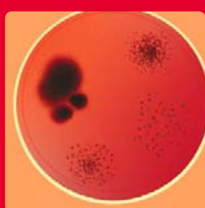
MACS & more
Vol 8 – 1/2004

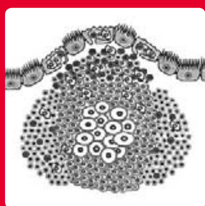
HIV MAGNETIZED

- DIRECT ISOLATION OF HIV-1 FROM PATIENT PLASMA
- NEW µMACS GFP ISOLATION KIT



CELLS' PARADISE

- A FULL RANGE OF MEDIA FOR HEMATOPOIETIC AND NONHEMATOPOIETIC STEM CELLS



RARE CELL ANALYSIS

- DENDRITIC CELLS FROM PEYER'S PATCHES
- HUMAN STEM CELL HOMING

NEW PRODUCTS

- NOVEL MOUSE PDCA-1 ANTIBODIES
- ISOLATION OF T CELL SUBSETS
- FASER KITS: ENHANCE YOUR FLUORESCENCE SIGNALS!
- DEAD CELL DISCRIMINATION WITH FIXED CELLS
- BUFFERS FOR CONVENIENT CELL SORTING
- $CD16^{+}$ MONOCYTE PURIFICATION MADE EASY

PERSPECTIVE: DISPLAY TECHNOLOGIES AND MAGNETIC SELECTION

Miltenyi Biotec
MACS

Dear customer,

The portfolio of MACS® products is steadily growing. In this issue you will find information about exiting new products as well as scientific reports on findings obtained with MACS® Technology.
A stimulating example is provided on pages 14 and 15 by Lupo and Butera who demonstrate the analytical power of magnetic cell separation by their report on direct analysis of HIV particles and their cellular origin, while Kadaoui and Corthésy provide further proof that MACS® Technology is the method of choice for isolating rare cells (p.10). On page 20, Johnston examines the perspectives of how to discover new protein-ligand interactions by combined display technology and magnetic selection. Last but not least, Lapidot and Kollet review the action of activating factors on the properties of translanted $CD34^+$ stem cells.
Specially formulated cell culture media for hematopoietic and nonhematopoietic stem cells are presented on pages 4 and 5. These media ensure optimal conditions during cell isolation, cultivation, and differentiation.
New kits for purification of $CD16^+$ monocytes (p. 8) and of T cell subsets (p.20) are now available, providing further options to isolate the cells of your choice even more conveniently.
Much more product information is available online – we look forward to your visit!

Your MACS&more team

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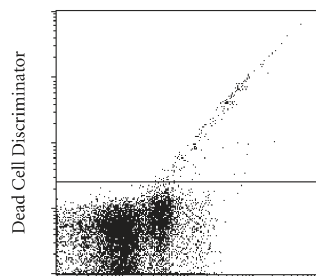
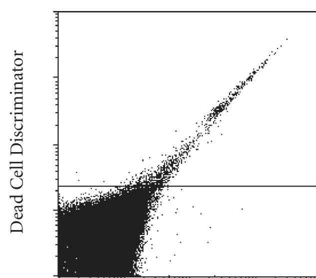
Fixation and Dead Cell Discrimination Kit

The Fixation and Dead Cell Discrimination Kit allows dead cell staining to be combined with cell fixation. After fixation, previously viable cells can be distinguished by flow cytometric analysis. The Fixation and Dead Cell Discrimination Kit is suitable for staining of all nucleated dead cells of any species.

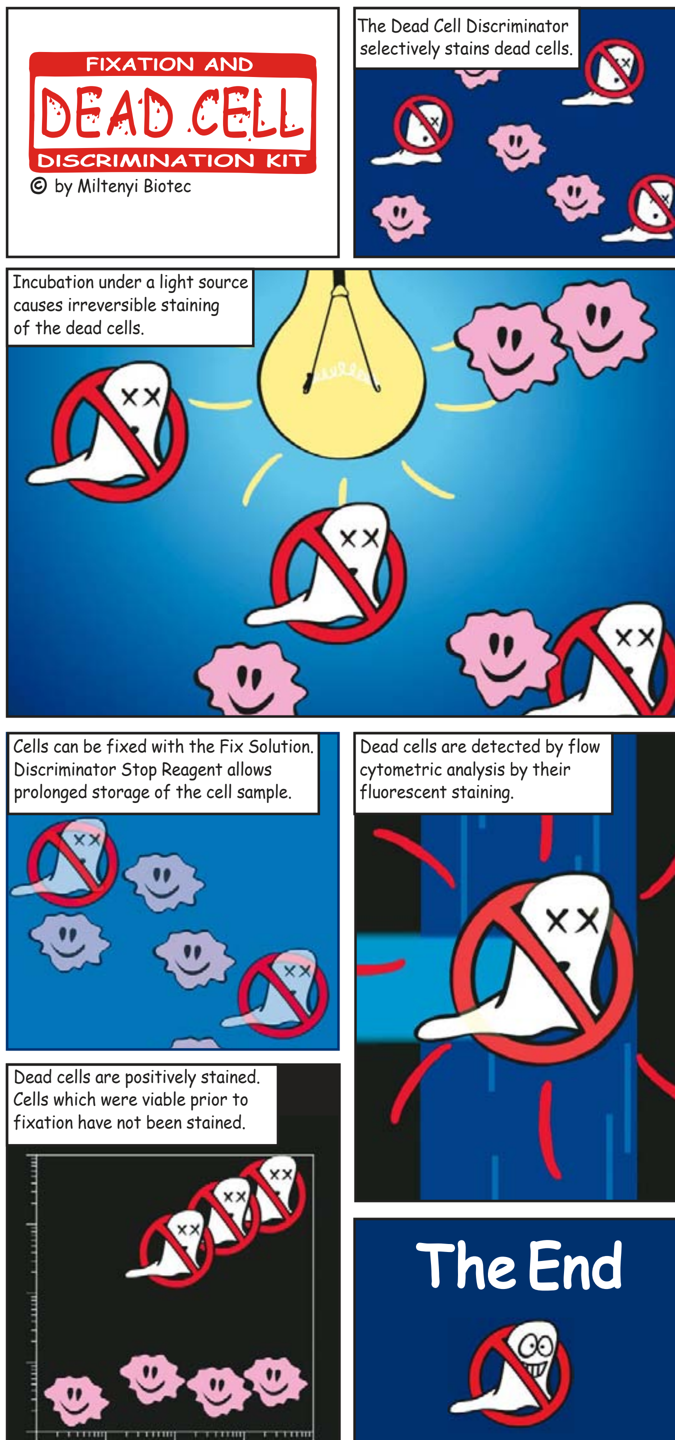
Principle

The Dead Cell Discriminator is a membrane-impermeant fluorescence dye which infiltrates selectively, into dead cells because of their damaged membranes. In the cell, it binds to nucleic acids of the respective cells. The samples are then exposed to visible light, e.g. under a 60 W light bulb, for a short time. This causes covalent binding of the Dead Cell Discriminator, and thus the staining becomes irreversible. After washing the cells can be fixed. The final addition of Discriminator Stop Reagent allows optimal dead cell discrimination even after prolonged storage (up to 24 hours) of the fixed cells at 4–8 °C. The Dead Cell Discriminator is excited by a laser emitting light at 488 nm and has a fluorescence emission maximum at 625 nm.





Peripheral blood mononuclear cells (PBMCs) were stained with Anti-CRTH2-PE (#130-091-238) or CD138-PE (#130-081-301), and Dead Cell Discriminator. The samples were exposed to visible light. Cells were washed and fixed, Discriminator Stop Reagent was added to the samples. The Dead Cell Discriminator allows exclusion of dead cells in the PE vs. Dead Cell Discriminator channel, by gating on the unstained cells (below the line).



Benefits

- Prolonged storage of cell samples (up to 24 hours)
- Reduced biohazard of infectious samples due to fixation of cells
- Highly sensitive analysis and excellent performance of rare cell analysis due to reduction of background staining caused by dead cells

HSC-CFU and NH media: products for hematopoietic and nonhematopoietic stem cells

Throughout the whole life cycle, the human body preserves adult stem cells as natural resources for the regeneration of a wide range of tissues. Therefore, these stem cells are a promising source to alleviate or cure hematopoietic diseases and tissue injuries (tissue replacement therapies).

HSC-CFU media

The hematopoietic system comprises constantly self-renewing stem cells, capable of differentiating into progenitor cells and mature blood cells of all hematopoietic lineages. Thus, it contains cells at various stages of maturation. Primitive hematopoietic stem cells (HSCs) with multi-lineage differentiation capacity give rise to identical daughter cells, whereas progenitor cells have restricted differentiation potential and lack self-renewal capacity. Stem cell transplants can help restore bone marrow function and rebuild the immune system for patients with inherited immunodeficiency or autoimmune diseases, or after immunosuppressive treatment.

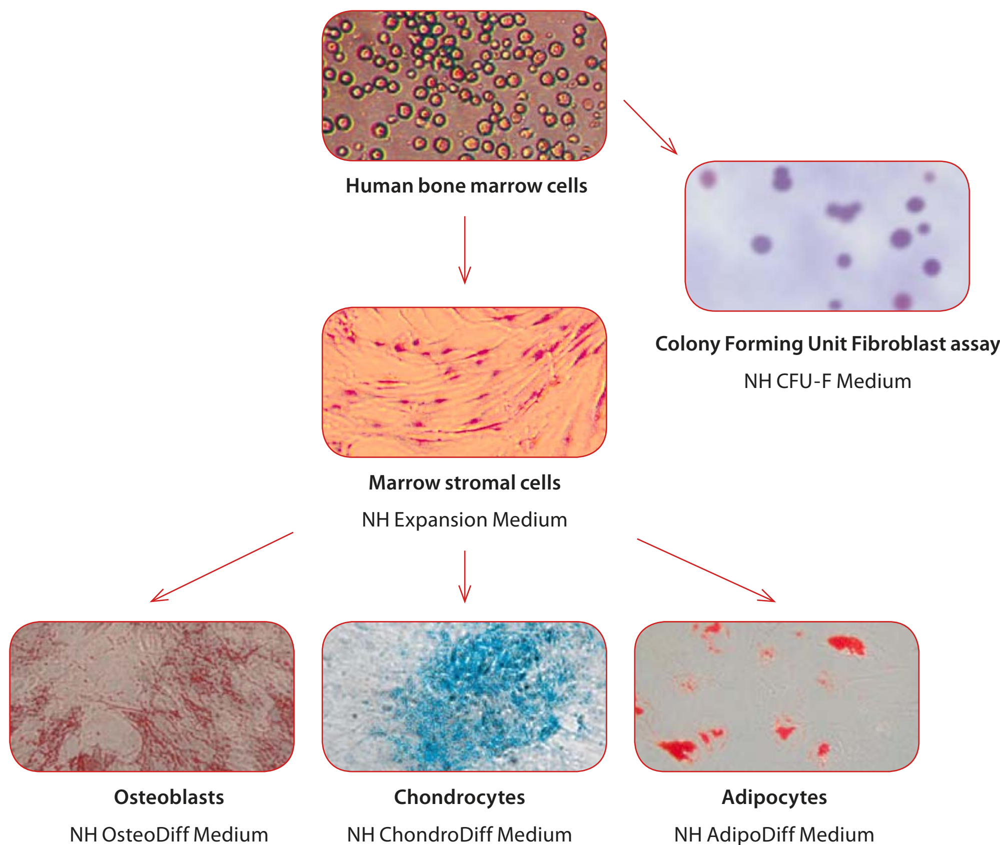
The HSC-CFU assay (hematopoietic stem cell-colony forming unit assay) is a reliable and reproducible test system to evaluate the differentiation potential of hematopoietic stem and progenitor cells *in vitro* and to estimate the engraftment potential of a transplant *in vivo*. The HSC-CFU assay is performed in HSC-CFU media, a product based on methylcellulose in IMDM and FCS, supplemented with several growth factors and cytokines in variable concentrations (for details see table below). These supplements support *in vitro* differentiation and proliferation of hematopoietic stem and progenitor cells. The semi-solid consistency of the media mimics the extracellular matrix. This reduces the migration of the clonal progeny of a single cell and causes it as a distinct cluster or colony. HSC-CFU media can be used for a variety of cell sources, such as mononuclear cells from peripheral blood, cord blood and bone marrow, as well as selected $CD34^+$, $CD117^+$ or $CD133^+$ cells.

NH media

Existing in close proximity to the hematopoietic stem and precursor cells in bone marrow the heterogeneous group of nonhematopoietic (NH) stem cells, named marrow stromal cells (MSCs), reveal broad nonhematopoietic differentiation potential to a variety of tissues. Therefore, MSCs show a high potential to support tissue regeneration, such as healing of non-union fractures and osteoarthritis (chondro- and osteogenesis). They also serve as a model for research on metabolic diseases (adipogenesis). The growing interest to apply

	HSC-CFU complete with Epo	**HSC-CFU** complete w/o Epo	**HSC-CFU** lite with Epo	**HSC-CFU** basic
The formulation supports growth of:				
CFU-G, CFU-M, CFU-GM	yes	yes	yes	yes / no*
BFU-E, CFU-E, and CFU-GEMM	yes	no	yes	yes / no*
Components:				
Methylcellulose in Iscove´s MDM	1%	1%	1%	1%
Fetal Bovine Serum	30%	30%	30%	30%
Bovine Serum Albumin	1%	1%	1%	1%
L-Glutamine	2 mM	2 mM	2 mM	2 mM
2-Mercaptoethanol	0.1 mM	0.1 mM	0.1 mM	0.1 mM
Stem Cell Factor	(50 ng/ml)	(50 ng/ml)	(50 ng/ml)	—
GM-CSF	(20 ng/ml)	(20 ng/ml)	(10 ng/ml)	—
G-CSF	(20 ng/ml)	(20 ng/ml)	—	—
IL-3	(20 ng/ml)	(20 ng/ml)	(10 ng/ml)	—
IL-6	(20 ng/ml)	(20 ng/ml)	—	—
Erythropoietin (Epo)	(3 U/ml)	—	(3 U/ml)	—

* Depending on added components



MSCs for clinical applications increases the necessity to better understand the fundamental processes that mediate the differentiation of MSCs into functional nonhematopoietic cell types and to identify the factors involved. Therefore, it is essential to establish efficient and reproducible procedures for the isolation, cultivation and differentiation of target cells. The optimization and standardization of experimental conditions, such as *in vitro* culture systems is an important first step. NH media are optimized for most reproducible and convenient quantification, quality control, expansion and differentiation of MSCs from human bone marrow.

The CFU-F (Colony Forming Unit Fibroblasts) assay is a well-established method based on the enumeration of the clonal progenies of MSCs – named CFU-F, after their spindle-like morphologic appearance. It is used for the quantification of MSCs in bone marrow samples or for the evaluation of the aspiration procedure used. Miltenyi Biotec therefore developed the convenient, pre-aliquoted NH CFU-F Medium.

As MSCs are present at low frequencies in bone marrow, it is usually necessary to expand these cells for further experiments, e.g. MSC transplantation studies (animal models), differentiation studies, or gene/protein expression profiling. The NH Expansion Medium is an optimized and standardized medium for the reproducible and reliable expansion of MSCs from human bone marrow.

For the evaluation of differentiation potential of expanded MSCs, NH differentiation media (NH AdipoDiff Medium, NH OsteoDiff Medium, NH ChondroDiff Medium) will soon be available.

MACS® basic media

The MACS basic media product line comprises classical formulations of RPMI and DMEM and modifications thereof with stable glutamine. RPMI and DMEM are used for a broad spectrum of applications. Through supplementation with vitamins, amino acids, cell growth factors, cytokines and serum, these media can be adapted for optimal culture of certain cell types. MACS basic media with stable glutamine are used for improved cell viability, yield and life span. They ensure standardization of culture conditions and are the first choice for long term culture of neoplastic cells. The media are produced under tightly controlled manufacturing conditions using high-quality ingredients of animal-free origin to guarantee an optimal and consistent lot-to-lot performance.

MACS® products

Product	Order no.
HSC-CFU complete with Epo	
100 mL	#130-091-280
24 × 3 mL	#130-091-278
HSC-CFU complete w/o Epo	
100 mL	#130-091-277
24 × 3 mL	#130-091-276
HSC-CFU lite with Epo	
100 mL	#130-091-281
24 × 3 mL	#130-091-282
HSC-CFU basic	
80 mL	#130-091-275
NH CFU-F Medium	
24 × 3 mL	#130-091-676
NH Expansion Medium	
500 mL	#130-091-680
NH AdipoDiff Medium	
100 mL	#130-091-677
NH ChondroDiff Medium	
100 mL	#130-091-679
NH OsteoDiff Medium	
100 mL	#130-091-678
RPMI 1640	
500 mL	#130-091-440
RPMI 1640 with stable Glutamine	
500 mL	#130-091-439
DMEM	
500 mL	#130-091-437
DMEM with stable Glutamine	
500 mL	#130-091-438

MACS® buffers for convenient manual and automated cell separation

MACS® products

autoMACS Running Buffer	
6 × 1.5 L	# 130-091-221
autoMACS Rinsing Solution	
6 × 1.45 L	# 130-091-222
MACS BSA Stock Solution	
6 × 75 mL	# 130-091-376

New MACS® cell separation buffers are sterile-filtered solutions prepared from high-quality ingredients and manufactured under tightly controlled conditions. MACS buffers are optimized for automated or manual magnetic cell sorting approaches using MACS Technology.
The autoMACS™ Separator is an automated bench-top magnetic cell sorter capable of sorting up to 10 million cells per second. It is compatible with any MACS cell separation reagent. The specially designed autoMACS™ Running Buffer and autoMACS Rinsing Solution bottles connect directly to the autoMACS Separator allowing standardization in automated high-throughput cell sorting. The combination of MACS® BSA Stock Solution and autoMACS Rinsing Solution allows the preparation of an optimal and preservative-free MACS Separation Buffer.

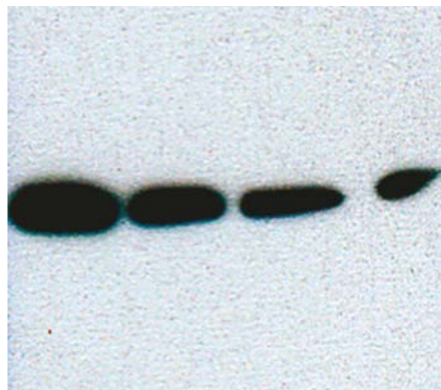
µMACS GFP Isolation Kit and Anti-GFP-HRP

Isolation of tagged proteins from eukaryotes made easy – with the second generation of the µMACS™ GFP Isolation Kit for even better results. They enable the isolation of epitope-tagged proteins whenever high sensitivity and minimum background are important, such as expression in eukaryotic cells and purification of unknown proteins or interaction partners.
The Anti-GFP MicroBeads coupled to new high-affinity monoclonal antibodies together with MACS® Column Technology allow the sensitive purification of GFP-tagged proteins in less than two hours. The kit contains all buffers for convenient working.
As a special feature, MACS® Column Technology allows enzymatic reactions with native bound proteins to be performed directly on the column. For this special application the new temperature-controlled thermoMACS® Separation Unit is ideal.

MACS® products

µMACS GFP Isolation Kit	# 130-091-125
Anti-GFP-HRP	# 130-091-833

The **new Anti-GFP antibody** is also available as a horseradish peroxidase conjugate and can be used for the single-step detection of wildtype and mutant GFP and GFP fusion proteins in Western blots and ELISA.



Aliquots of recombinant GFP fusion protein (20, 10, 5, and 1 ng) were subjected to gradient SDS-PAGE and transfered to a PDVF membrane. Bands were detected with Anti-GFP-HRP (1:5000, 1 hr, RT) and chemiluminescent signal capture.

New developments for B cell research

MACS® Technology offers a wide range of MicroBeads, Cell Isolation Kits, MultiSort Kits, manual and automated separators and antibodies for the purification and detection of B cells from any kind of cell source and species. Optimized and ready-to-use products are available for human, non-human primate, mouse and rat B cells.

MACS® products

Untouched B cells	
B Cell Isolation Kit II	# 130-091-151
Naive B Cell Isolation Kit II	# 130-091-150
CD43 MicroBeads	# 130-091-333
B cells directly from whole blood	
Whole Blood CD19 MicroBeads	# 130-090-880
Analysis of human B cells	
CD19-FITC	# 130-091-328
CD19-PE	# 130-091-247
CD19-APC	# 130-091-248
Analysis of mouse B cells	
CD45R (B220)-FITC	# 130-091-829
CD45R (B220)-PE	# 130-091-828
CD45R (B220)-APC	# 130-091-843
CD43-FITC	# 130-091-580
CD43-PE	# 130-091-585
CD43-APC	# 130-091-581

Purification and analysis of B cells

B cell type	Cell source	Example for analysis (MACS® control*)	MACS Separation	Separation strategy	Order no.
HUMAN					
B cells	e.g. PBMC, lymphoid tissue, bone marrow	CD19^{+}CD20^{+}	CD19 MicroBeads CD20 MicroBeads CD22 MicroBeads	positive selection positive selection positive selection	130-050-301 130-091-104 130-046-401
	whole blood, bone marrow	CD19^{+}CD20^{+}	Whole Blood CD19 MicroBeads	positive selection	130-090-880
	PBMC	CD19^{+}CD20^{+}	B Cell Isolation Kit II	untouched isolation	130-091-151
Naive B cells	PBMC	CD19^{+}CD20^{+}IgD^{+}CD27^{-}	Naive B Cell Isolation Kit II	untouched isolation	130-091-150
Resting B cells	e.g. PBMC, lymphoid tissue	CD19^{+}CD20^{+}CD43^{-}	CD43 MicroBeads	untouched isolation	130-091-333
Memory B cells	e.g. PBMC, lymphoid tissue	CD19^{+}CD20^{+}CD27^{+}IgD^{-}	*combination of* B Cell Isolation Kit II or CD19 MultiSort Kit and CD27 MicroBeads	sequential sorting	130-091-151 130-055-301 130-051-601
Memory B cells, IgG expressing	e.g. PBMC, lymphoid tissue	CD19^{+}CD20^{+}IgG^{+}CD27^{+}	*combination of* CD19 MultiSort Kit and Mouse Anti-Human IgG MicroBeads	sequential sorting	130-055-301 130-047-501
Activated B cells	e.g. PBMC, lymphoid tissue	CD19^{+}CD20^{+}CD25^{+} CD19^{+}CD20^{+}CD30^{+} CD19^{+}CD20^{+}CD69^{+}	*combination of* CD19 MultiSort Kit and CD25 MicroBeads or CD30 MicroBeads or CD69 MicroBeads	sequential sorting	130-055-301 130-090-445 130-051-401 130-051-501
Plasma cells, myeloma cells	e.g. PBMC, lymphoid tissue, bone marrow	CD138^{+}	CD138 MicroBeads	positive selection	130-051-301
B cell progenitors	bone marrow	CD19^{+}	CD19 MicroBeads	positive selection	130-050-301
Pro-B cells	bone marrow	CD19^{+}CD34^{+}CD133^{+}	*combination of* CD34 MultiSort Kit *and* CD19 MicroBeads	sequential sorting	130-056-701 130-050-301
NON-HUMAN PRIMATE					
B cells	e.g. PBMC, lymphoid tissue	CD20^{+}	CD20 MicroBeads	positive selection	130-091-105
MOUSE					
B cells	lymphoid tissue, e.g. spleen	CD19^{+}CD45R/B220^{+}	CD19 MicroBeads CD45R (B220) MicroBeads	positive selection positive selection	130-052-201 130-049-501
Resting B cells	lymphoid tissue, e.g. spleen	CD19^{+}CD45R/B220^{+}CD43^{-}	B Cell Isolation Kit	untouched isolation	130-090-862
	e.g. PBMC, lymphoid tissue, bone marrow	CD19^{+}CD45R/B220^{+}CD43^{-}	CD43 MicroBeads	untouched isolation	130-049-801
B-1 cells	lymphoid tissue, e.g. spleen	CD5^{+}CD19^{+}CD45R/B220^{+}	CD5 MicroBeads	positive selection	130-049-301
RAT					
B cells	e.g. PBMC, lymphoid tissue, bone marrow	CD45R^{+}CD45RA^{+}	CD45R MicroBeads CD45RA MicroBeads Mouse Anti-Rat Kappa MicroBeads	positive selection positive selection positive selection	130-090-495 130-090-494 130-047-401
Resting B cells	e.g. PBMC, lymphoid tissue, bone marrow	CD45R^{+}CD45RA^{+}CD43^{-}	CD43 MicroBeads	untouched isolation	130-090-742
ANY CELL TYPE					
B cells	e.g. PBMC, lymphoid tissue, bone marrow	own antibody	Anti-Ig MicroBeads (various) Anti-Fluorochrome MicroBeads (various) Anti-Biotin MicroBeads	positive selection positive selection positive selection	please inquire please inquire please inquire
B cell subsets	e.g. PBMC, lymphoid tissue, bone marrow	own antibody	Anti-Fluorochrome MultiSort Kits (various)	sequential sorting	please inquire

* MACS fluorochromes (green color) are optimized for the evaluation of MACS-separated cell fractions (MACS control). The convenient format and protocol allows staining of cells during or after magnetic cell isolation using MACS Technology. In addition, the combination of MACS fluorochromes and Anti-Fluorochrome MicroBeads allows labeling for magnetic cell sorting and subsequent cell analysis in a single step. These products are for research use only.

Separation of monocyte subsets: The new CD16+ Monocyte Isolation Kit

Human monocytes do not represent a homogenous cell population in peripheral blood. They can at least be subdivided into two distinct subpopulations: CD14^{high} CD16$^{–}$ monocytes, which account for about 90–95% of all blood monocytes, and CD14^{low} CD16^{+} monocytes, which account for about 5–10% of all blood monocytes. These two distinct monocyte subsets differ in phenotype and immunological function. For example, CD16^{+} monocytes are supposed to be more mature and have a higher T cell stimulatory capacity than conventional CD16$^{–}$ monocytes. They are lower in phagocytic activity and cytokine production but exhibit high expression of MHC class I and II and costimulatory B7 molecules. An increase in CD16^{+} monocyte subsets is associated with different acute inflammatory diseases and infections.

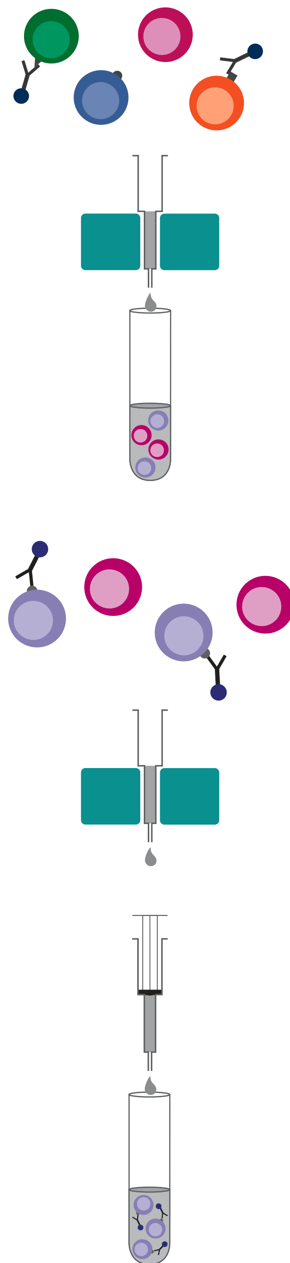
With the new CD16^{+} Monocyte Isolation Kit, CD16^{+} monocytes can easily be isolated to high purity in a two-step procedure (see figure).

With the new kit, Miltenyi Biotec expands its unique product portfolio of antibodies and kits for identification and isolation of monocytes and dendritic cells.

More information about fluorochrome-conjugated antibodies against human CD14, CD15, CD16, CD56 and Biotin is available on our website, www.miltenyibiotec.com.

MACS® product

CD16^{+} Monocyte Isolation Kit, human (2×10^{9} total cells) #130-091-765



1. Depletion of granulocytes and NK cells

Preparation of PBMC and magnetic labeling of NK cells and granulocytes using a depletion cocktail (CD15 and CD56 MicroBeads)

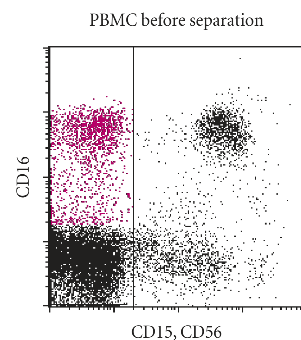
Depletion of labeled cells by magnetic separation

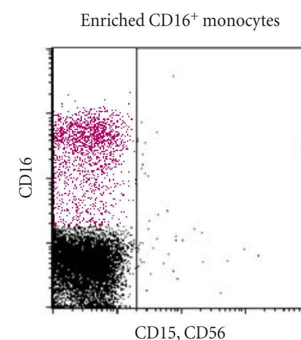
2. Positive selection of CD16^{+} monocytes

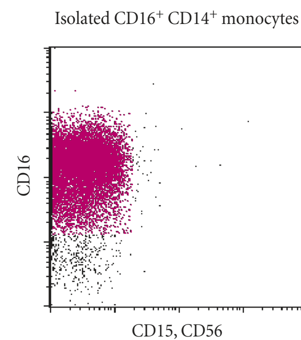
Magnetic labeling of CD16^{+} monocytes with CD16 MicroBeads

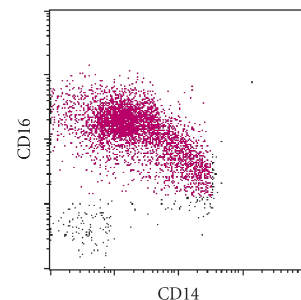
Magnetically labeled CD16^{+} monocytes are retained on the MACS column upon magnetic separation.

The column is removed and CD16^{+} monocytes are eluted.









CD16^{+} monocytes were isolated from PBMC using the CD16^{+} Monocyte Isolation Kit, an LD Column and a MidiMACS™ Separator for the depletion step, and an MS Columns and a MiniMACS™ Separator for the positive selection step *(schematic on the left)*. Aliquots of each cell fraction were analyzed by flow cytometry *(right)*.

FASER Kits – Beam up your cells!

Weak fluorescence signals?
Difficult-to-define cell populations?
Beam up your cells!
With the new FASER Kits, fluorescence intensity of cells labeled with virtually any FITC-, PE- or APC-conjugated antibody can be amplified. The Kits are suitable for fresh or formaldehyde-fixed cells in suspension of any type and species. Analysis is performed by flow cytometry. The higher sensitivity provided by FASER Kits makes flow cytometric analysis easy and more reliable.

Applications

- for amplification of fluorescence signals caused by weakly expressed antigens, low antibody affinity, or immunomagnetic and immunofluorescent labeling of one target epitope,
- for more clearly defined cell populations,
- for amplification of magnetic labeling in combination with MACS® Anti-FITC, -PE, or -APC MicroBeads.

MACS® products	
FASER Kit – FITC	#130-091-763
FASER Kit – PE	#130-091-764
FASER Kit – APC	#130-091-762

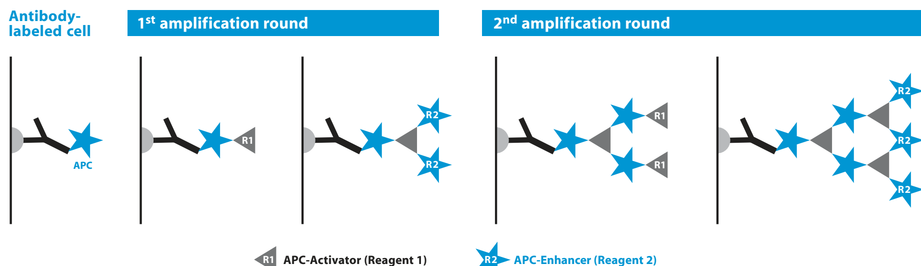


Figure 1. Fluorescence amplification is based on sequentially adding two reagents, the fluorochrome-specific *Activator (Reagent 1)* and the fluorochrome-conjugated *Enhancer (Reagent 2)*. Sequential addition of the reagents can be repeated as often as required.

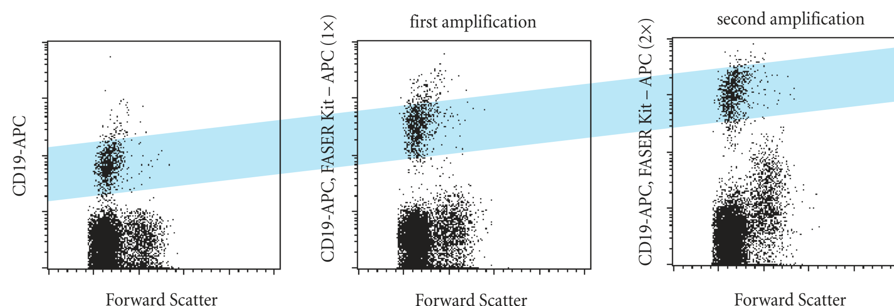
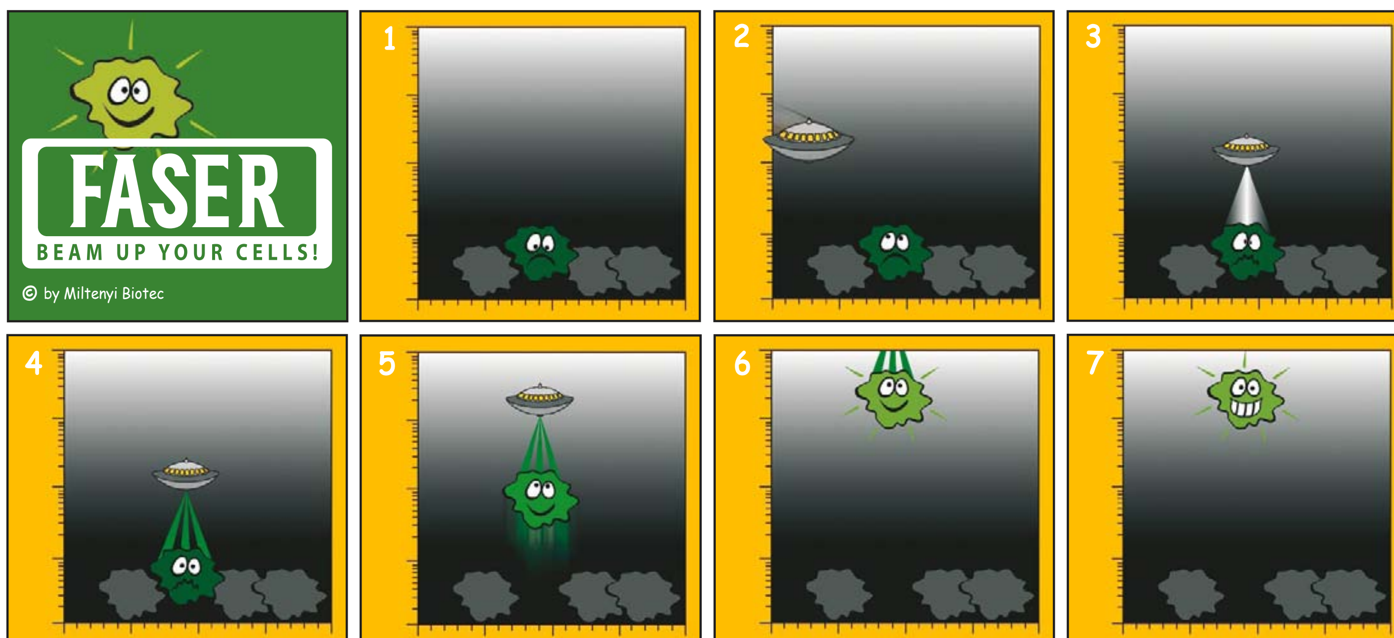


Figure 2. Human peripheral blood mononuclear cells were stained with CD19-APC, and the APC fluorescence was increased by two rounds of amplification using the FASER Kit – APC. The mean fluorescence intensity of the $CD19^+$ cell population is amplified in the first round of amplification by a factor of 5 and in the second round by a factor of 13 compared to the non-amplified staining (indicated by blue bar).



Maintaining optimal viability and preventing artifactual maturation are two hallmarks of successful cell isolations. In the case of rare cells – especially if no simple isolation procedures exist – it is often difficult to reach these qualitative aims. In such cases, magnetic cell sorting is the method of choice, as exemplified here for the purification of antigen-presenting cells from mouse intestine.

Isolation of dendritic cells from mouse Peyer's patches using magnetic cell sorting.

Khalil A. Kadaoui and Blaise Corthésy
Laboratoire R&D du Service d'Immunologie et d'Allergie, CHUV-Lausanne, Switzerland.

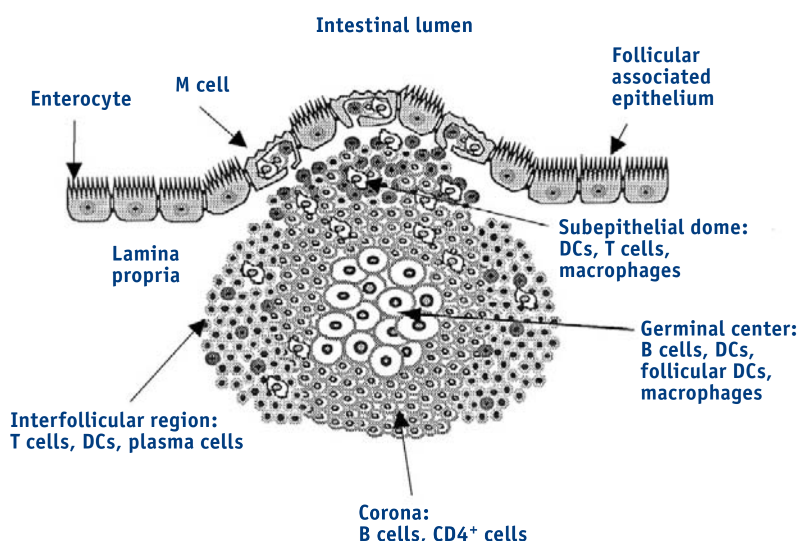


Figure 1 Schematic drawing of an intestinal mouse Peyer's patch (PP). Most of dendritic cells (DCs) are localized in the subepithelial dome, and represent about 1–2% of the total cells in a PP (taken and adapted from Kraehenbuhl and Neutra[11]). Antigens are transported across M cells and captured by DCs, leading to the initiation of the many processes resulting in mucosal immune responses[12].

Introduction

In the mouse intestine, Peyer's patches (PP) represent the primary site for uptake and presentation of antigens (figure 1). Following transport across M cells[1], antigens are processed by dendritic cells (DCs), the most potent antigen-presenting cells[2]. Because of their very low abundance[3] (1–2%) and due to the limited overall number of cells in the tissue (≥10^6 cells per PP), the isolation of PP DCs remains a challenging task. Furthermore, the properties and function of these cells are likely to differ from their spleen or skin counterparts[4]. Likewise, DCs derived from bone marrow[5] might not exhibit all the characteristics and function of PP DCs. In addition, the state of differentiation and activation might not reflect the intrinsic nature of the DC isolated from its natural context. Therefore, we have set up a purification procedure based on magnetic cell sorting which permits to recover highly viable PP DCs within one hour after tissue removal from the animal, with enrichment reaching up to 96% without any pre-depletion steps. Furthermore, this protocol is suitable to recover enough PP DCs (≥150,000) from one single animal.

Material and methods

Solutions.

Buffer A: Phosphate-buffered saline (PBS; pH 7.3), 5% fetal calf serum (FCS; Gibco-BRL), 5 mM EDTA (pH 8.0). Buffer B: PBS, 0.5% bovine serum albumin (Fluka 05477), 5 mM EDTA (pH 8.0). Complete MEM-α medium: plain MEM-α medium (Gibco-BRL), 10% FCS, 10 mM Hepes (Gibco-BRL), 1×penicillin/streptomycin (1:100 of a 5 mg/ml stock solution; Gibco-BRL).

Recovery of PP cells.

BALB/c female mice (4–6 weeks old) were obtained from Harlan (The Netherlands). Freshly collected PP in complete MEM-α medium were digested with 0.5 mg/ml prewarmed (37 °C) collagenase (Sigma) in plain MEM-α medium for 15 min at room temperature under gentle shaking. After enzymatic treatment, PP were crushed and forced with a 5 ml syringe pestle through a

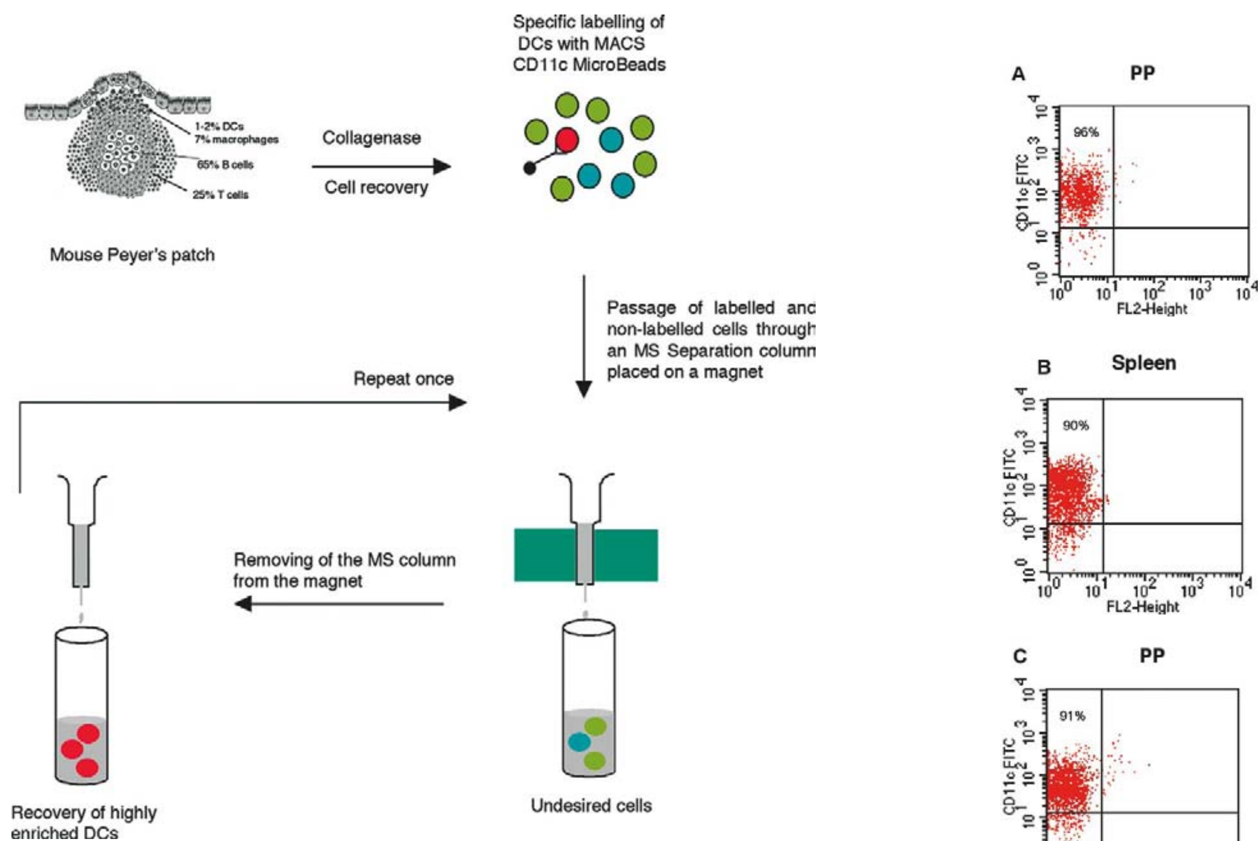


Figure 2 Schematic representation of the MACS® Technology-based isolation procedure for mouse PP DCs. Total cells were mechanically and enzymatically recovered from PP, then blocked with anti FcγII/III receptor Ab prior to incubation with CD11c MicroBeads. Non-labeled cells passed with the flow- through on a MS column placed on a magnet. To obtain highly enriched DCs, the passage over the column has to be performed twice.

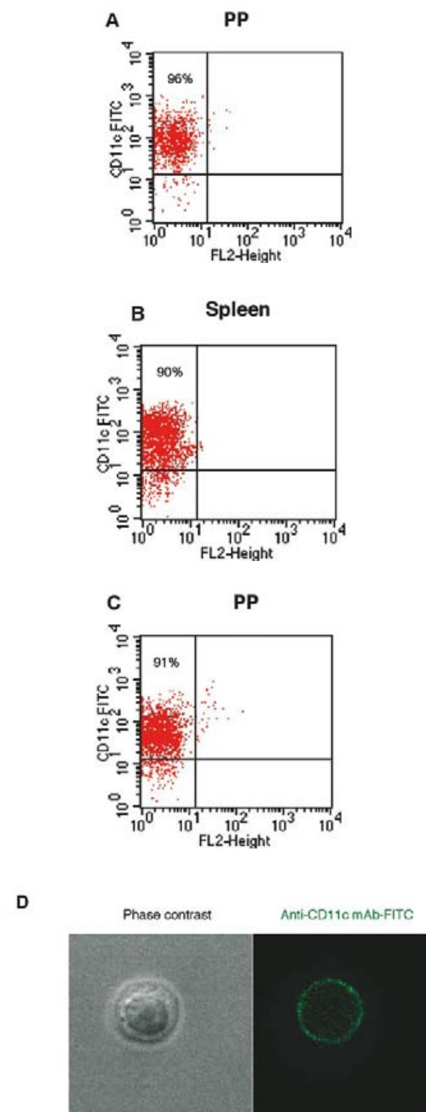


Figure 3 Isolation of PP and spleen DCs by magnetic cell sorting. DCs from PP (A and C) or spleen (B) were stained with anti-CD11c mAb-FITC and analyzed by flow cytometry. Panels A and C show two independent experiments. (D): Analysis of PP DCs by laser-scanning confocal microscopy.

70 µm-pore nylon mesh cell strainer (Falcon; BD Biosciences). 5×2 ml of complete MEM-α medium were used to guarantee the passage of most cells through the strainer. Cells were then passed through a 40 µm-pore nylon mesh cell strainer (Falcon; BD Biosciences) with two subsequent washings with 2×2.5 ml of complete MEM-α medium. Cells were recovered by centrifugation for 5 min at 400×g at 4 °C. The supernatant was discarded, and the pellet was suspended in cold buffer A at a concentration of 5×10^6 cells/100 µl. To avoid non-specific labeling, Fcγ II/III receptors were blocked using rat anti-mouse CD16/CD32 antibodies (Ab) (1: 100 dilution; Pharmingen) for 15 min at 4 °C. The excess of blocking Ab was washed away using buffer A, and the cells were pelleted by centrifugation for 5 min at 400×g at 4 °C prior to suspension in 100 µl of buffer A.

Magnetic separation.

$10–25\times10^6$ cells were incubated with MACS® CD11c MicroBeads (Miltenyi Biotec), using 10 µl beads per 10^7 cells. The tube containing the mixture of cells and beads was kept on ice for 15 min, with gentle tapping performed every 5 min. In the meantime, an MS Separation Column (Miltenyi Biotec) was mounted on a magnet, and equilibrated with 500 µl of buffer A (one MS column fits up to 2×10^8 total cells). The mixture of cells and beads was then applied to the column, the non-binding cells were allowed to flow through, and the column was washed twice with 500 µl of buffer B. To recover bound cells, the column was removed from the magnet and placed on a 5 ml polystyrene round-bottom tube (Falcon; BD Biosciences). Elution was carried out with 500 µl of buffer B using the plunger supplied with the column. To ensure enrichment values shown in figure 3A–C (see Results and Discussion), the magnetic separation had to be repeated using a second column under the same conditions with the exception that the column was washed twice with 250 µl of buffer B instead of 500 µl as indicated just above. A schematic representation of the procedure is depicted in figure 2.

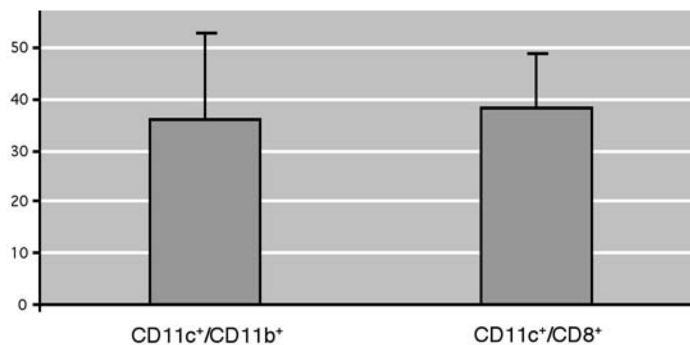


Figure 4 Surface phenotype of myeloid ($CD11c^+/CD11b^+$) and lymphoid ($CD11c^+/CD8^+$) DC subtypes. Percentage of double-labeled DCs isolated from mouse PP. Sorted DCs were stained with a combination of anti-CD11c/CD11b (myeloid phenotype) or anti-CD11c/CD8 (lymphoid phenotype) mAbs, and analyzed by flow cytometry. Mean results of four independent experiments are presented. Bars show standard deviation.

Flow cytometry analysis of $CD11c^+$-enriched DCs.

Post-elution, 5,000–30,000 purified DCs were washed and suspended in 200 µl of cold buffer B, and incubated for 25 min on ice with a 1:100 dilution of FITC-conjugated anti-CD11c mAb (clone N-418; Pharmingen). The excess of antibody was washed with 1 ml of buffer B and dead cells were excluded from the analysis by selection in the presence of 1 µg/ml propidium iodide solution (Sigma). Flow cytometry analysis was performed on a FACScan flow cytometer (BD Biosciences) and data were processed using the CellQuest software (BD Biosciences).

To track the percentage of myeloid and lymphoid DC subtypes in PP, DCs were labeled for 25 min on ice with a combination of either FITC-conjugated anti-CD11c mAb (1:100) and PE-conjugated anti-CD11b mAb (1:200; Pharmingen), or FITC-conjugated anti-CD11c mAb (1:100) and PE-conjugated anti-CD8α mAb (1:200; Pharmingen), respectively. Flow cytometry was performed as above.

Laser-scanning confocal microscopy.

Post-elution, 5,000 purified DCs were washed and suspended in 50 µl of cold buffer B, and incubated for 25 min on ice with a 1:50 dilution of FITC-conjugated anti-CD11c mAb (clone N-418; Pharmingen). The excess of antibody was washed away, and cells were mounted on glass slides according to the protocol described in the Immunology Methods Manual[6]. Confocal microscopy pictures were obtained using a Leica TCS NT microscope (Glattbrugg, Switzerland). Excitation was obtained with an Argon-Krypton laser, with lines set at 488 nm for FITC excitation, the emitted light was filtered through a BF 530/30 filter. Images were taken with a 63× objective and processed using the Leica TCS NT software.

Results and Discussion

In order to prevent artifactual maturation and ensure optimal viability, procedures to isolate cells must guarantee appropriate yield and speed. This holds true for DCs, and particularly for PP DCs whose abundance is very low, and for which no simple isolation procedure exists. The tissue from which the cells originate represents a sensitive issue as it is highly likely that PP DCs exhibit functional properties and degree of maturation different to those from spleen for instance[7].

We have thus developed a two-step protocol to isolate DCs from PP using magnetic cell sorting. The isolation procedure relies on incubation with CD11c MicroBeads, and sorting using two successive MS columns. When analyzed by flow cytometry on the basis of CD11c expression, we demonstrate that the purity of sorted DCs reached up to 96% (figure 3A). The high purity obtained is close to the enrichment we and others could obtain using fluorescence-activated cell sorting[8,9], yet the FACS procedure is burdensome, often based on negative selection, and prone to trigger DC activation. In addition, the protocol described herein is adapted to the isolation from one single mouse, and provides the investigator with as many as 150,000 cells for pilot experiments. Similar mean percentages of enrichment were achieved when the procedure was applied to spleen (90%, figure 3B) used as a control in comparison with 91% for PP (figure 3C). Interestingly, purified DCs can be easily stained with anti-CD11c fluorescent mAb to permit visualization by confocal microscopy (figure 3D). Ongoing experiments in the laboratory indicate that PP DCs are suitable to study the possible internalization of antigens and track internal processing *ex vivo*.

For those interested in studying the DC subtype in PP, we confirmed that the procedure is adequate to recover all members of the DC population (figure 4). Indeed, double-labeling for the myeloid ($CD11c^+/CD11b^+$) or the lymphoid subtype ($CD11c^+/CD8^+$) yielded percentages of cells strikingly similar to previous reports using more complicated methods of isolation, requiring dozens of animals. Both double-labeled DCs ranged around 35% of the overall DCs in PP, the last third being contributed by the so-called double-negative DCs[10] (data not shown).

In conclusion, we have established a simple, fast, small-scale procedure that permits to recover highly enriched PP DCs appropriate to address open questions in the field of mucosal immunity such as the functional properties of PP DC subtypes and the processing capacity of such DC in the context of PP.

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mPDCA-1: A presumably novel antigen exclusively expressed by murine plasmacytoid dendritic cells

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In mice as well as in humans, dendritic cells (DC) do not represent a homogenous cell population but rather a mixture of distinct DC subsets differing in function and phenotype. In mouse spleen, $CD8a^{+}CD11b^{-}$ DC were originally distinguished from $CD8^{-}CD11b^{+}$ DC. Later, splenic $CD8^{-}CD11b^{+}$ DC were further subdivided into a $CD4^{+}$ and $CD4^{-}$ subset (Shortman, 2000). All these splenic DC populations express MHC II and high levels of CD11c. More recently, murine plasmacytoid DC (mPDC) have been identified in mouse lymphoid organs as $CD45R(B220)^{+}$, $Ly\text{-}6C^{+}$, $CD11c^{+}$, $CD8a^{+/-}$, $CD11b^{-}$ cells (Asselin-Paturel, C. *et al.*, 2001). Like their human counterpart, murine plasmacytoid DC exhibit a plasmacytoid morphology and are able to produce large amounts of type I interferon (IFN-α and IFN-β) in response to bacterial DNA containing particular unmethylated CpG motifs (CpG-DNA) or upon viral challenge. In humans, PDC have been shown to specifically express BDCA-2 and BDCA-4 (Dzionek *et al.*, 2000). In mouse, no such specific markers are available to date. New markers specific for mPDC would, however, be of great benefit, in order to monitor, characterize and isolate mPDC and also to study their *in vivo* function by mAb-mediated *in vivo* depletion of mPDC. We have generated a panel of monoclonal antibodies (mAb) recognizing a single, presumably novel antigen, which we have termed murine plasmacytoid dendritic cell antigen-1 (mPDCA-1). In murine spleen, bone marrow and lymph nodes, mPDCA-1 is exclusively present on cells, which are $CD11c^{int}$, $CD45R(B220)^{+}$, $Ly\text{-}6C^{high}$, $Gr\text{-}1^{int}$ (Ly-6C/G), $CD3^{-}$, $CD8a^{dim}$, $CD11b^{-}$, $CD19^{-}$, CD49b $(DX\text{-}5)^{-}$, $Th\text{-}1.2^{-}$, $MHC\text{-}II^{int}$ cells, $CD40^{-}$, $CD80^{dim}$ and $CD86^{-}$, and therefore phenotypically identical to mPDC. In fact, multi-color staining reveals that all $CD11c^{int}$, $CD45R^{+}$(B220), $Ly\text{-}6C^{high}$ mPDC are $mPDCA\text{-}1^{+}$ and that there are no other $mPDCA\text{-}1^{+}$ cells apart from $mPDCA\text{-}1^{+}$ mPDC (figure 1). Injection of Anti-mPDCA-1 mAb (rat $IgG_{2b/\kappa}$; 200–500 µg i.p.) almost completely *in vivo* depletes mPDCs (more than 80% are depleted within 24 h in spleen, bone marrow and lymph nodes) (figure 2). Thus, the Anti-mPDCA-1 mAbs are not only extremely useful for single-color identification of mPDCs by flow cytometry, but are also of great value for elucidating the *in vivo* role of these cells.

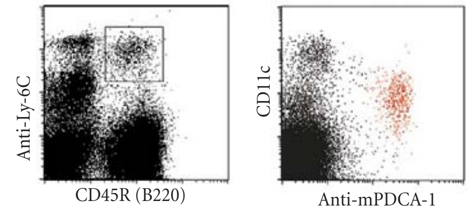

Figure 1 Identification of $mPDCA\text{-}1^{+}$ mPDCs. (1) mPDC were gated based on $CD45R^{+}$(B220) and $Ly\text{-}6C^{high}$ expression. (2) mPDCs gated as shown in (1) are depicted in red. Note that all $CD11c^{int}CD45R^{+}$(B220) $Ly\text{-}6C^{high}$ mPDCs are $mPDCA\text{-}1^{+}$ and that there are no other $mPDCA\text{-}1^{+}$ cells apart from mPDCs.

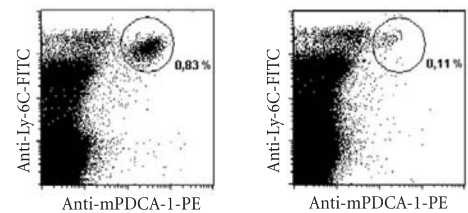

Figure 2 Specific *in vivo* depletion of mPDCs by injection of Anti-mPDCA-1 mAb. The frequency of $Ly\text{-}6C^{+}$ $mPDCA\text{-}1^{+}$ mPDCs is dramatically reduced within 24h after i.p. injection of 200–500 µg anti mPDCA-1 mAb as compared to a control mouse injected i.p. with diluent only (one representative experiment of four is shown).

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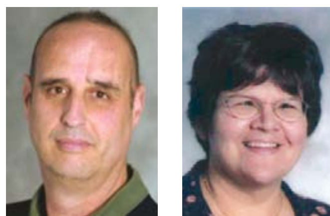
In their review, Lapidot and Kollet discuss the properties of transplanted CD34⁺ stem cells and the action of various factors mediating their activity, especially Stromal Derived Factor 1 and its receptor, CXCR4.

Mechanism of human $CD34^+$ stem cell homing and mobilization in transplanted immune-deficient NOD/SCID mice.



Tsvee Lapidot and Orit Kollet
Dept. of Immunology, The Weizmann Institute, Rehovot, Israel.

Transplanted human $CD34^+$ stem cells migrate via the circulation, home to and engraft the host bone marrow (BM) and continuously release maturing blood cells into the circulation. This process is essential for successful clinical transplantations of blood forming stem cells. In addition, G-CSF-induced $CD34^+$ stem cell mobilization from the BM into the blood circulation is the major source of stem cells for transplantation. During late embryonic development hematopoietic stem cells migrate from the liver to the BM. Murine embryos which lack the chemokine Stromal Derived Factor 1 (SDF-1) or its receptor CXCR4 have multiple lethal defects, including lack of bone marrow hematopoiesis, suggesting a major role for SDF-1/CXCR4 interactions during stem cell homing and/or BM engraftment.

SDF-1 is highly expressed in human bone marrow endothelium and the stem cell-rich endosteum region[1]. CXCR4 is expressed by many cell types, including primitive human $CD34^+/CD38^-$ and murine $Sca\text{-}1^+/c\text{-}kit^+/lin^-$ stem cells. Human and murine SDF-1 differ in one amino acid and are cross reactive, enabling murine SDF-1 in the BM of immune-deficient NOD/SCID mice transplanted with enriched, human $CD34^+$ progenitors, to signal via CXCR4. We demonstrated that SDF-1/CXCR4 interactions are essential for homing to the murine bone marrow[2] and high-level multilineage repopulation in NOD/SCID and serially transplanted $B2m^{null}$ NOD/SCID mice transplanted with MACS-enriched human $CD34^+$ stem and progenitor cells, isolated from cord blood, adult bone marrow and G-CSF induced mobilized peripheral blood from healthy donors[3]. These interactions, mediated activation of the major adhesion molecules expressed by immature human $CD34^+/CXCR4^+$ cells, leading to firm adhesion to their ligands expressed by $SDF\text{-}1^+$ human endothelial cells, under shear flow *in vitro*, mimicking *in vivo* interactions between transplanted human progenitors migrating in the blood circulation and the bone marrow endothelium, which is an essential first step in the multi-step homing and repopulation process[4].

In addition, SDF-1 mediated activation of the major adhesion molecules LFA-1, VLA-4, VLA-5[5] and CD44 on MACS-enriched human $CD34^+$ /$CXCR4^+$ cells which are essential for human stem and progenitor cell migration and *in vivo* homing, retention and repopulation of the BM in transplanted NOD/SCID mice. MACS-enriched human cord blood $CD34^+$ cells which do not express CXCR4 by cell sorting harbor low levels of intracellular CXCR4 which can rapidly oscillate *in vitro* and *in vivo* and mediate SDF-1-dependent homing and repopulation *in vivo*[6]. Short-term *in vitro* stimulation with the cytokines SCF and IL-6 for 24–48 hr increase surface CXCR4 expression, *in vitro* directional migration in response to a gradient of SDF-1, and *in vivo* homing and repopulation[2,3,6].

Since all clinically transplanted patients have only low levels of immature long-term culture-initiating cells (LTC-IC), increasing CXCR4 expression before transplantation and/or increasing levels of SDF-1 in the bone marrow[1] could improve the outcome of clinical stem

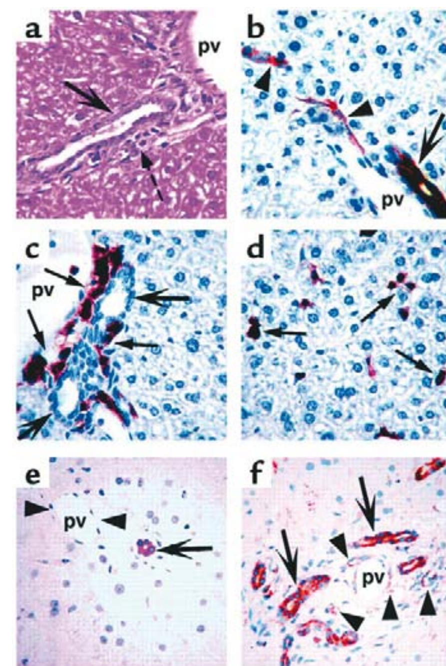


Figure 1 SDF-1 expression and engrafting cell accumulation within the liver. (**a–d**) NOD/SCID mice transplanted with CB CD34+ cells. (**a**) Hematoxylin and eosin staining 5–6 weeks after transplantation shows a bile duct (large arrow) adjacent to a large portal vein (pv) with cells surrounding the duct (dashed arrow) that are not observed in normal mouse liver. (**b**) Identification of SDF-1 in the bile ducts of NOD/SCID mouse liver. All epithelial cells in the bile ducts are strongly positive for SDF-1 (large arrow); scattered bile ductule cells (arrowheads) are also SDF-1-positive. (**c**) Human $CD45^+$ hematopoietic cells (small arrows) are present in large numbers surrounding the bile ducts (large arrows) and accumulate to very high density between the ducts and the adjacent portal veins. (**d**) Human $CD45^+$ cells are also observed as single cells or small clusters in random distribution in the hepatic sinusoids. Original magnification for a–d, ×400. (**e and f**) SDF-1 expression in normal adult human liver and in the liver of a patient with chronic hepatitis resulting from HCV infection was detected by immunohistochemistry. (**e**) Normal liver shows a single mature bile duct stained positive for SDF-1 (arrow) and absence of SDF-1 expression in endothelial cells lining a venous channel (arrowheads). (**f**) Liver from a patient with chronic liver disease from HCV infection shows extensive proliferation of bile ducts positive for SDF-1 expression (arrows) as well as expression of SDF-1 in bile ductule epithelium and/or canal of Hering or oval cells (arrowheads). Magnification for e and f, ×200.

cell transplantation and SDF-1/CXCR4 interactions could also be used to navigate cells *in vivo* for a wide variety of clinical protocols.
Stem cell mobilization in response to daily stimulations with G-CSF mimics stress-induced release and recruitment of stem and progenitor cells from the bone marrow reservoir in response to stress signals during inflammation and injury.
This process involves a transient increase in SDF-1 levels within the bone marrow after each injection which is followed by a profound decrease, mediated by activated myeloid cells such as neutrophils and osteoclasts which secrete the proteolytic enzymes elastase, cathepsin G, CD26 and MMP-2/9 reaching lowest levels during the time of mobilization. In parallel, after each injection there is a slight decrease of surface CXCR4 on BM hematopoietic cells including CD34 cells, followed by an increase, reaching peak levels of surface CXCR4 expression during the mobilization period, demonstrating for CXCR4 signaling during stress induced stem and progenitor cell egress[7,8]. Recruitment of MACS-enriched immature human cord blood or mobilized PBL $CD34^+$ cells to the irradiated NOD/SCID mouse liver or to the inflamed human liver of transplanted patients due to infection with hepatitis virus also involve increase in SDF-1 levels in this organ and a shift in membrane-bound SDF-1. Liver injury in previously transplanted NOD/SCID mice leads to stress-induced recruitment of human stem and progenitor cells also by shifting surface SDF-1 expression, to the release of the metalloprotei-nases MMP-2 and MMP-9 which increase surface CXCR4 expression on human progenitors in the murine BM of established chimeras, and to the release of cytokines such as HGF, SCF and IL-6 which also increase surface CXCR4 expression and SDF-1-mediated directional migration of human progenitors which are recruited to the damaged liver as part of organ repair[9].
In summary, human $CD34^+$ stem and progenitor cells migrate in response to stress signals which are mediated by an interplay between cytokines, chemokines, proteolytic enzymes and adhesion molecules, demonstrating a central role for SDF-1/CXCR4 interactions in both stem cell homing to the bone marrow and stress-induced mobilization, or recruitment to the irradiated or injured liver.

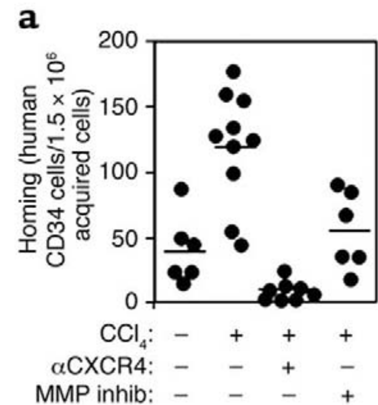


Figure 2 SDF-1-mediated 4-hour homing of human MPB $CD34^+$ cells to the liver of nonirradiated mice injected 24 hours earlier with 15 µl of CCl_4. Anti-CXCR4 pretreatment of transplanted cells or intraperitoneal injection of MMP-2/MMP-9 inhibitor (1.5 hours before transplantation). Bars indicate the mean number of homing cells per 1.5×10^6 acquired cells (n = 3 experiments).

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The questions

Test yourself!

Frequently asked questions regarding regulatory T cells

1

How are regulatory T helper cells characterized?

a) There is no known marker to distinguish between regulatory T helper cells and 'normal' T helper cells.
b) Regulatory T helper cells express CD25.
c) In contrast to other T helper cells, regulatory T cells do not secrete cytokines.

2

Is it important to enrich the $CD25^+$ regulatory T cells over two columns?

Yes / No

3

Which isolation strategy does Miltenyi Biotec use to isolate regulatory T cells?

a) First, T helper cells are enriched using a depletion strategy and then CD25 MicroBeads are used to enrich $CD4^+CD25^+$ regulatory T cells.
b) Pure regulatory T cells can be enriched using CD25 MicroBeads.
c) After isolation with CD25 MicroBeads, $CD4^+CD25^+$ regulatory T cells can be enriched by performing an additional separation with CD4 MicroBeads.

4

What is the function of regulatory T helper cells?

a) Regulatory T helper cells kill reactive T cells.
b) Regulatory $CD4^+CD25^+$ T helper cells seem to suppress harmful immunological reactions to self or foreign antigens.
c) They down-regulate the functionality of NK cells.

Find the answers on page 19.

When cells are infected by viruses and are forced to synthesize viral progeny, the budding particles take with them components of the host cell membrane. It is therefore possible to identify susceptible cell types by purification using appropriate markers. With MACS Technology, this can be achieved directly – without prior blood plasma processing.

Application of µMACS™ Streptavidin MicroBeads for the analysis of HIV-1 directly from patient plasma

L. Davis Lupo and Salvatore T. Butera
HIV and Retrovirology Branch, Division of AIDS, STD, and TB Laboratory Research, National Center for HIV, STD, and TB Prevention, Centers for Disease Control and Prevention, Atlanta, Georgia, USA

Introduction

It is established that host membrane proteins are incorporated into the retroviral envelope as HIV-1 virions bud from human cells (for a review see Tremblay *et al.*[1]). Direct or indirect mechanisms of host protein inclusion or exclusion from the retroviral envelope may be involved. This fact has permitted the ability to discriminate host cell types supporting viral replication by a targeted capture of virions directly from HIV-infected patient plasma via a previously described method[2–5]. However, this method required extensive processing of patient plasma to permit immunomagnetic capture of HIV-1 with high efficiency. In this report, we now describe marked improvements in the immunomagnetic capture protocol that permits analysis of virions directly from patient plasma without prior processing. These improvements not only allow direct analysis of patient plasma but they also open new approaches to the characterization of virions from HIV-infected individuals.

Materials and Methods

Preparation of samples.
Laboratory stocks of HIV-1 (strain Ba-L, subtype B), grown in either purified macrophages or $CD4^+$ T lymphocytes, were spiked into tissue culture medium or plasma at known viral particle numbers[2]. Either a commercial source of normal human plasma or a subtype B HIV-1-infected patient plasma with high anti-HIV antibody titers but undetectable viral load was used. Where indicated, HIV-1 spiked into specific antibody-containing plasma was processed by ultracentrifugation, salt dissociation, and spin column filtration, as described[2–5]. HIV-1-infected plasma samples with detectable viral loads were obtained from patients enrolled in a longitudinal TB therapy cohort under study in Kampala, Uganda, via collaboration with Dr. Zahra Toossi at Case Western Reserve University (Cleveland, Ohio). Appropriate informed consent was obtained by the Case Western Reserve Makerere Research Collaboration.

Immunomagnetic capture of HIV-1.
HIV-1 spiked into medium or plasma (processed or unprocessed) was first incubated with 0.5 µg of various antibodies (all obtained biotinylated from commercial sources) for 30 min at room temperature. To each sample, 20 µl µMACS™ Streptavidin MicroBeads (Miltenyi Biotec, Auburn, CA) were added and the binding reaction was incubated an additional 10 min at room temperature. Antibody-bound virus was then captured by magnetic separation with slight modifications from the manufacturer's protocols. Briefly, µ columns were placed in the µMACS™ magnetic separator attached to a MultiStand under a biological safety cabinet. Each column was prepared by prewetting with 100 µl protein equilibration buffer and rinsing twice with 100 µl PBS containing 2% fetal bovine serum (PBS/FBS). The entire volume of the virus capture reaction mixture (approximately 200 µl) was then applied to the column and allowed to drain completely. The columns were washed four times with 200 µl volumes of PBS/FBS. Fifty microliters of an appropriate lysis buffer (see below) were then added to the column, allowed to stand for 5 minutes at room temperature, and then followed by an additional 150 µl of the same buffer. All lysate elution fractions were collected as a pool. When capture efficiencies using MACS™ Technology were compared to the previous protocol using Dynal magnetic beads (Great Neck, NY), 2×10^7 streptavidin-conjugated Dynabeads (M-280) were used and the capture reactions were performed as previously described[2–5].

Analysis of captured virus.
For quantitation of captured HIV-1, two commercially available kits were used and assays were performed as directed. For the HIV-1 p24 antigen enzyme immunoassay (Coulter/ Immunotech Inc., Westbrook, Maine), the lysis buffer consisted of PBS containing 0.5% NP-40. Whereas, for the more sensitive HIV-1 RNA quantitation assay, the lysis buffer was obtained from the commercial source of the NucliSens QT viral load assay (Organon Teknika/

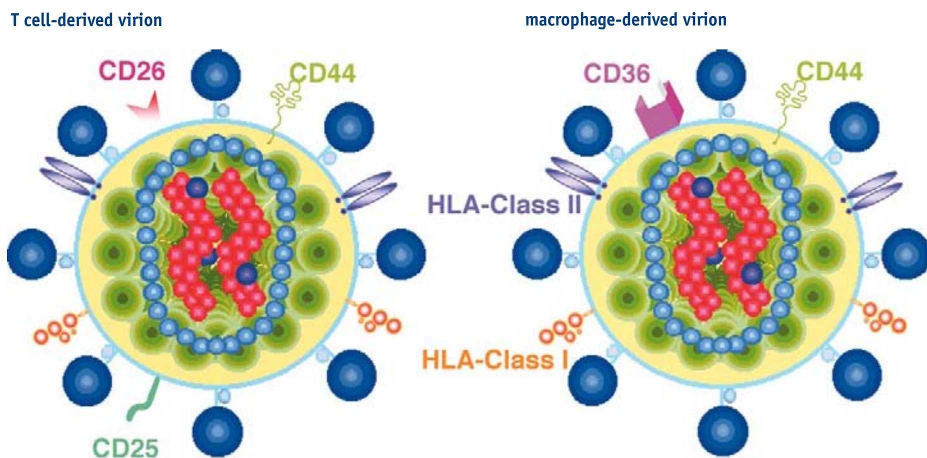

Figure 1 HIV-1 replication in different target cell populations results in selective incorporation of distinguishing host cell proteins. Virions derived from T cells (schematic on left) incorporate distinct T cell markers (i.e. CD26, CD25) while those derived from macrophages (schematic on right) acquire macrophage markers (i.e.. CD36). HIV-1 replication in either cell type allows for incorporation of dual markers, CD44 and HLA-DR. (modified from Lawn, S.D. (2004) J. Infect. 48: 1–12)

bioMérieux, Inc., Durham, NC). Viral RNA isolation and RT-PCR quantitation were performed using manufacturer's reagents and protocols. In some experiments, intact captured virus was recovered by removing the column from the magnetic separator and eluting with complete tissue culture medium. The infectivity of captured virus was then determined by direct inoculation onto purified $CD4^+$ T lymphocyte cultures that had been stimulated with PHA for 3 days. Cultures were fed with fresh media on day 7 and HIV-1 replication was monitored in cell-free culture supernatants by virion-associated, magnesium-dependent reverse transcriptase (RT) activity, as described[6].

Results and Discussion

Comparison of HIV-1 capture efficiency from processed and unprocessed plasma.

The ability to capture HIV-1 from plasma of infected persons has novel applications for understanding cellular compartments of viral replication (figure 1) and the impact of opportunistic infections. However, as we developed this technique[2–5], the need for extensive processing of plasma to overcome inhibition of capture by anti-HIV antibodies and serum reactive proteins was laborious, time-consuming, and damaging to the virus. Furthermore, plasma processing reduced the overall sensitivity of the technique due to aggregation or loss of virus. Because of their small size and behavior in suspension, we hoped that µMACS™ MicroBeads would show improved efficiency of virus capture directly from unprocessed plasma as compared to the much larger magnetic beads (Dynabeads) on which the original technique was developed.

Indeed, when the efficiency of capture with a positive control antibody (CD44) was determined for 10,000 HIV-1 particles spiked into plasma containing anti-HIV antibodies, the µMACS™ MicroBeads captured 100% of the input virions from unprocessed plasma while, as previously observed[2], capture by Dynabeads was markedly inhibited (figure 2). When plasma spiked with 10,000 HIV-1 particles was first processed, the input level of virions per capture fell (to approx. 4,000 copies) due to viral loss. However, the efficiency of capture by either µMACS™ MicroBeads or Dynabeads was similar, reaching about 50–60% of input (figure 2). The lack of complete capture by either system after plasma processing probably reflects virion aggregation, incomplete resuspension, or physical damage as a consequence of the ultracentrifugation. However, the use of µMACS™ Streptavidin MicroBeads permits the direct viral capture from plasma without the need for previous processing and therefore avoids this loss of input copy number. This also allows for the application to be transferred to patient material having lower viral loads, while saving the time and expense of plasma processing.

The efficiency of virus capture directly from the plasma was determined using the positive control marker (CD44) on samples from a HIV-1-infected cohort. As expected, the efficiency of virus capture varied among the 28 samples tested, with an average efficiency of 60% of input virus (figure 3). However, this variation was not dependent upon the patient viral load (ranging between 8×10^3 and 2×10^6 virion copies per ml in this sample set, figure 3) or the input amount of patient plasma (ranging between 100 and 150 µl in this study, data not shown).

Recognition of distinguishing host cell proteins incorporated in HIV-1.

To confirm that the use of µMACS™ MicroBeads with this technique can provide comparable results in regard to identifying host cell origins of viral replication (figure 1), HIV-1 stocks from the same strain (Ba-L) that were grown in either purified macrophage or $CD4^+$ T lymphocyte cultures were captured using a panel of antibodies against distinguishing host cell surface proteins. In accordance with the cellular

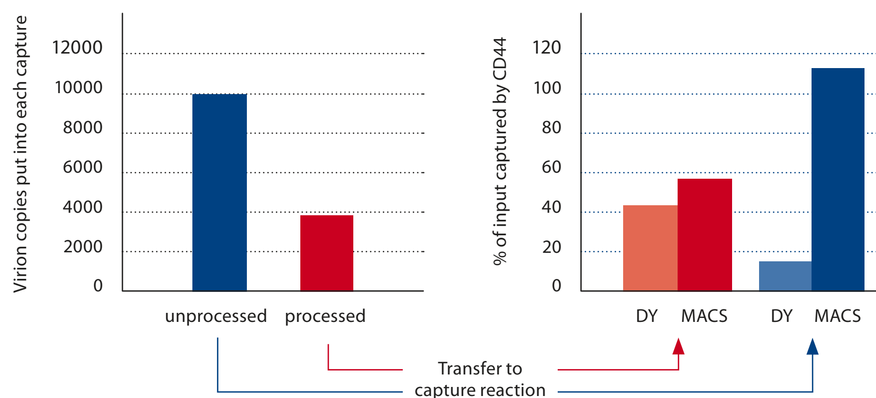

Figure 2 Efficiency of HIV-1 immunomagnetic capture when spiked into HIV antibody-positive plasma; with and without plasma processing. Ten thousand HIV-1 particles were spiked into human plasma with a high titer of anti-HIV antibodies and left unprocessed or taken through a plasma purification algorithm, as previously described[2]. These preparations were then subjected to the virus capture procedure using the universal marker CD44 and comparing efficiency of capture with Streptavidin-conjugated Dynal Dynabeads (Dy) and Miltenyi Biotec µMACS™ MicroBeads (MACS). Capture efficiency reflecting greater than 100% of input is due to the minor degree of quantitative variability inherent to the HIV-1 viral load (RT-PCR) assay.

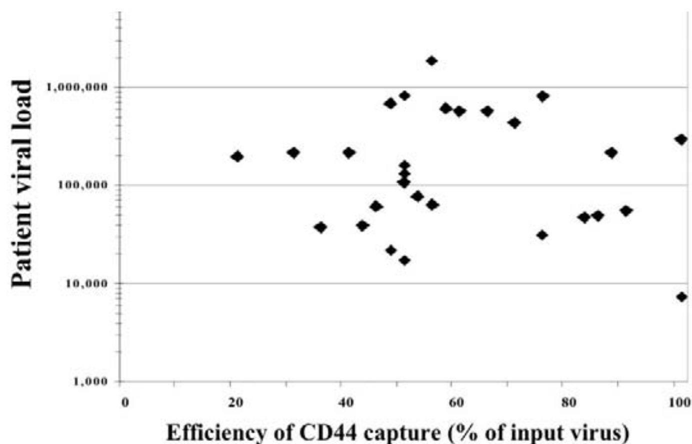

Figure 3 Efficiency of viral capture directly from HIV/TB-patient plasma. HIV-1-infected patient plasma (28 samples from 7 individuals over several time points) was used directly in the viral capture procedure. The efficiency of capture (percentage of virus input) by the universal marker CD44 was compared over the range of patient viral load determinations.

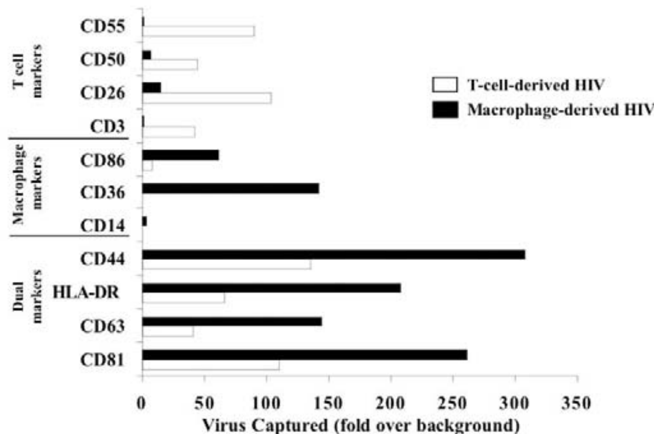

Figure 4 HIV-1 capture by antibodies targeted against cell surface markers selective for macrophages or CD4+ T lymphocytes. Antibodies against cell surface markers that had previously been shown to distinguish macrophage-derived and T cell-derived HIV-1, or present on both viral stocks (dual markers), were tested using µMACS beads in the capture procedure. Levels of captured virus were determined by HIV-1 p24 antigen immunoassay and compared to capture by a negative control antibody (CD19) to derive fold-over background values, as described[2].

origin of viral replication, HIV-1 stocks were selectively captured with host markers specific for the cell lineage (figure 4). These results are similar to those previously obtained using Dynabeads[2] except that the fold capture over background was substantially increased with µMACS™ MicroBeads due to low levels of nonspecific capture.

Analysis of captured virions for infectivity.
The previous method for HIV-1 capture directly from plasma included high salt treatment during processing to dissociate the outer viral envelope protein and any bound antibodies or other potential inhibitors of magnetic capture[2]. This treatment negated infectivity of captured virions. Furthermore, it seems unlikely that HIV-1 attached to a significantly larger magnetic bead could efficiently bind its co-receptors and fuse with a target cell. Since the processing steps were circumvented with the use of the much smaller and biodegradable µMACS™ Streptavidin MicroBeads, we directly examined the issue of infectivity of MicroBead-labeled virions. Known copy numbers of HIV-1 were spiked into and subsequently captured from normal human plasma and plasma containing anti-HIV antibodies. As previously observed (figure 2), using the positive control host cell marker (CD44) the efficiency of viral capture by µMACS™ MicroBeads from both plasma preparations approached 100% of input (data not shown).
Captured virions were eluted from the separation column and placed directly onto activated CD4+ T lymphocyte cultures. Over the course of the following two weeks, HIV-1 replication was observed in these cultures with as few as 2.4×10^5 virions spiked into plasma prior to capture (figure 5). Therefore, this application could be used to derive viral isolates from plasma or other biologic fluids, especially when cryopreserved patient peripheral blood cells are not available. Lower numbers of spiked virions also gave rise to positive infection cultures, but the successful outcome was variable (data not shown). Virions captured from anti-HIV antibody-containing plasma showed a delayed rise of viral replication but were not completely neutralized during the acute infection (figure 5).

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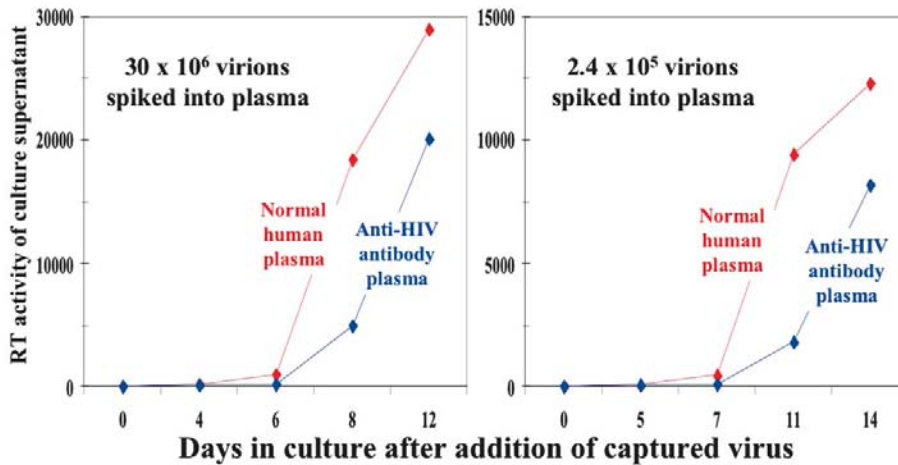

Figure 5 **Acute infection of $CD4^+$ T lymphocytes with HIV-1_{Ba-L} captured (with anti-CD44) from spiked plasma.** Virions were spiked into plasma at the numbers indicated, captured by CD44 antibody recognition, eluted without lysis, and cultured overnight with activated $CD4^+$ T lymphocytes. Cultures were continued for two weeks to determine extent of HIV-1 replication.

Conclusions and future applications

Study of HIV-1 directly from patient plasma or other biologic fluids can provide novel insight into cellular compartments supporting viral replication and aspects of viral pathogenesis. The improved ability to apply viral capture technology without extensive sample processing, due to the unique properties of µMACS™ MicroBeads, can advance this technique into new applications. By eliminating the need to remove inhibitors (antibodies, acute serum proteins etc.) from biologic fluids by processing, this will allow the approach described here to be utilized for bodily fluids for which extensive processing would not be feasible (breast milk, semen, etc.). A dual labeling technique could also be feasible using magnetic beads from different commercial sources. Furthermore, to study antibody neutralization, this technique could be modified to culture captured HIV-1 in the presence of increasing concentrations of autologous or heterologous plasma.

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The extraordinary efficiency of MACS Technology offers new perspectives for research using phage, ribosome, or yeast display techniques.

Efficient target molecule identification by combining display technology with magnetic selection

Ian Johnston
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Display technology

Display technology[1] classically refers to the generation and screening of a large library of expressed proteins or peptides using an immobilized ligand to characterize or discover new protein-ligand interactions.

There are a number of characteristics that have ensured that display technology is the method of choice when a new biomolecular interaction is to be identified. Firstly, the structure of the expressed protein (phenotype) is linked to the genetic information encoding it (genotype). This allows immediate identification and further characterization of the novel binding partner once it has been isolated from the library. Secondly, very large libraries can be generated and displayed to the ligand of interest. The larger the library, the greater is the chance of finding a binding partner. Thirdly, most established display systems enable amplification of the library without the introduction of a bias for a particular type of DNA fragment or protein. This allows successive unbiased rounds of screening to take place against a particular ligand, increasing the sensitivity. Finally, the amplification of the library can be used to introduce random errors into the encoding DNA sequence to enable mutant proteins with altered binding affinities or binding kinetics to be isolated (affinity maturation).

Popular display systems

Phage display

The display of foreign peptides and proteins on the surface of filamentous bacteriophages, "phage display", is now a widely-used technique to investigate molecular interactions. Normally the protein library to be screened is expressed as a fusion with the gene 3 protein product at one end of the bacteriophage particle. Infection of bacteria with this phage library allows very efficient library amplification. However, as the gene 3 protein is also required for bacteriophage assembly and infectivity, there are some limits to the size of protein that can be successfully expressed and displayed without introducing a bias during bacteriophage replication.

Ribosome display

Ribosome display[2] is a technology that enables the selection and evolution of large protein libraries completely *in vitro*. The only biological component required is a bacterial cell extract that contains the factors required for the translation of *in vitro*-generated transcripts encoding the protein sequences. Genotype and phenotype are linked through ribosomal complexes, consisting of messenger RNA (mRNA), ribosome, and encoded protein. After selection via the encoded protein, the isolated mRNAs are amplified by RT-PCR and can be freshly transcribed and translated for another round of selection.

Yeast display

Yeast display is a powerful new technique to study protein-protein interactions that has been developed by Prof. Dane Wittrup of the Massachussetts Institute of Technology[3]. This surface expression system can be used to express recombinant proteins on the surface of *S. cerevisiae* as a fusion with the a-agglutinin yeast adhesion receptor. Due to correct post-translational modification, processing and folding of mammalian proteins, this system provides an extremely attractive alternative to phage display for the discovery, characterization and affinity maturation of protein-ligand interactions, especially where full-length proteins are to be studied and not isolated protein fragments.

Display technology and magnetic sorting

In all of the display systems described, magnetic sorting with MACS® Technology enables an efficient and rapid isolation of interacting proteins. Orders of magnitude more events can be sorted than with other technologies and a very low non-specific enrichment is obtained compared to conventional panning techniques due to the highly efficient washing steps that are possible on the column. As a general strategy, the ligand can be labeled (for example biotinylated) and then after incubation with the target library, the interacting complex can be isolated using Streptavidin or Anti-Biotin MicroBeads as shown in figure 1. Alternatively, if the ligand is an antibody, µMACS Protein A or Protein G MicroBeads can be used.

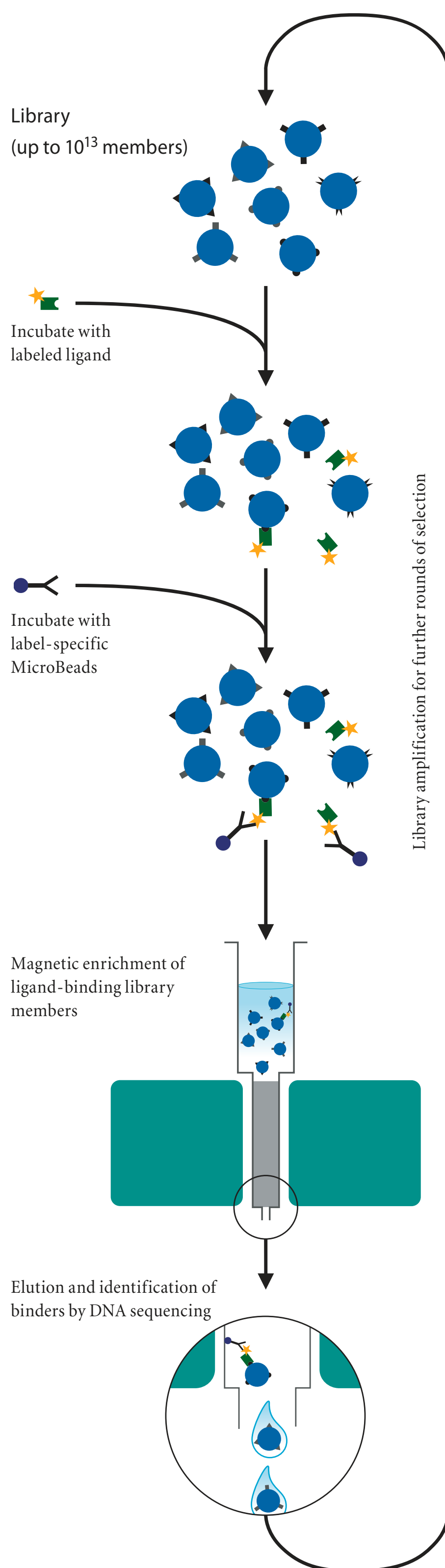

Figure 1 Screening a display library for ligand-binding activity using MACS Technology

Examples of library sorting using MACS® Technology

A phage library expressing human fab fragments was sorted with MACS® Technology using biotinylated human red blood cell proteins to isolate specific fab fragments against the Rhesus D protein[4].

Michael Feldhaus[5] of the Pacific Northwest National Laboratory has isolated antigen-specific human scFv fragments using a yeast display library with over 10^9 unique members. He has also developed a magnetic sorting protocol[6] on LS columns using biotinylated target molecules and a library of 10^{10} yeast cells. After sorting first with Streptavidin MicroBeads, the library is expanded and further sorted with Anti-Biotin MicroBeads. The enriched cell population is finally sorted and characterized by FACS.

Thomas Weichhart[7] incubated a *S. aureus* peptide library, expressed by ribosomal display, with serum from healthy volunteers. Incubation with µMACS Protein G MicroBeads then allowed the efficient isolation of antigenic peptides from the library after only 2–3 rounds of selection.

Further information and detailed protocols concerning display technologies and MACS® products can be found on our web site under: http://www.miltenyibiotec.com/index.php?site=macs-techno-display

High sorting capacities using MACS® Technology

Yeast display:
10^{10} yeast cells can be sorted in one LS Column[5,6]
Phage display:
10^{11} bacteriophage particles can be sorted in one µ Column[4]
Ribosome display:
10^{13} ribosome-mRNA complexes can be sorted in one µ Column[7]

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For sorting of molecular and phage libraries:

µMACS Protein A MicroBeads	# 130-071-001
µMACS Protein G MicroBeads	# 130-071-101
µMACS Streptavidin Kit	# 130-074-101

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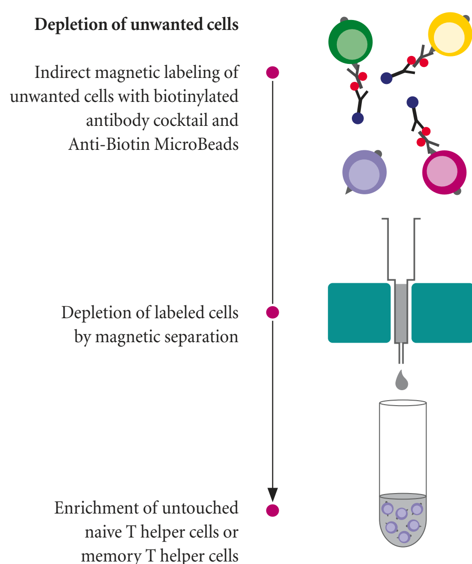
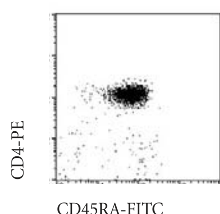
Isolation of T cell subsets: New kits for naive and memory T cells

The newly defined kits for T cell isolation extend the large panel of T cell products, providing the optimal tools for isolation of distinct human and murine T cell subsets.
For isolation of untouched human CD4+ naive and memory T cells we have developed the Naive CD4+ T Cell Isolation Kit and the Memory CD4+ T Cell Isolation Kit. Both kits are based on magnetic labeling and depletion of unwanted cells (see figure 1). Non-CD4+ T cells and either memory or naive T cells are indirectly magnetically labeled and then depleted by magnetic separation. Thereby pure unlabeled CD4+ naive or CD4+ memory cells are isolated in only 45 minutes.
The new Naive CD4+ CD62L+ T Cell Isolation Kit for mouse cell isolation contains all needed antibodies and MicroBeads to facilitate a faster and convenient isolation of naive CD4+CD62L+ T cells from lymphoid organs. The isolation of highly pure naive T cells is performed in just two steps (see figure 2) by depleting non-CD4+ cells, followed by positive selection of CD62L+ cells. (eh, mm)

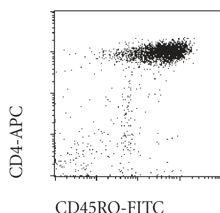
Isolation of naive or memory CD4+ T cells (human)



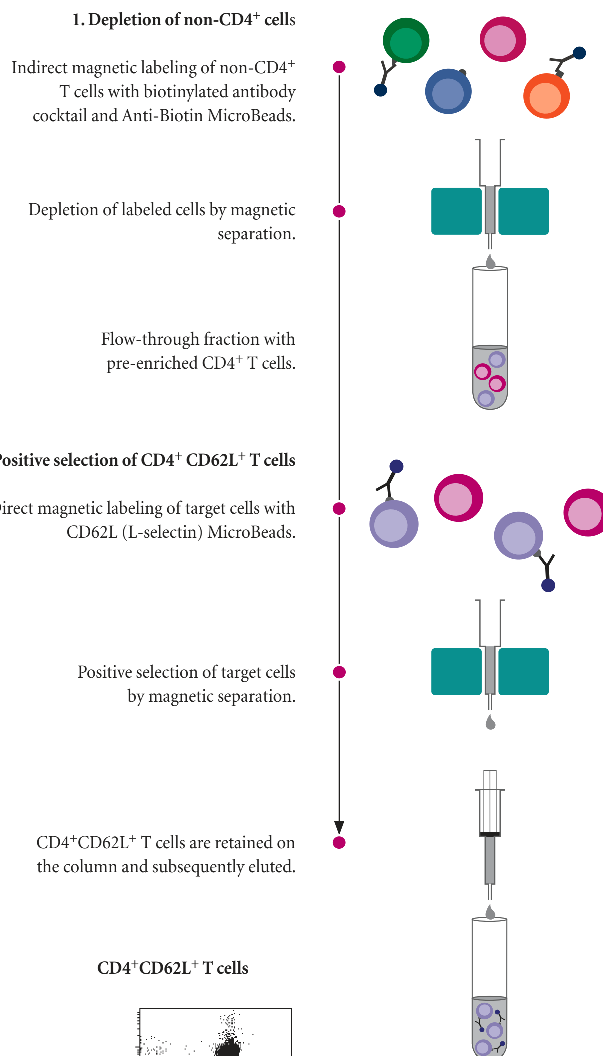
Isolated naive CD4+ T cells



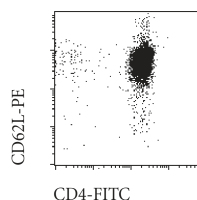
Isolated memory CD4+ T cells



Isolation of naive CD4+CD62L+ T cells (mouse)



CD4+CD62L+ T cells



Central players in immune suppression: interaction of regulatory T cells with dendritic cells

"Modulation of tryptophan catabolism by regulatory T cells"
Francesca Fallarino, Ursula Grohmann, Kwang Woo Hwang, Ciriana Orabona, Carmine Vacca, Roberta Bianchi, Maria Laura Belladonna, Maria Cristina Fioretti, Maria-Luisa Alegre & Paolo Pucetti (2003) Nat. Immunol. 4(12): 1206–12.

Research on CD4$^+$CD25$^+$ regulatory T cells (T regs) has generated widespread interest over the last years. However, many aspects of their biological properties are still controversial.
Some of the immune suppressive effects of T regs seem to be cell-contact dependent, relying on the direct interaction of T regs with responding T cells and antigen-presenting cells, whereas others appear to be mediated mainly by secretion of inhibitory cytokines.
Fallarino and co-workers investigated the role of CTLA-4 expressed on resting or activated T regs in priming of dendritic cells (DC) for tolerance induction. T regs isolated from lymph node cells of C57BL/6 mice were co-cultured with splenic DC to analyse the induction of suppressive effects in different experimental settings. The T regs used in this study, were isolated with the CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit or by flow cytometric cell sorting (in both cases the purity was: >90%). Splenic DC were isolated with CD11c MicroBeads (purity: >98%).
The authors provide evidence that CD4$^+$CD25$^+$ T regs can condition DC *in vitro* to express suppressive properties *in vitro* and *in vivo*. They propose distinct pathways by which T regs might affect the properties of DC. Depending on the stimuli used for activation of T regs, the process is supposed to be either CTLA-4-dependent, requiring additionally IFN-γ produced by DCs, or CTLA-4-independent, mediated by cytokines produced by regulatory T cells (IFN-γ, IL–10). (mm)

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CD11c MicroBeads, mouse	# 130-052-001
CD25 MicroBead Kit, mouse	# 130-091-072

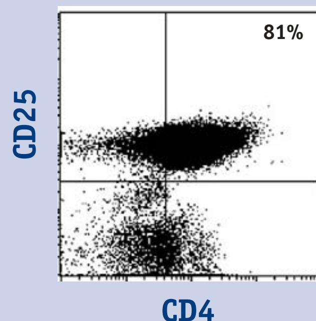
The answers
Test yourself!

1 1. Answer b) is correct.

2 Enrichment over two columns enhances the purity of CD4$^+$CD25$^+$ T cells. In humans, regulatory T cells highly express CD25 antigen whereas activated T cells express CD25 only dimly. Enrichment over two columns reduces the amount of activated T cells while regulatory T cells are enriched.

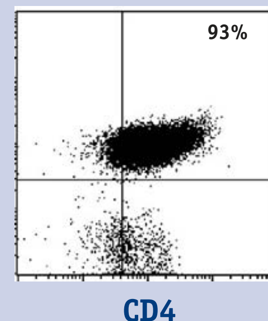
3 Answer a) is correct.

Human PBMCs after depletion of non-T cells and positive selection of CD25$^+$ cells over one MS Column (or program POSSEL on the autoMACS™ Separator).



4 Answer b) is correct.

Human PBMCs after depletion of non-T cells and positive selection of CD25$^+$ cells over two consecutive MS Columns (or program POSSELD on the autoMACS™ Separator).



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