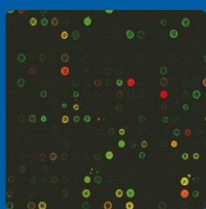


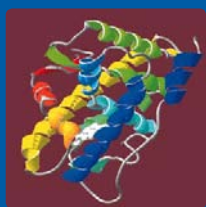
DENDRITIC CELLS

- MYELOID AND PLASMACYTOID CELLS IN LUNG CANCER TISSUE
- CCL19/21-INDUCED MIGRATION REQUIRES PROSTAGLANDIN E₂
- MOUSE DENDRITIC CELL SUBSETS



MACS'N'CHIPS

**PURIFIED CELLS FOR SUPERIOR RESULTS
IN GENE EXPRESSION PROFILING**



DNA-BINDING PROTEINS

**TARGET IDENTIFICATION
IN MAMMALIAN CELLS**

**RAPID PURIFICATION OF
PROKARYOTIC REGULATORY PROTEINS**



NEW PRODUCTS

T-REGS. - ISOLATION OF REGULATORY T CELLS

**MOUSE DENDRITIC CELL SUBSET
ISOLATION KITS**

**MICROBEADS FOR
NON-HUMAN PRIMATE CELLS**

CRTH2: NOVEL MARKER FOR TH2 CELLS

T CELLS IN CELLULAR THERAPY (CD8/CD25)

Dear MACS user,

This journal is entitled “MACS&more”, and for issue no. 7–2 this means: more pages, more research reports, more new products, more scientific perspectives.

As indicated on the cover, a major topic of this issue are the fascinating properties of dendritic cells, potent elicitors of the immune response – with two reports on **pages 12 and 14** and information about relevant MACS products on **page 3**.

The isolation of DNA-binding proteins, transcription factors for example, becomes an easy-to-solve task by using biotin-conjugated oligonucleotides as target sequences and the appropriate MicroBeads to magnetically isolate the protein-DNA complexes. The other way round, MACS® Technology also makes it possible to identify regulatory sequences on DNA by magnetic labeling of the binding protein – for more details see the reports on **pages 8 and 10**.

As mentioned above, “more” also means: more perspectives for your research. Miltenyi Biotec has broadened its product range to cover all aspects of downstream applications in cellular research and therapy – in addition to the wide range of high-quality products for the separation and isolation of specific cell types. Purification of cells prior to detailed analysis is a crucial requirement for successful research strategies – the report by Gerstmayer and colleagues provides an excellent example. They employed a combination of magnetic cell sorting and gene expression profiling to distinguish between cells from healthy donors and primary diagnosed Ankylosing Spondylitis patients – a promising way to understand how specific cell populations contribute to this inflammatory rheumatic disease.

Last, but not least, all information about MACS® (and more) products can be found on our web site – we look forward to your visit!

Your MACS team

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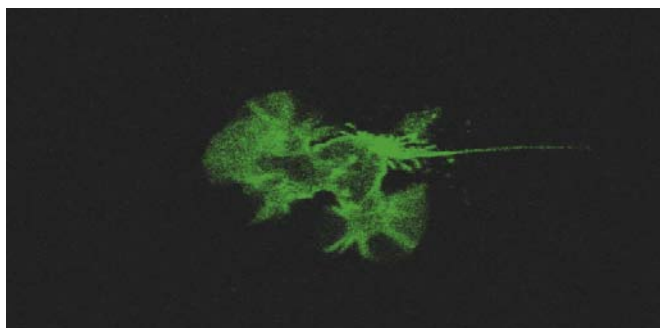
Mouse Dendritic Cell Subset Isolation Kits

In mice, the CD11c antigen is specifically expressed on the majority of dendritic cells (DC). Distinct subtypes of CD11c⁺ DC have been defined based on the expression of CD8, CD4, CD45R(B220) and anti-Ly-6G/C(Gr-1). In order to evaluate their functions and biological properties, highly pure and viable cell populations are needed.

With the new MACS® Dendritic Cell Isolation Kits, a fast and easy method for the isolation of these rare DC populations is provided.

For the isolation of the main conventional CD11c⁺CD45R(B220)⁻ DC from lymphoid organs the **CD8⁺ Dendritic Cell Isolation Kit** and the **CD4⁺ Dendritic Cell Isolation Kit** were developed. CD11c⁺CD45R(B220)⁺anti-Ly-6G/C(Gr-1)⁺ plasmacytoid DC can be easily isolated with the new **Plasmacytoid Dendritic Cell Isolation Kit**.

The kits are based on a two-step MACS separation strategy. DC are first enriched by depletion of unwanted cells and subsequently positively selected with direct MicroBeads. (mm)

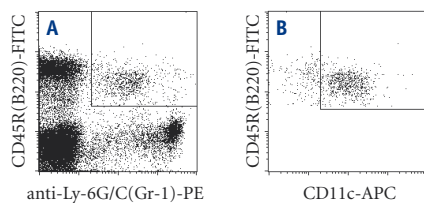


MACS-isolated CD8⁺CD11c⁺ dendritic cell from spleen stained with CD11c-FITC.

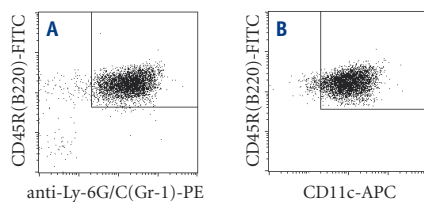
Dendritic cell detection and isolation

What type of dendritic cell are you looking for? Which product is best for its detection or isolation? Check the table below, or ask your "Web Decision Assistant" for help: <http://www.miltenyibiotec.com/dcda>

Spleen cells before separation



Isolated CD11c⁺CD45R(B220)⁺Ly-6G/C(Gr-1)⁺ plasmacytoid dendritic cells



A: cells are gated on CD45R(B220)⁺Ly-6G/C(Gr-1)⁺ cells;
B: only cells as gated in A are shown.

Isolation of plasmacytoid dendritic cells from a mouse spleen cell suspension using the Plasmacytoid Dendritic Cell Isolation Kit, an LD and two MS Columns, a MidiMACS and a MiniMACS Separator. The cells were stained with CD11c-APC, anti-Ly-6G/C(Gr-1)-PE and CD45R(B220)-FITC and analyzed by flow cytometry.

MACS® products

CD8⁺ Dendritic Cell Isolation Kit # 130-091-169
CD4⁺ Dendritic Cell Isolation Kit # 130-091-262
Plasmacytoid Dendritic Cell Isolation Kit # 130-091-263

DC type	Cell source	Detection by using antibody/kit against:	MACS® Separation	Analysis after MACS® Separation by using antibody/kit against:
HUMAN				
DC	Whole blood PBMC	Blood Dendritic Cell Enumeration Kit	Blood Dendritic Cell Isolation Kit II	Blood Dendritic Cell Enumeration Kit
Plasmacytoid DC (PDC, IPC, pre-DC2)	PBMC BM-MNC Leukapheresis*	BDCA-2	BDCA-4 (Neuropilin-1) Cell Isolation Kit	BDCA-2
	Tissue	BDCA-2	BDCA-2 Cell Isolation Kit	BDCA-4 (Neuropilin-1)
Myeloid DC1 (CD11c⁺ DC, DC1)	PBMC BM-MNC Leukapheresis*	CD1c (BDCA-1) CD19	CD1c (BDCA-1) Cell Isolation Kit	CD1c (BDCA-1) via Biotin CD19
Myeloid DC2	PBMC BM-MNC	BDCA-3	BDCA-3 Cell Isolation Kit	BDCA-3
Langerhans cells	Skin	CD1c (BDCA-1)	CD1a MicroBeads	CD1c (BDCA-1)
Monocytes for DC generation	Whole blood PBMC Leukapheresis*	CD14	CD14 MicroBeads Monocyte Isolation Kit II CD14 Whole Blood MicroBeads	CD14
Hematopoietic progenitor cells for DC generation	Whole blood PBMC BM-MNC	CD34	CD34 Progenitor Cell Isolation Kit	CD34
	Whole blood PBMC BM-MNC	CD133/1	CD133 Cell Isolation Kit	CD133/2
	after culture	CD1c (BDCA-1)	CD1a MicroBeads	CD1c (BDCA-1)
			*CliniMACS® plus System	
MOUSE				
DC	Spleen, LN, Thymus, BM	CD11c	CD11c MicroBeads	CD11c
Plasmacytoid DC	Spleen, LN, BM	CD11c B220 Ly-6C	Plasmacytoid Dendritic Cell Isolation Kit	CD11c B220 Ly-6C
CD4⁺ DC	Spleen	CD11c CD4	CD4 ⁺ Dendritic Cell Isolation Kit	CD11c CD4
CD8⁺ DC	Spleen, LN	CD11c CD8	CD8 ⁺ Dendritic Cell Isolation Kit	CD11c CD8
RAT				
DC	Spleen	OX62	Anti-DC (OX62) MicroBeads	OX62
CD4⁺ DC CD4⁻ DC	Spleen	OX62 CD4	Anti-OX62-FITC, Anti-FITC Multisort and CD4 MicroBeads	OX62 CD4

(kp)

MACS® Technology for the isolation of non-human primate cells

A panel of MACS® products designed for use in non-human primate research is now available. Pure cell populations can now be easily and quickly isolated and enriched by taking advantage of MACS Technology.

Due to their close relationship with humans, other primates play a significant role as animal models in medical research. Rhesus monkeys are most important for the development of vaccines against AIDS. They are not susceptible to HIV but to the simian HIV homologue, SIV. Valuable



Photography courtesy of Kathy West, California National Primate Research Center, Davis, CA, USA

information is gained by infecting vaccinated rhesus monkeys with SIV or with hybrid human/simian viruses (SHIV), which cause simian AIDS. Some vaccination strategies prevent an infection using neutralizing antibodies, while others focus on disease prevention by triggering killer-cell responses.

In order to determine a vaccination strategy that provides the best protection against the virus, obtaining reliable results is crucial and requires pure cell populations. The new MACS non-human primate products are optimized for the separation of cells from rhesus monkeys (*Macaca mulatta*). Please contact us for information about cross-reactivity with other non-human primates like cynomolgus (*Macaca fascicularis*), pigtail macaque (*Macaca nemestrina*) or chimpanzee (*Pan troglodytes*).

Rhesus monkey T cells, B cells, monocytes or NK cells can now be separated to excellent purity and recovery. Sample separation is performed over manual MACS Separators and Columns, or by using the autoMACS™ Instrument. The autoMACS Instrument is especially useful as it reduces handling of virus-infected samples to a minimum.

CD2 MicroBeads can be used for the separation of T cells and co-enrichment of rhesus monkey NK cells. T helper cells or cytotoxic T cells are separated according to the expression of the TCR co-receptors CD4 or CD8 using CD4 MicroBeads or the CD8 MicroBead Kit. CD45RA MicroBeads in combination with MultiSort Kits allow rapid separation of naive CD4⁺CD45RA⁺ T cells, whereas activated T and B cells are easily depleted by using CD25 MicroBeads. For the isolation of regulatory

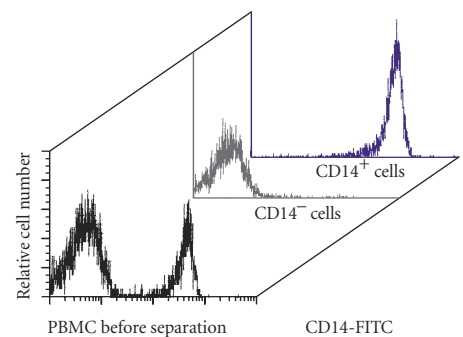


Figure 1: Separation of rhesus monkey PBMC using CD14 MicroBeads and an MS Column.

MACS® products for non-human primate cell separations

Rhesus monkey T cells	Order no.
CD2 MicroBeads, non-human primate	# 130-091-113
CD4 MicroBeads, non-human primate	# 130-091-102
CD8 MicroBead Kit, non-human primate	# 130-091-112
CD25 MicroBeads, non-human primate	# 130-091-095
CD45RA MicroBeads, non-human primate	# 130-091-092
Rhesus monkey NK cells	
CD16 MicroBeads, non-human primate	# 130-091-145
Rhesus-monkey B cells	
CD20 MicroBeads, non-human primate	# 130-091-105
Rhesus monkey monocytes	
CD11b MicroBeads, non-human primate	# 130-091-100
CD14 MicroBeads, non-human primate	# 130-091-097
CD56 MicroBeads, non-human primate	# 130-091-094

MACS® Antibodies for fluorescent staining of non-human primate cells

Antigen	Clone	Format	Order no.
CD1c	AD5-8E7	FITC	# 130-090-507
		PE	# 130-090-508
		APC	# 130-090-903
CD2	LT2	PE	# 130-091-115
CD4	M-T321	FITC	# 130-080-501
CD8	BW135/80	FITC	# 130-080-601
		PE	# 130-091-084
		APC	# 130-091-076
CD11b	M1/70.15.11.5	FITC	# 130-081-201
CD14	TÜK4	FITC	# 130-080-701
		PE	# 130-091-242
		APC	# 130-091-243
CD20	LT20	FITC	# 130-091-108
		PE	# 130-091-109
		APC	# 130-091-024
CD25	4E3	PE	# 130-091-024
CD56	AF12-7H3	PE	# 130-090-755
		APC	# 130-090-843

Antibodies are tested to recognize cell-surface antigens of rhesus monkey and cynomolgus monkey cells.

T cells from rhesus monkey PBMC, depletion of B cells is followed by positive selection with CD25 MicroBeads. B cell depletion as well as positive selection are performed by using CD20 MicroBeads.

CD11b MicroBeads or CD14 MicroBeads can be used to isolate monocytes from rhesus monkey PBMC (fig. 1). Human CD14 MicroBeads are frequently used to generate human monocyte-derived DCs, and similar protocols have been applied to generate rhesus monkey DCs by culturing them with GM-CSF and IL-4 [1]. In contrast to human cells, rhesus monkey monocytes but not NK cells are reported to express CD56 [2]. Thus, rhesus monkey monocytes can also be isolated with CD56 MicroBeads.

Rhesus monkey NK cells are characterized by a CD16⁺CD8⁺CD2⁺CD3⁻ phenotype. A specific NK cell marker still remains to be defined. Separation of NK cells is achieved by positive selection with CD16 MicroBeads. However, CD16 is also expressed on a subset of rhesus monkey monocytes which will be co-enriched with the NK cells. The co-isolated monocytes can be depleted in a preceding step using CD14 MicroBeads or CD56 MicroBeads if their presence might interfere with subsequent NK cell experiments.

MACS Indirect MicroBeads enable cell separations with any antibody of non-human primate reactivity. In a first step, cells are labeled with the primary antibody, which can be fluorochrome-conjugated, biotinylated or unconjugated. Magnetic labeling of the primary antibody is achieved in a second step using MACS Anti-Fluorochrome, Anti-Biotin or Anti-Immunoglobulin MicroBeads.

Finally, a variety of MACS antibodies with non-human primate cross-reactivity is available for flow cytometric analysis or quality control of MACS separations.

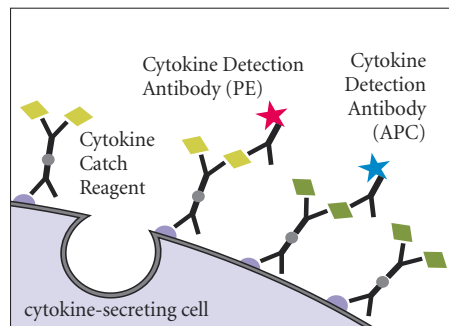
Please note that the non-human product line is being constantly expanded. Visit our website regularly to see what's coming up. (dh) ■

References

1. Barratt-Boyes, S. M. *et al.* (2000) *J. Immunol.* 164: 2487–2495.
2. Carter, D. L. *et al.* (1999) *Cytometry* 37: 41–50.

Mouse Cytokine Secretion Assay – Detection Kits (APC)

Now murine antigen-specific T cells can be monitored for secretion of IFN- γ , IL-2, IL-5 and IL-10 using the bright blue color of allophycocyanin (APC, emission at about 680 nm). Mouse Cytokine Secretion Assay – Detection Kits (APC)



can be combined with another Mouse Cytokine Secretion Assay – Detection Kit (PE) to monitor coexpression of two different cytokines. The new kits may also be used in combination with PE-conjugated tetramers to obtain functional information about the antigen-specific T cells. Cytokine-secreting cells can be analyzed simultaneously for the secretion of two different cytokines, combining the Mouse Cytokine Secretion Assay – Detection Kit (APC) with another Mouse Cytokine Secretion Assay (PE). This provides information on the production of different cytokines by single cells. (eh) ■

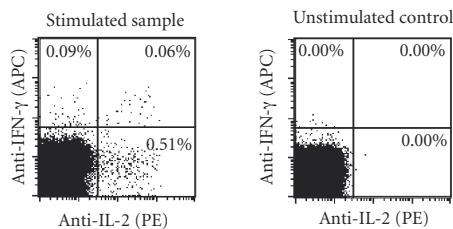


Figure 1: BALB/c mice were immunized i.p. with KLH (keyhole limpet hemocyanin) in Incomplete Freund's Adjuvant and Pertussis Toxin. After 21 days, the spleen cells were incubated for 15 hours with or without KLH. The responding cells were stained according to secretion of IL-2 and IFN- γ using the Mouse IL-2 Secretion Assay – Detection Kit (PE) in combination with the Mouse IFN- γ Secretion Assay – Detection Kit (APC). Cytokine secretion by viable CD4⁺ lymphocytes is displayed. (eh) ■

MACS® products

Detection Kits (APC) for:

Mouse IFN- γ Secretion Assay	# 130-090-984
Mouse IL-2 Secretion Assay	# 130-090-987
Mouse IL-5 Secretion Assay	# 130-091-174
Mouse IL-10 Secretion Assay	# 130-090-939

Mouse IL-5 Secretion Assays – Detection Kits

We have now further extended the Mouse Cytokine Secretion Assay products to include the Mouse IL-5 Secretion Assays. These kits were developed for detection and optional magnetic enrichment of IL-5-secreting cells, enabling advanced research on Th2 cell development and commitment. The Mouse IL-5 Secretion Assays will expand research opportunities on animal models, in such fields as allergy and parasitic infections.

IL-5 is a cytokine predominantly secreted by CD4⁺ T cells. It promotes growth and activation of eosinophils and has been implicated in a range of allergic reactions. It mediates immune responses against parasites, induces the proliferation of activated B cells and promotes differentiation of cytotoxic T cells from thymocytes.

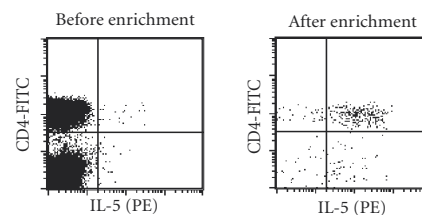


Figure 1: BALB/c mice were immunized i.p. with KLH (keyhole limpet hemocyanin) in Incomplete Freund's Adjuvant and Pertussis Toxin. After 15 weeks, the spleen cells were restimulated for 15 hours with KLH. The responding cells were stained and magnetically enriched, according to secretion of IL-5 using the Mouse IL-5 Secretion Assay – Cell Enrichment and Detection Kit. IL-5 secretion by viable lymphocytes is displayed. (eh) ■

Different Kit configurations are available for the Mouse IL-5 Secretion Assay. The Detection Kits include either PE or APC-conjugated Detection Antibodies for flow cytometric analysis IL-5 secreting cells. These kits can also be used for analysis of cytokine coexpression. The Cell Enrichment and Detection Kit includes PE-conjugated IL-5 Detection Antibody and Anti-PE MicroBeads for the magnetic isolation of IL-5-secreting cells. (eh) ■

MACS® products

Mouse IL-5 Secretion Assay Kits:

– Cell Enrichment and Detection Kit (PE)	# 130-091-175
– Detection Kit (PE)	# 130-091-166
– Detection Kit (APC)	# 130-091-174

CD8 and CD25 MicroBeads: Novel tools for selection or depletion of T cells in cellular therapy

T cells play critical roles in the regulation of immune responses, which might be wanted but also be a threat. For instance, T cells are held responsible for causing autoimmune diseases and graft-versus-host disease (GvHD), which is a major and often fatal complication in the transplant setting. Thus, the depletion of T cells is increasingly performed in the allogeneic transplantation setting, where the alloreactive, cytotoxic T cells are depleted from the graft or donor lymphocyte infusions (DLI), respectively, thereby diminishing the GvHD while not ablating the graft-versus-tumor (GvT) effect. Miltenyi Biotec has developed CliniMACS®

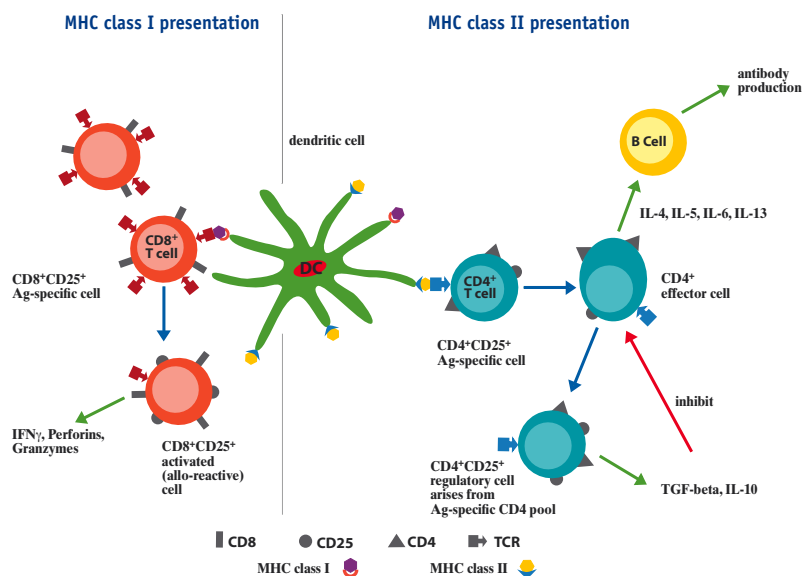
CD8 and CD25 MicroBeads for research use which allow for selection and depletion of different T cell subsets thus providing the basis for a variety of different protocols. Manufacturing and quality control follow cGMP guidelines.

CD8, the co-receptor for MHC class I molecules, is expressed strongly on cytotoxic T cells and dimly on a subset of NK cells. CliniMACS® CD8 MicroBeads for research use allow for the positive or negative selection of cytotoxic T cells. One vial contains 7.5 mL sterile, non-pyrogenic solution for labeling of up to 2×10^9 CD8⁺ cells from up to 20×10^9 total cells.

CD25, the low-affinity interleukin-2 receptor alpha chain (IL-2R α), is expressed on activated T and B cells. In addition, CD25 is highly expressed on regulatory T cells. CliniMACS® CD25 MicroBeads are suitable for positive selection or depletion of activated and alloreactive T cells.

In conjunction with e.g. CliniMACS CD8 MicroBeads for research use or CliniMACS CD19 MicroBeads for research use, to deplete unwanted CD25 expressing populations, they can be used for the enrichment of regulatory T cells.

One vial contains 7.5 mL sterile, non-pyrogenic solution for labeling of CD25 cells from up to 40×10^9 total cells. (ji)



References:

- CD8: Baron, F. *et al.* (2002) *Haematologica* 87: 78-88.
 Dudley, M. E. *et al.* (2002) *Science* 298: 850-854.
 Savoldo, B. *et al.* (2002) *Blood* 100: 4059-4066.
 CD25: Solomon, S. *et al.* (2002) *Cytotherapy* 4: 395-406.
 Fehse, B. *et al.* (2000) *Br. J. Haematol.* 109: 644-651.
 Amrolia, P. *et al.* (2003) *Blood* 102: 2292-2299.
 Jonuleit, H. *et al.* (2002) *J. Exp. Med.* 196: 255-260.

MACS® products

CliniMACS products for research use*

- CliniMACS CD8 MicroBeads for research use # 308-01
 CliniMACS CD25 MicroBeads for research use # 325-01

*In the United States, CliniMACS® products for clinical use are available only under an approved Investigational Device Exemption (IDE).



Now available from Miltenyi Biotec!

The complete portfolio of quality cellular therapy products from

Baxter Oncology

- **Isolex 300i** Magnetic Cell Selection System (Version 2.5)
- **CytoMate** Cell Processing System
- **Cryocyte** Preservation Bags
- **Lifecell** Culture Bags

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Anti-CRTH2 conjugates: Novel tools to detect and isolate Th2 cells

CRTH2, a receptor for prostaglandin D₂, is a novel surface marker which allows identification of T helper subtype 2 (Th2) cells. So far, Th2 cells could only been distinguished by their production of cytokines, like IL-4, IL-5, or IL-13.

On CD4⁺ T cells, CRTH2 is exclusively expressed on Th2 cells, and is not present on Th1 cells. The CRTH2 antigen is also highly expressed on peripheral blood basophils and eosinophils, and on a small population of CD8⁺ T cells. CRTH2 was also shown to be expressed by a subpopulation of dendritic cells.

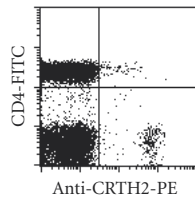
PE-conjugated and biotinylated Anti-CRTH2 antibodies allow the sensitive detection of CRTH2 cells by flow cytometry or microscopy.

The Anti-CRTH2 MicroBead Kit enables CRTH2⁺ cells to be isolated magnetically. Th2 cells can be isolated by depletion of non-T helper cells using the CD4⁺ T Cell Isolation Kit II followed by positive selection of CRTH2⁺ cells with the Anti-CRTH2 MicroBead Kit (see figure). (eh)

Reference:

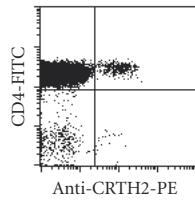
Cosmi et al. (2000) Eur. J. Immunol. 30: 2972–2979.

PBMC before separation

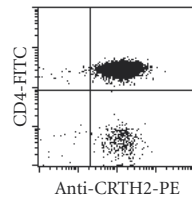


Isolation of human peripheral blood CD4⁺CRTH2⁺ cells using the CD4⁺ T Cell Isolation Kit II and the CRTH2 MicroBead Kit, an LD and two MS Columns.

Pre-enriched CD4⁺ T cells after depletion of non-CD4⁺ T cells



Isolated CD4⁺CRTH2⁺ Th2 cells



MACS® products

Anti-CRTH2-PE	# 130-091-238
Anti-CRTH2-Biotin	# 130-091-239
Anti-CRTH2 MicroBead Kit	# 130-091-274
CD4 ⁺ T Cell Isolation Kit II	# 130-091-155

Challenge for Imagineers 2004

A Cell.
Magnetized.
Purified.
Photogenic?

for online information about Miltenyi Biotec's cell imaging contest "Challenge for Imagineers" see

www.miltenyibiotec.de/imagineers

Miltenyi Biotec



MACS New T Cell Products

T-REGS.

Isolation of CD4⁺CD25⁺ Regulatory T Cells

- For human, murine, non-human primate cells
- Optimal yields and purities
- Easy and convenient handling

Miltenyi Biotec

www.miltenyibiotec.com



Regulatory proteins that specifically bind to a particular DNA sequence play a key role in signal transduction. If the putative operator sequence is known, such transcription factors can easily be purified with μ MACS™ Streptavidin MicroBeads.

Rapid purification of a prokaryotic regulatory protein with μ MACS™ Streptavidin MicroBeads



Kathryn A. Kalivoda^a, Kirt W. Braun^b, Eric R. Vimr^a

^aDiv. of Microbiology and Immunology, Dept. of Pathobiology, University of Illinois, Urbana, IL

^bMiltenyi Biotec Inc., Auburn, CA

Introduction

The availability of overexpression vectors allowing the addition of in-frame fusion tags to overproduced polypeptides facilitates protein purification by a variety of affinity chromatography techniques. In many cases, the tagged proteins retain biological activity and are useful for functional analyses. However, in the case of DNA binding proteins, tag placement or composition may be critical as it could interfere with operator recognition, effector binding, oligomerization or information transfer (allostery) [1]. Although the tags may be removed or at least be shortened before attempting further protein characterization, it may be desirable to

of complete microbial genomic DNA databases, a sequence of 10 amino acids will probably be sufficient in most cases to identify a previously unknown regulatory protein. Here we describe a rapid and simple technique for purifying native DNA binding proteins using μ MACS™ Streptavidin MicroBeads.

Material and methods

Figure 1 shows the basic protocol for purifying DNA binding proteins using MACS® Technology. Biotinylated operator DNA is incubated briefly with the protein extract followed by the addition of μ MACS Streptavidin MicroBeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to bind the protein-DNA complexes. This mixture is applied to a μ Column where the MicroBeads, biotinylated target DNA and specifically bound protein(s) are retained in the magnetic field of a μ MACS Separator (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Non-specifically bound molecules are removed by stringently washing the column, and the bound target protein is eluted in conditions under which the biotinylated DNA remains in the column. After the initial binding to target DNA, the entire purification scheme can be completed in 10 minutes, yielding pure DNA binding protein for subsequent procedures such as protein sequencing and mobility gel shift analysis.

To verify the experimental concept shown in figure 1, a PCR fragment encoding the complete *nanR* (*yhkK*) structural gene was ligated into the arabinose-inducible vector pBAD23 [3] to generate plasmid pSX675. *E. coli* strain JM109 harboring pSX675 was grown in 100 mL of Luria-Bertani media and induced by the addition of L-arabinose. After 3 hours of induction with L-arabinose *E. coli* JM109/pSX675 were harvested by centrifugation at 3,000×g for 10 minutes and resuspended in 1 mL of binding buffer. Comparable results were obtained when 10 mM Tris-HCl, pH 8.0 was used instead of binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5% (v/v) glycerol, 1 mM DTT, 0.01% (v/v) Triton X-100, 100 mM NaCl). Cells were disrupted by sonication. Cell membranes and debris were removed by centrifugation at 50,000×g.

The target DNA for NanR purification was amplified by PCR from plasmid pNan1-3 [4] using synthesized and gel-purified forward (5'-CGACGCAGACTCGCTTTATC-3') and reverse (biotin-5'-GGCACGCTTTCGGTCAGACC-3') primers. The forward primer begins 39 bp from the translational stop codon of *nanR*, the reverse primer 86 bp from the translational start codon of *nanA*, with a biotin group added at the 5' end. These primers amplify a 246-bp DNA fragment that includes the *nanA* promoter and NanR binding sites. The soluble protein extract was incubated at room temperature for 10 minutes with the biotinylated target DNA to form the NanR-DNA complex. 100 μ L of μ MACS Streptavidin MicroBeads were added to the suspension containing the NanR-DNA complexes.

Prior to magnetic separation, the μ Column was placed in the magnetic field of the μ MACS Separator and equilibrated with 100 μ L of the Protein Equilibration Buffer provided in the μ MACS Streptavidin Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After rinsing the column with 2×100 μ L of binding buffer the reaction mixture was applied to the column. The solution was allowed to pass through the column by gravity and the magnetically labeled NanR-DNA complexes were retained on the column. The column was then rinsed with 6×100 μ L of binding buffer to remove non-specifically interacting molecules. Elution of NanR was accomplished with 150 μ L of binding buffer containing 1M NaCl.

The molecular identity of NanR was confirmed by automated Edman degradation of the N-terminus at the Protein Sequencing Facility at the University of Illinois at Champaign-Urbana using the sequencer Procise 494HT

MACS® products

μ MACS Streptavidin Kit	# 130-074-101
μ MACS Streptavidin Starting Kit	# 130-074-287
μ Columns	# 130-042-701
μ MACS Separation Unit	# 130-042-602

begin with a native polypeptide when working with previously uncharacterized DNA binding proteins. If the putative operator sequence is known, it should be possible to purify unknown regulatory proteins in sufficient quantities (10 picomol) for sequencing by Edman chemistry, thus facilitating identification of the regulator by a reverse genetic approach. With the burgeoning

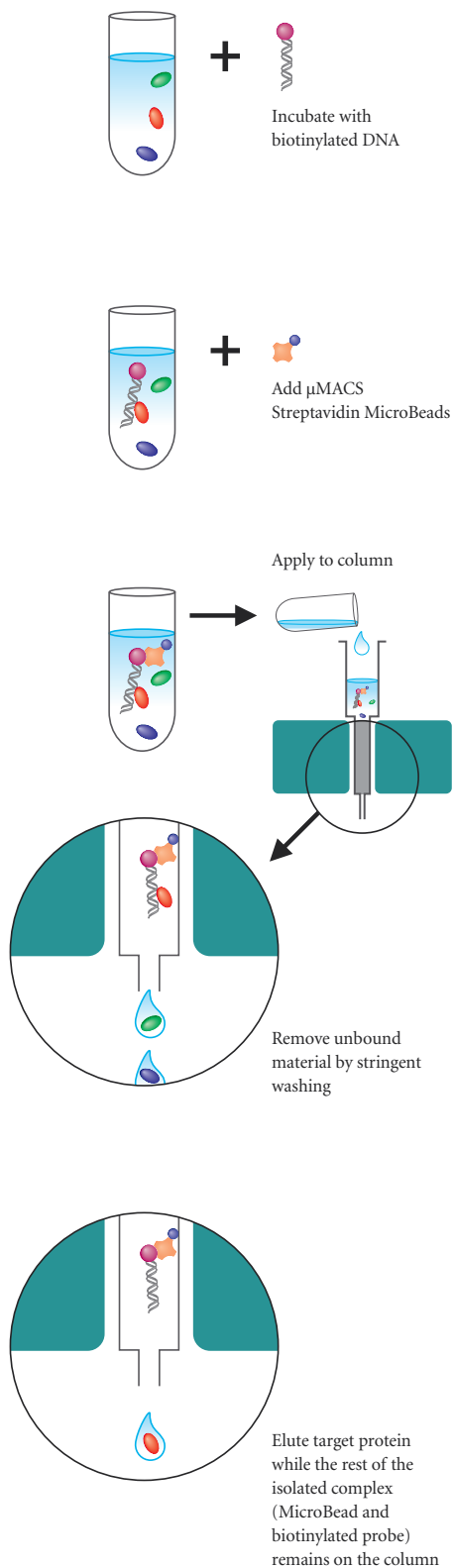


Figure 1: Isolation of DNA binding proteins with biotinylated probes. Biotinylated target DNA (red) is incubated with an extract containing the DNA binding protein. Addition of μ MACS Streptavidin MicroBeads (orange) bind the complexes and when this mixture is applied to a μ Column the complexes are retained in the paramagnetic field of the separator (blue). After removing unbound material, target protein is eluted in a high-salt buffer

(PerkinElmer ABD, Foster City, CA).

To determine whether the microbead method could be used to purify the basal amount of NanR in *E. coli*, we repeated the isolation with a soluble extract of strain MC4100. MC4100 (3 L) was grown to an A_{600} of 0.8 and the cells harvested by centrifugation at $3,000\times g$ for 10 minutes. The pelleted cells were resuspended in 27 mL 10 mM Tris-HCl, pH 8.0, and subjected to French Pressure Cell disruption at 10,000 psi. Membrane fragments and cell debris were removed by an initial centrifugation at $12,000\times g$ for 10 minutes followed by ultracentrifugation as described above. Solid ammonium sulfate was added to the supernatant to 60% saturation at 4 °C. The protein precipitate was collected by centrifugation at $12,000\times g$ for 15 minutes as above and then resuspended in 3 mL 10 mM Tris-HCl, pH 8.0. The solution was dialyzed for 8 hours against 4×1 L of 10 mM Tris-HCl, pH 8.0.

Results and discussion

NanR eluted in the 2nd through 5th drop that emerged from the column. From the first purification scheme, where an arabinose-inducible vector was used, a total of 9.5 μ g of pure NanR protein was isolated (fig. 2, lane 3). Automated Edman degradation of the N-terminus showed that, with exception of the predicted N-terminal methionine residue, which *in vivo* is most likely removed from NanR, the succeeding 9 amino acid residues perfectly matched the predicted NanR N-terminal primary sequence [(M)NAFDSQTED].

For the isolation of basal NanR, three column purifications representing the original 3 L of culture were performed (1 mL extract/column) and the 300 μ L of eluted protein were concentrated 5-fold prior to denaturing polyacrylamide electrophoresis (fig. 2, lane 4), demonstrating isolation of a protein with the same relative mobility as overproduced NanR. The isolated protein was confirmed to be NanR by gel mobility shift assay (data not shown) [2]. It was determined that 3.36 μ g of pure NanR protein were isolated, representing basal expression of NanR. The additional protein band at approximately 19 kDa may be a contaminant or a breakdown product of NanR.

The innovative technology from Miltenyi Biotec combined with the specificity of DNA binding proteins for corresponding target DNA sequences provides a powerful new tool for identifying uncharacterized transcription regulators. A putative operator sequence is all that is necessary to isolate proteins that potentially regulate gene expression. The

specificity of the protein/DNA interaction and stringent wash buffers are sufficient to ensure that any protein isolated is likely to be specific for the particular DNA target sequence. μ MACS MicroBead purification should facilitate the isolation of uncharacterized DNA binding proteins, and work is in progress to identify novel regulators of sialic acid metabolism in other bacterial species.

We are grateful to Eric Deszo for help with artwork. This work was supported by National Institutes of Health Grant AI42015 to E.V. ■

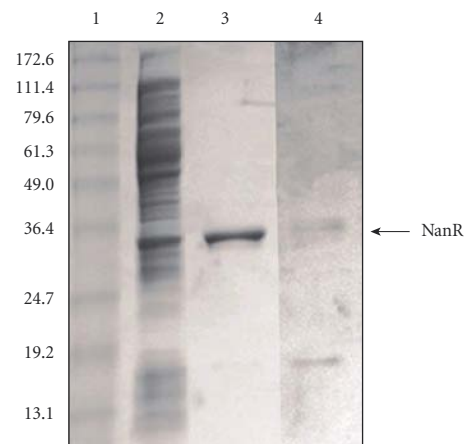


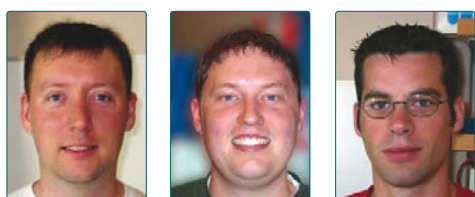
Figure 2: Purification of NanR. Lanes show Coomassie brilliant blue-stained samples fractionated by denaturing polyacrylamide gel (4–20%) electrophoresis. Lane 1, Benchmark Prestained Markers (Invitrogen) with sizes in kDa given on the left; lane 2, soluble protein fraction from JM109 harboring pSX675 after induction with 0.2% L-arabinose (30.9 μ g); lane 3, purified recombinant NanR (3.5 μ g); lane 4, NanR from *E. coli* MC4100 (1.68 μ g).

References

- Buning, H. *et al.* (1996) *Anal. Biochem.* 234: 227–230.
- Carey, J. (1991) *Gel retardation. Meth. Enzymol.* 208: 103–112.
- Guzman, L. M. *et al.* (1995) *J. Bacteriol.* 177: 4121–4130.
- Plumbridge, J. and Vimr, E. (1999) *J. Bacteriol.* 181: 47–54.

As reported by David Mulholland and co-workers, MACS® Column Technology is the method of choice for co-immunoprecipitation, chromatin immunoprecipitation, and isolation of DNA-binding proteins. It allows for faster as well as more efficient isolations when compared to standard immunoprecipitation techniques.

Enhanced target identification of the androgen receptor in mammalian cells and tissue



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Introduction

Androgens and dihydrotestosterone (DHT) control the development, differentiation, and function of male reproductive tissue. Androgen also affects other organs and tissues, such as skin, brain and bone marrow. The principle action of androgen is to regulate gene expression via the androgen receptor (AR), which belongs to the superfamily of nuclear receptors. Nuclear receptors are ligand-inducible transcription factors that mediate the signals of a broad variety of fat-soluble hormones.

In this report, super-paramagnetic μ MACS Protein G MicroBeads together with MACS Column Technology were used to immunoprecipitate activated AR as well as the associated ligand Beta-Catenin. Further, the Protein G MicroBeads were used for chromatin immunoprecipitation (ChIP) to identify the binding site of AR. Using μ MACS Streptavidin MicroBeads we isolated a specific DNA-bound protein, which was identified as a phosphoprotein.

MACS products

μ MACS Protein G MicroBeads	# 130-071-101
μ MACS Streptavidin MicroBead Kit	# 130-074-101
μ Columns	# 130-042-701
μ MACS Starting Kit	# 130-042-602

Materials and methods

Immunoprecipitation from LNCaP cultured cells. LNCaP cells were subjected to lysis using either RIPA buffer (0.1 % SDS, 150 mM NaCl, 50 mM Tris pH 8.0) or NP40 lysis buffer (1 % NP40, 150 mM NaCl, 50 mM Tris pH 8.0). A total of 500 μ g of cell lysate per IP were used to immunoprecipitate AR using anti-AR antibodies (Pharmingen) with μ MACS Protein G Microbeads (Miltenyi Biotec) or Protein A/G plus agarose beads (Santa Cruz). Proteins were detected by Western blotting using anti-AR and anti-Beta-Catenin (Santa Cruz) antibodies.

Chromatin immunoprecipitation from LNCaP cultured cells and tumors. LNCaP prostate cancer cells, grown in vitro, were treated with 10 nM R1881 (DHT) for 48 hours, were crosslinked with 1 % formaldehyde, and placed on a shaker for 10 min at room temperature. Excised tumors were minced in cold phosphate buffer followed by immersion fixation with formaldehyde (60 min) for 15 min at 37 °C. In both instances, Chromatin/protein crosslinking was stopped by adding 0.125 M glycine and rocking for 5 min. Cultured LNCaP cells were scraped from the dish or minced tumor cells were collected and centrifuged in cold PBS (protease inhibitors optional) and resuspended in cold cell lysis

buffer with inhibitors (5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % NP40, Roche complete protease inhibitors) for 10 min. Nuclei were pelleted by centrifuging at 5,000 rpm for 5 min. The cytosolic portion was removed and resuspended in 300 μ L nuclear lysis buffer with inhibitors (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1 % SDS, protease inhibitors) for 10 minutes. Chromatin was sonicated to an average of 500 bp (assessed by gel) by using 5 x 10 seconds pulses at ~30 % max. setting on a sonicator in a 15 mL conical tube. In order to reduce SDS concentration to ~0.1 %, lysate was diluted ten-fold (i.e. 150 μ L of lysate to 1500 μ L) with IP dilution buffer (0.01 % SDS, 1 % NP40, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167 mM NaCl). To clear non-specific binding proteins and DNA, supernatants were pre-cleared for 30 min using 20 μ L of the respective protein G beads and sheared salmon sperm DNA. Supernatants were then microfuged for 5 min at 15,000 rpm and lysate was harvested. Either 2.5 μ g of anti-AR antibody or an equivalent amount of non-immune control IgG (Santa Cruz) were added to the lysate and inverted overnight. The lysate was combined with 20-30 μ L of μ MACS Protein G MicroBeads and incubated for 1 hour.

The complete volume of cell lysate/ μ MACS MicroBeads was pipetted onto a MACS Column, allowed to flow through, and washed 4x500 μ L with ChIP wash buffer (100 mM Tris-Cl pH 8.0, 500 mM LiCl, 1 % NP40, 1 % deoxycholic acid). ChIP product was then eluted by applying 2x150 μ L of ChIP elution buffer (50 mM NaHCO₃, 1 % SDS) to each column. To reverse the crosslinking reaction, 1 μ L of RNase A (10 μ g/ μ L) and 5 M NaCl (0.3 M final) was added to the eluate and incubated in a 67 °C waterbath for 4 hours. DNA was precipitated by adding 2.5 volumes of ethanol and incubation at -20 °C overnight. DNA was concentrated by microfuging at 14,000 rpm at 4 °C for 15-20 min, washed with ethanol, and resuspended in 175 μ L of TE. Residual salts were removed by a phenol/chloroform clean-up step. 750 μ L of EtOH and 5 μ g of glycogen (painted) were added and suspensions were again precipitated overnight at -20 °C. Samples were microfuged at 14,000 rpm for 20 min (4 °C) and DNA was resuspended in 30 μ L of TE.

PCR was performed on samples using 1-2 μ L of sample for 35 cycles at 58 °C. For PCR, oligonucleotide primers specific for the prostate-specific antigen (PSA) gene were used: 5'-TCTGCCTTTGTCCCCTAGAT-3', 5'-AACCTTCATCCCCAGGACT-3' and others within the gene for further verification of pull-down specificity.

Results

In order to evaluate the efficiency of μ MACS MicroBeads, we used prostate cancer cells (LNCaP) cultured *in vitro*. LNCaP cells were treated with dihydrotestosterone (DHT), the physiological ligand for the androgen receptor (AR), to promote activation of AR transcription and to complete nuclear translocation of the receptor within 60 minutes. Utilizing the μ MACS Protein G MicroBeads, we set out with two initial goals in mind: (1) to compare the ability of the MicroBeads with conventional Protein A/G agarose beads in immunoprecipitation of the AR and (2) to co-immunoprecipitate a known AR binding partner such as Beta-Catenin¹.

Immunoprecipitation results (fig. 1) showed that using μ MACS Protein G MicroBeads at half the bead volume of the agarose beads, we were able to immunoprecipitate 3-4 times more AR. Under NP-40 lysis conditions, we validated that the μ MACS MicroBeads were also more efficient in isolating Beta-Catenin (fig. 2).

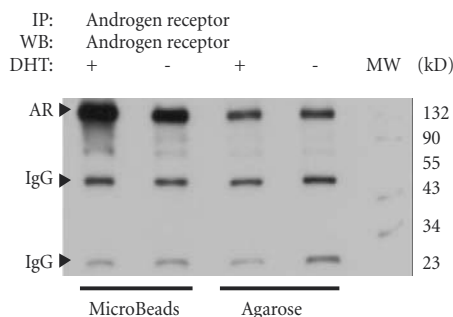


Figure 1: Western Blot of immunoprecipitated AR using anti-AR antibody. AR was immunoprecipitated from DHT-stimulated (lane 1, 3) or unstimulated LNCaP cells (lane 2, 4) with μ MACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4).

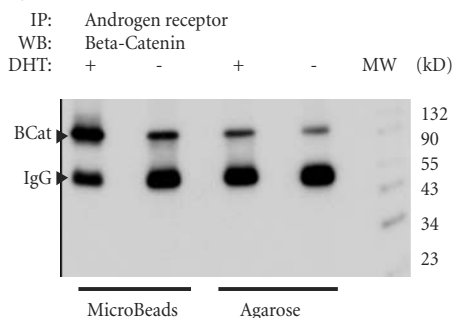


Figure 2: Western Blot of AR-co-immunoprecipitated Beta-Catenin using anti-Beta-Catenin antibody. AR was immunoprecipitated from DHT-stimulated (lane 1, 3) or unstimulated LNCaP cells (lane 2, 4) with μ MACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4).

The AR/Beta-Catenin interaction is known to be ligand-sensitive as is evident from the result obtained using the agarose beads. Immunoprecipitation with μ MACS Protein G MicroBeads

showed a four- to five-fold increase in ligand dependency, as opposed to a two-fold increase as seen with the agarose beads. The ability for Protein G MicroBeads to isolate complexes prompted us to evaluate the efficiency of the MicroBeads with more difficult isolation techniques.

Chromatin immunoprecipitation (ChIP), a technique that isolates chemically fixed protein/DNA complexes by standard immunoprecipitation procedures, is a powerful tool for studying protein-DNA interactions. A fundamental question in prostate cancer research revolves around the discovery of novel transcriptional regulators that may be directly bound to promoter regions of the PSA gene. Assaying for transcriptional regulator/DNA complexes in solid prostate tumors is likely to be an area of abundant discovery. After isolation of the complexes, the cross-linking is reversed, the DNA is ethanol precipitated and subsequently identified by PCR. In the presence of androgen, the AR translocates to the nucleus and binds cooperatively to promoter regions. LNCaP cells not exposed to androgen should theoretically not have large amounts of the target PSA gene bound to the AR. One of the technical challenges of performing ChIP experiments is to produce a clear positive PCR signal while minimizing the presence of non-specific PCR products. In work done previously, we used a panel of AR antibodies in combination with agarose beads and were consistently able to generate a moderate level of PCR product using primers targeted towards the AR binding regions of the PSA gene in androgen-induced LNCaP cells.

However, PCR products were also amplified from the negative control with yields approaching 60 % of those of the androgen-induced samples. When we used μ MACS MicroBeads, we not only observed enhanced immuno-precipitation under fixed conditions but we also observed reduced levels of non-specific PCR product (fig. 3).

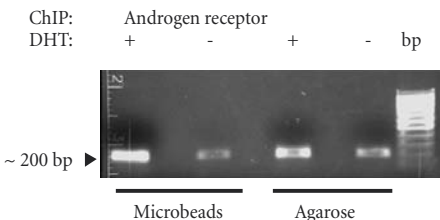


Figure 3: PCR reaction of the PSA gene using ChIP obtained DNA from cultured cells. Immunoprecipitation was carried out with μ MACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4). Anti-AR antibody (lane 1, 3) or as a control anti-IgG (lane 2, 4) were used for ChIP.

A more difficult extension of ChIP is to precipitate target genes from tissues. Using LNCaP cells, that were grown in culture, injected subcutaneously to the dorsal side of mice and harvested as a capsulated tumor, we evaluated the use of the μ MACS MicroBeads. When using agarose beads, we had considerable difficulty reducing the amount of non-specific PSA gene product signal in the IgG negative control (fig. 4).

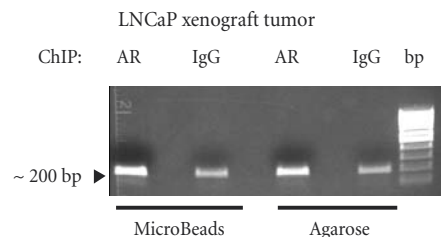


Figure 4: PCR reaction of the PSA gene using ChIP obtained DNA from tumor cells. Immunoprecipitation was carried out with μ MACS Protein G MicroBeads (lanes 1-2) or with Protein A/G agarose beads (lanes 3-4). Anti-AR antibody (lane 1, 3) or as a control anti-IgG (lane 2, 4) were used for ChIP.

In many cases our experimental signal was the same intensity as the negative control. Using Protein G MicroBeads we were able to reduce the non-specific product significantly with an equal or lower volume of beads.

Given the ability of μ MACS Protein G MicroBeads to specifically precipitate protein species in greater amounts than agarose beads, we decided to use MACS Technology for protein discovery. We designed a biotinylated DNA probe to attempt to isolate LNCaP nuclear protein associated specifically with a promoter region in the PSA gene.

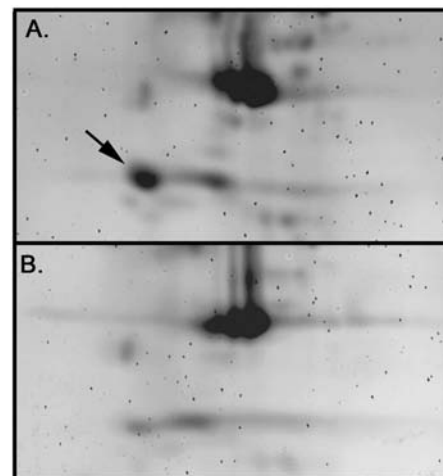


Figure 5: 2D-gel electrophoresis of proteins bound to the biotinylated target DNA (A) or a non-specific biotinylated control DNA (B).

To isolate DNA binding proteins, μ MACS Streptavidin MicroBeads were used to pull biotinylated DNA/protein complexes from LNCaP lysate. Following elution, the samples were separated using 2D gel electrophoresis and stained with Cypro red (Molecular Probes) (fig 5). Our DNA probe clearly shows a major protein product (1, arrowhead) while a non-specific DNA probe (2) shows little sign of a corresponding species. The labeled spot was excised from the gel and sequenced by mass spectrometry (MALDI-TOF) and subsequently identified as a phosphoprotein.

Discussion

Several unique features of the μ MACS MicroBeads, together with the Column Technology, likely contribute to their ability to increase the yield and specificity of a precipitated protein. Among these are the high surface area-to-volume ratio, the colloidal nature of the MicroBeads as well as their super-paramagnetic property. When we used conventional Protein A/G agarose beads we were, at best, only able to achieve semi-quantitative analysis. At only a slight cost increase compared to the agarose beads, the combination of the Microbeads with the μ Columns allowed us to achieve increased efficiency and, therefore, evaluate our immunoprecipitations in a more quantitative manner. Increased yields may also ease protein identification and even facilitate protein discovery.

References:

1. Mulholland, D.J. *et al.* (2002) *J. Biol. Chem.* 277: 17933-17943.

Taking advantage of unique MACS® antibodies, Jacek Tabarkiewicz and co-workers were able to distinguish between well-defined different dendritic cell subsets and support the idea that these cells are specifically accumulated in cancer tissue.

BDCA-1⁺ myeloid and BDCA-2⁺ plasmacytoid dendritic cells in non-small cell lung cancer tissue

Jacek Tabarkiewicz, Kamila Wojas, Jacek Rolinski

Department of Clinical Immunology, University Medical School, Lublin
Head of Department: Jacek Rolinski

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells that take up, process and present antigens, and prime primary as well as secondary immune responses [2, 5, 6, 7]. From studies of DCs it became evident that they do not represent a homogeneous cell population, but rather a mixture of at least two populations: myeloid DCs (MDCs) and plasmacytoid DCs (PDCs). These populations differ in function, morphology and immunophenotype [2, 5, 7]. In our study, we investigated the tissue distribution of PDCs and MDCs in patients with non-small cell lung cancer (NSCLC) [4, 7, 8].

Patients and Methods

Patients. We examined subpopulations of circulating DCs in peripheral blood, draining lymph nodes and cancer tissue in 13 NSCLC patients. In six of these patients, we also examined pleural effusions. The investigation was approved by the local bioethical committee and informed consent was obtained from each individual.

Isolation of the mononuclear cells. Peripheral blood samples were collected in sterile heparinized tubes. Draining lymph nodes and cancer tissue were taken during surgeries. Pleural effusion was obtained during therapeutic drainage. Solid tissue samples were homogenized in MediMachine (DAKO) to cell suspension. Mononuclear cells were separated by gradient centrifugation (Gradisol-L, Aqua Medica) and washed twice in phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} , containing 0.5% bovine serum albumin and 2 mM EDTA.

Immunophenotyping of the cells. The cell surface antigens were determined directly after receiving the fresh samples. The following directly conjugated monoclonal antibodies were used: mouse anti-human CD1c (BDCA-1)-FITC, mouse anti-human BDCA-2-FITC (Miltenyi Biotec), CD123-PE, CD19-CyChrome (BD-Pharmingen). Immunofluorescent staining was prepared according to manufacturers' protocol. Cells were analyzed using a flow cytometer. Each time, 300,000 total events were collected.

MACS products

CD1c (Anti-BDCA-1)-FITC	# 130-090-507
CD1c (Anti-BDCA-1)-PE	# 130-090-508
Anti-BDCA-2-FITC	# 130-090-510
CD123-PE	# 130-090-899

Results and discussion

BDCA-1 and BDCA-2 are two novel antigens, which have been recently described as being specifically expressed on subsets of human blood DCs [4,9,10]. BDCA-1 is expressed on MDCs and a subset of B cells, whereas the expression of BDCA-2 is highly restricted to PDCs, in both blood and peripheral tissues. Phenotypical characterization of BDCA-1⁺ blood MDCs revealed that these cells are CD11c^{bright}, CD4⁺, CD45RO⁺, CD123^{dim}, CD2⁺ and negative for lineage markers (lin), including CD3, CD16, CD19 and CD56. In contrast PDC are lin⁻, CD11c⁻, CD4⁺, CD45RA⁺, CD123^{bright} and CD2⁻ [4]. In blood, both DC populations appear immature: they show poor stimulatory capacity and do not express CD80 and CD83.

In our study, we identified circulating MDCs as BDCA-1⁺ and CD19⁻ cells, and circulating PDCs as BDCA-2⁺ cells. To verify their identity, PDCs were also analyzed for CD123 expression. The count of DCs was expressed as a percentage of DCs in the mononuclear cell (MC) gate. The MC analysis region for PBMCs, draining lymph nodes MCs, tumor infiltrating MCs and pleural effusion MCs was applied to light scatters (fig. 1 and fig. 2). Our results show that the highest percentage of BDCA-1⁺ CD19⁻ MDCs was present in pleural effusions (table 1). In contrast, BDCA-2⁺ CD123⁺ PDCs were most frequent in lymph nodes and cancer tissue. Interestingly, the lowest numbers of both MDCs and PDCs were detected in peripheral blood. In lymph nodes and cancer tissue more PDCs than MDCs were present, whereas in pleural effusion MDCs were more prominent.

In summary, we found an increased percentage of immature dendritic cells in tumor tissue, draining lymph nodes and pleural effusion. Similar results were shown by Diana Bell *et al.* [3], who investigated breast carcinoma tissue by using immunohistochemistry and immunofluorescence. Other investigators have suggested that tissues involved in neoplastic process represent a “black hole” for DCs and that they succumb to apoptotic death at those sites, explaining the lack of DCs in case of poor tumor prognosis [7].

Further studies on function and maturation of tumor-infiltrating DCs will be necessary to completely understand the influence of tumor tissue on the behavior of DCs and how important this fact may be for the tumor to circumvent the immune system.

Acknowledgments. This work was supported by a grant from Committee of Scientific Research (Warsaw, Poland) No. 3 PO5B 073 23.

	PBMCs	Draining lymph nodes	Cancer tissue	Pleural effusion
MDCs	0.24% SD ± 0.20%	0.28% SD ± 0.18%	0.45% SD ± 0.44%	1.69% SD ± 1.40%
PDCs	0.19% SD ± 0.16%	0.77% SD ± 0.89%	0.69% SD ± 0.95%	0.52% SD ± 0.93%

Table 1: Frequence of myeloid (MDCs) and plasmacytoid (PDCs) dendritic cells in different tissues.

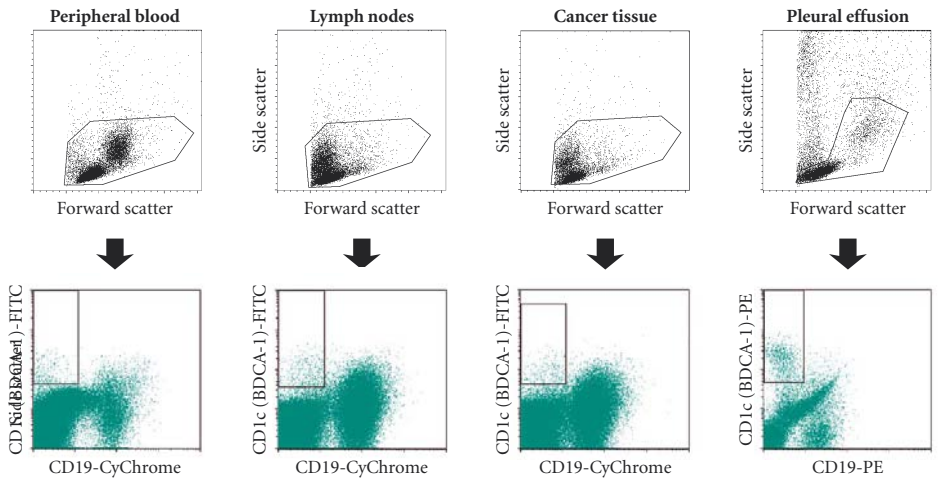


Figure 1: Analysis of circulating myeloid dendritic cells (BDCA-1⁺CD19⁻) in PBMCs, draining lymph nodes, cancer tissue and pleural effusion in patients with NSCLC. Cells were gated according to their light scatter characteristics to exclude dead cells and debris from the flow cytometric analysis.

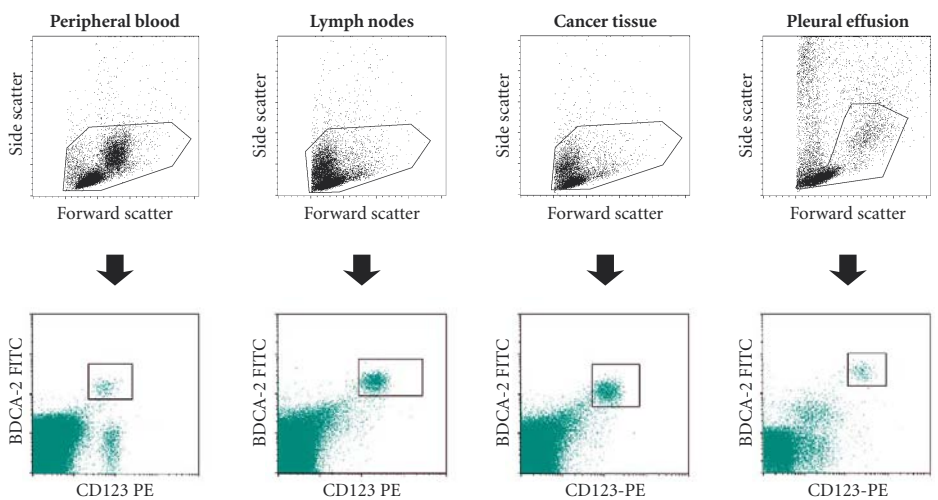


Figure 2: Analysis of circulating lymphoid dendritic cells (BDCA-2⁺/CD123⁺) in PBMCs, draining lymph nodes and cancer tissue in patients with NSCLC. Cells were gated according to their light scatter characteristics to exclude dead cells and debris from the flow cytometric analysis.

References

- Almand, B. (2000) Clin. Canc. Res. 6: 1753-1766.
- Avigan, D. (1999) Blood Reviews 30: 51-64.
- Bell, D. *et al.* (1999) J. Exp. Med. 190: 1417-1425.
- Dzionek, A. *et al.* (2000) J. Immunol. 165: 6037-6046.
- Hart, D. N. J. (1997) Blood 90:3245-3287.
- Lipscomb, M. and Masten, B. (2002) Physiol. Rev. 82: 97-13.
- Lotze, M. T., Thomson, A. W. (2001) Dendritic cells, 2nd ed. New York, Academic Press.
- Penna, G. *et al.* (2001) J. Immunol. 167: 1862-1866.
- Krug, A. *et al.* (2001) Eur. J. Immunol. 31: 3026-3037.
- Dzionek, A. *et al.* (2002) Human Immunol. 63: 1133-1148.

The pro-inflammatory mediator prostaglandin E_2 is crucial for monocyte-derived dendritic cells to acquire the capacity to stimulate T helper cells. Moreover, it increases the chemotactic responsiveness to lymph node-derived chemokines.

CCL19/21-induced migration of monocyte-derived dendritic cells requires PGE₂

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Institute of Immunology, Hannover Medical School, Hannover, Germany

Introduction

Dendritic cells (DCs) are professional antigen-presenting cells and are hence exploited as cellular vaccines for immunotherapy^{1,2}. Migration of DCs from peripheral tissues to the T cell areas of draining lymph nodes is crucial to elicit primary immune responses^{3,4}. We investigated how the maturation of human monocyte-derived DCs (MoDCs) by several different stimuli in combination with prostaglandin (PG) E_2 affected expression of chemokine receptors, T cell stimulatory function, and migratory behavior of MoDCs in response to the lymph node-derived chemokines CCL19 and CCL21.

Materials and Methods

Generation of MoDCs

PBMCs were separated by standard density gradient centrifugation on Ficoll-Paque (Pharmacia, Sweden). Subsequently, monocytes were purified by positive selection using magnetic CD14 MicroBeads and LS columns (Miltenyi Biotec). 1×10^6 cells/mL were cultivated in AIM V medium containing GM-CSF (Leukomax, Novartis) and IL-4 (Strathmann). On day 6, cells were harvested and re-cultured in cytokine-

containing medium in the absence or presence of either a cocktail of proinflammatory cytokines (TNF- α , IL-1 β , IL-6; Strathmann), sCD40L (Immunex Corporation) or polyI:C (Sigma, Switzerland) to induce maturation for additional 48 hours. Where indicated, PGE₂ (Prostin E2, Pharmacia & Upjohn), was added simultaneously.

T cell purification and MoDC co-cultures

Naive CD4⁺CD45RA⁺ T cells were purified from monocyte-depleted PBMCs by negative selection using the CD4⁺/CD45RO⁻ MultiSort Kit and LS or MS columns (Miltenyi Biotec). This method yielded purified (>98%) CD4⁺/CD45RA⁺/CD45RO⁻ T cells as assessed by flow cytometry (data not shown). For MLR experiments mature and immature allogeneic MoDCs were washed, irradiated (30 Gray) and cultured at different numbers in duplicates with 1×10^5 CD4⁺CD45RA⁺ T cells in 96-well flat-bottom plates. On day 4 of co-culture, ³H-thymidine was added and incorporation was tested after 14–16 hours.

FACS analysis of MoDCs

MoDCs were analyzed on a FACScan, flow cytometer (Becton Dickinson) after staining with following mAbs: anti-CCR5 (clone 2D7, Pharmingen), anti-CXCR4 (clone 12G5, R&D

Systems, Germany) and anti-CCR7 (clone 3D12)⁵. FITC-conjugated sheep-anti-mouse IgG (Silenus) and FITC-conjugated goat-anti-rat IgG (Jackson Immunoresearch) were used to detect unlabeled mAbs.

Chemotaxis assay

Chemotaxis of MoDCs was measured by migration through a polycarbonate filter with 5 mm pore size in 24-well Transwell chambers (Corning Costar) using AIM V as assay medium. 600 μ L of assay medium containing CCL19, CCL21 (R&D Systems) or assay medium alone as a control for spontaneous migration were added to the lower chamber. 1×10^5 MoDCs, unstimulated or stimulated for 48 hours with the indicated reagents, were added to the upper chamber in a total volume of 100 μ L and incubated for 3 hours at 37 °C. A 500 μ L aliquot of the cells that migrated to the bottom chamber was counted by flow cytometry acquiring events for a fixed time period of 60 seconds using CellQuest software (Becton Dickinson). Each experiment was performed in duplicate. The mean number of spontaneously migrated cells was subtracted from the total number of migrated cells. Values are given as the mean number of migrated cells \pm SEM.

The questions Test yourself!

1

With the new Anti-Tag MicroBeads not only His-, HA-, c-myc-, GFP- and GST-tagged recombinant proteins can be isolated, but also their interacting partners or whole protein complexes. Elution yields the recombinant or interacting protein only, while the MicroBeads remain on the column in the magnet.

Correct/Incorrect?

2

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Correct/Incorrect?

3

During purification, the target proteins are generally retained on the column in a native confirmation. Therefore, enzymatic reactions, e.g. kinase assays, can be performed on the column before elution of the processed molecules.

Correct/Incorrect?

Find the answers on page 19.

μ MACS Epitope-Tagged Protein Isolation Kits

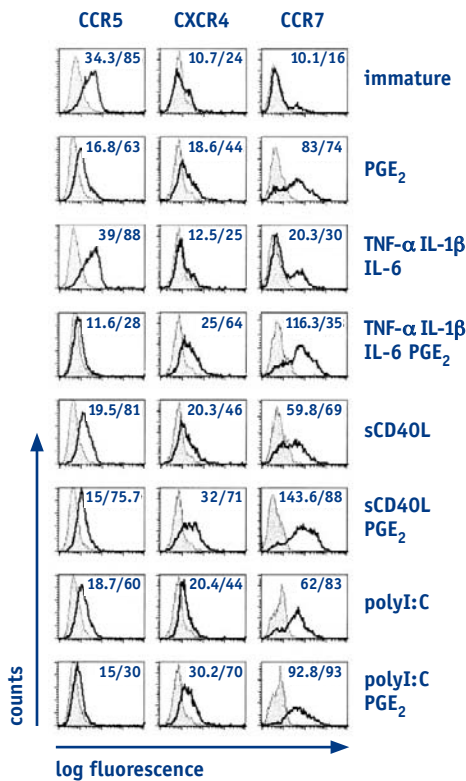


Figure 1: MoDCs were matured by treatment with a cocktail of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6), sCD40L or polyI:C in the presence or absence of PGE₂. After 48 hours, MoDCs were analyzed by flow cytometry for surface expression of the chemokine receptors CCR5, CXCR4, and CCR7 (black lines). Filled gray histograms represent staining with isotype-matched irrelevant mAbs. The inserted numbers indicate the mean fluorescence intensity (MFI)/percent positive cells. The data shown are from one out of six experiments which gave similar results.

Results

PGE₂ modulates chemokine receptor expression on MoDCs.

Co-stimulation with PGE₂ induced the expression of the CCL19/CCL21 receptor CCR7 on MoDCs stimulated with proinflammatory cytokines, and slightly enhanced the expression of CCR7 on the cell surface of MoDCs when they were matured with soluble CD40L. However, PGE₂ did not affect CCR7 expression of polyI:C stimulated MoDCs (fig. 1).

PGE₂ is required for efficient migration of MoDCs towards CCL19 and CCL21.

To investigate the migratory potential of MoDCs, immature MoDCs, and MoDCs stimulated with sCD40L, polyI:C, or the cytokine cocktail in the absence or presence of PGE₂ were examined in a transwell migration assay (fig. 2). Surprisingly, we found that for all maturation stimuli tested,

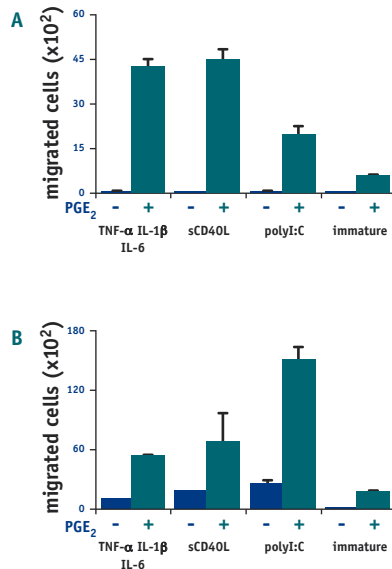


Figure 2: MoDCs were matured by treatment with a cocktail of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6), sCD40L, or polyI:C in the presence or absence of PGE₂ for 48 hours. Unstimulated and stimulated MoDCs were analyzed for their chemotactic response to CCL19 (A) or CCL21 (B). The data are shown as the mean of duplicate cultures \pm SEM and are from one representative experiment out of three performed. The mean number of spontaneously migrated cells was subtracted from the number of cells which migrated in response to chemokines.

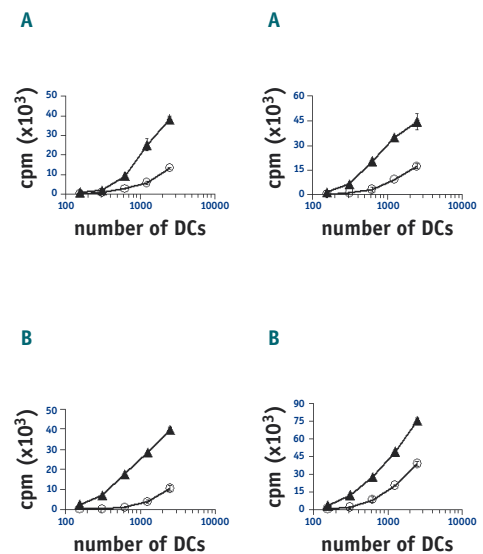


Figure 3: Naive CD4⁺CD45RA⁺ T cells were incubated with graded numbers of allogeneic MoDCs which had been either left untreated (A) or stimulated with polyI:C (B), a cocktail of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) (C), or sCD40L (D) in the presence (triangles) or absence (open circles) of PGE₂. After 4 days of co-culture, the proliferative response was measured by ³H-thymidine incorporation. The data are shown as the mean of duplicate cultures \pm SEM and represent one experiment out of four with similar results.

the addition of PGE₂ was required for effective migration of MoDCs toward the lymph node-derived chemokines CCL19 and CCL21, irrespective of their expression of CCR7.

MoDC capacity to stimulate T cells is enhanced by PGE₂.

Furthermore, the capability of MoDCs to stimulate allogeneic naive CD4⁺ T cells was significantly increased after treatment of MoDCs with PGE₂ (fig. 3). Interestingly, PGE₂ augmented the stimulatory functions of MoDC matured in the presence of sCD40L and polyI:C as well (fig. 3B, D), although PGE₂ had no effect on the phenotypic maturation of these MoDCs (data not shown).

In conclusion, our results suggest that signals provided by the proinflammatory mediator

PGE₂ are crucial for MoDCs to acquire potent capacity to stimulate T helper cells as well as substantial chemotactic responsiveness to lymph node-derived chemokines. This is a new and important parameter for the preparation of MoDCs as cellular vaccines in tumor immunotherapy.

Some of the data presented are reported in Scandella et al. (2002) *Blood*. 100:1354-1361

References

1. Banchereau J and Steinman RM (1998) *Nature* 392: 245-252.
2. Thurner B, et al. (1999) *J. Exp. Med.* 190:1669-1678.
3. Förster R, et al. (1999) *Cell* 99:23-33.
4. Sallusto F, et al. (1999) *Eur. J. Immunol.* 29: 1617-1625.
5. Sallusto F, et al. (1999) *Nature* 401:708-712

MACS® technology has been employed to purify leukocyte subpopulations from Ankylosing Spondylitis patients and control individuals. By comparison of these cells using cDNA microarray analysis, Gerstmayer and colleagues were able to identify differentially expressed genes that allowed the distinction of most patients from healthy donors.

Gene expression profiling of highly purified peripheral blood monocytes in chronic rheumatic inflammation

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Introduction

Ankylosing Spondylitis (AS) represents an inflammatory rheumatic disease of the spine and the joints of the lower extremities [1]. AS pathogenesis has been associated with the HLA class I allele HLA-B27 antigen, which has been found in more than 95% of patients. In the past, the presence of T cells in affected joints has been demonstrated, indicating the involvement of cellular immune responses in the pathogenesis of the disease. So far, the role of other immune cell types is rather unclear and the onset and pathogenesis of AS is still poorly understood. Here we show the analysis of expression profiles from highly purified immune cells of untreated, primary diagnosed Ankylosing Spondylitis patients and healthy volunteers as controls. In the first “proof-of-principle study” we concentrated on CD14⁺ cells of eleven patients and four healthy donors from which RNAs were isolated. A pool of RNAs from CD14⁺ cells was used as a control. All RNAs were linearly amplified, labeled and subsequently hybridized to customized PIQOR™ rheumatology cDNA arrays comprising 900 carefully selected disease-relevant genes. Although the expression patterns from AS patients and normal volunteers revealed inter-individual variations, we were able to identify genes that were differentially

regulated between patients and healthy individuals. Our study shows that the combination of MACS® separation and cDNA microarray analysis represents a powerful approach to elucidate the role of specific cell populations in AS patients for a better understanding of the disease.

Methods

AS cases: 40 mL of peripheral blood was taken from each of eleven drug-untreated AS patients. All patients were positive for HLA-B27 and showed active disease with elevated levels of C-reactive protein (CRP), increased rate for erythrocyte sedimentation and inflammatory spinal pain or swollen joints.

Controls: Peripheral blood of nine healthy donors was prepared; five samples were pooled and served as a control for the hybridization experiments.

Isolation of highly purified leukocyte populations (see fig. 1): Blood was treated with erythrocyte-lysing solution at 4 °C to obtain leukocytes deprived of erythrocytes. Granulocytes were separated by magnetic cell sorting using a MACS®-conjugated anti-CD15 monoclonal antibody (Miltenyi Biotec). CD15-negative cells were stained for monocytes (CD14), for lymphocytes (CD4 and CD8) and for NK cells (CD56). Labeled cells were separated in parallel by 6-color 4-way high-speed sorting with a MoFlo cytometer (Cytomation).

Comparison of protein and gene expression in CD14⁺ and CD15⁺ cells (n=134)

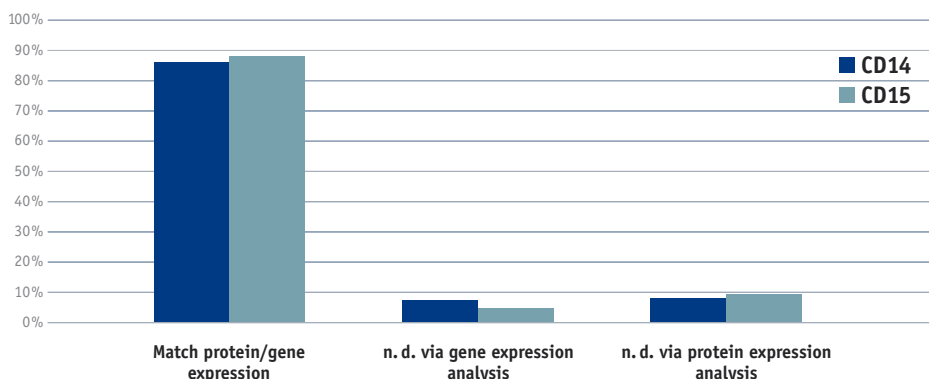
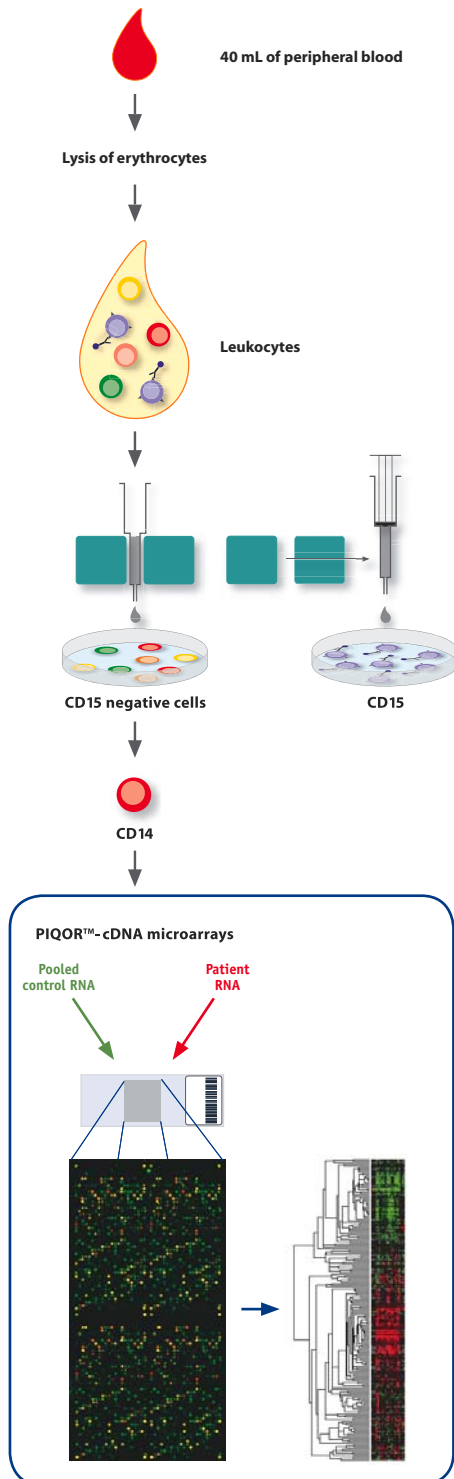


Figure 2: Qualitative comparison of the expression of all CD genes present on the array (n=134) with the predicted surface expression of the respective proteins on CD14⁺ and CD15⁺ cells

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Figure 1: Principle of expression profiling by using PIQOR™ rheumatology arrays. Cy3-labeled control cDNA and Cy5-labeled patient cDNA, derived via reverse transcription from the respective RNA sources, are combined and simultaneously hybridized on a PIQOR™ cDNA array. After overnight hybridization and subsequent washing, the array can be scanned and analyzed.



RNA preparation and PIQOR™ microarray analysis: Total RNA was isolated and the quality was checked using the 2100 Bioanalyzer system (Agilent Technologies). First and second strand cDNA was generated (Invitrogen) from total RNA using oligo(dT) primer conjugated to the sequence of the T7 promoter. Finally, aRNA was derived from the double-stranded cDNA via an *in vitro* transcription reaction (Ambion). Fluorescence-labeled cDNA was prepared by reverse transcription of aRNA (Invitrogen). Reference RNA sample (pool of five controls) was Cy3-labeled, while single controls and patient samples were Cy5-labeled. Custom-made PIQOR™ cDNA microarrays (Memorec Biotec) consisted of 900 spotted cDNAs (200–400 bp) [2] including ten positive controls (housekeeping genes) and two negative controls (herring sperm DNA and buffer only). Genes were spotted as quadruplicates. For each patient analysis, 2 µg of amplified control RNA and patient RNA were co-hybridized on a single chip (fig. 1). Fluorescence signals were detected by a laser scanner (ScanArray Lite, Packard Biosciences) [3].

Results

The sorting of individual cell populations in average yielded a purity of 98%. The purity of cell populations was confirmed at the transcriptional and translational levels (table 2 and fig. 2). Here, we focussed on all CD (Cluster of differentiation) molecules of CD14⁺ and CD15⁺ cells, checked whether a gene was detectable in microarray analysis and compared the result to its predicted expression on the cell surface. A high correlation between RNA expression and respective CD antigen presentation was observed. Finally, the expression profiles of CD14⁺ monocytes from healthy individuals and AS patients were compared via two-dimensional cluster analysis. Eight out of eleven patients were grouped in a separate cluster apart from the healthy control individuals (fig. 3). Furthermore, a number of genes could be identified that were found to be almost exclusively regulated in AS patients but not in control individuals.

Conclusions

In this study a combined approach using cell sorting protocols for the isolation of highly purified leukocyte subpopulations and subsequent gene expression analysis via the PIQOR™ platform has been successfully applied. The purity of the cells could be confirmed via FACS re-analysis of isolated cells.

As detailed in table 1, the cellular composition of blood from AS patients showed considerable variations. Consequently, the use of whole blood as a source for gene expression analysis would result *a priori* in the detection of

cell population	% of leukocytes	factor of variation
monocytes	4 – 15	3.8
granulocytes	33 – 83	2.5
NK cells	1 – 12	12
B lymphocytes	1 – 7,5	7.5
CD4 lymphocytes	3 – 30	10
CD8 lymphocytes	1 – 13	13

Table 1: Cellular composition of blood from AS patients (n=24).

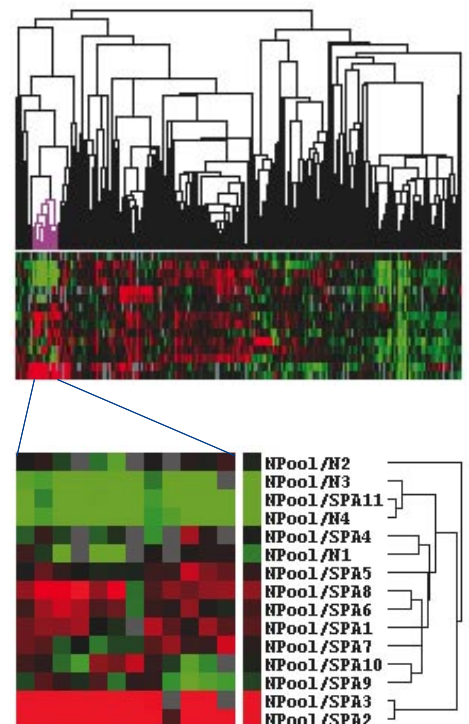


Figure 3: Average linkage cluster analysis of 15 expression profiles derived from CD14⁺ monocytes of different control individuals and patients. Only those genes that are expressed in 70% of all experiments were chosen as input for the cluster analysis (this corresponds to a selection of 210 cDNAs).

Abbreviations: Npool, pool of 5 control individuals; N, normal control individual; SPA, patient.

Table 2: Comparison of the detection of certain marker molecules for T cells (T), B cells (B), Dendritic cells (DC), NK cells (NK), Stem cells (SC), Macrophages/Monocytes (Mac/Mono), Granulocytes (Granu), Platelets (P), Erythrocytes (E), Endothelial cells (Endo), Epithelial cells (Epi) on the gene expression and protein expression level with the focus on CD14⁺ monocytes and CD15⁺ granulocytes.

CD	surface protein expression										gene expression		
	T	B	DC	NK	SC	Mac/Mono	Granu	P	E	Endo	Epi	Mac/Mono	Granu
CD3E	+		-	-	-	-	-	-	-	-	-	-	-
CD3Z	+		-	-	-	-	-	-	-	-	-	-	-
CD8A	+	-		+	-	-	-	-	-	-	-	-	-
CD8B	+	-		-	-	-	-	-	-	-	-	-	-
CD154	+*	-	-	-	-	-	-	-	-	-	-	-	-
CD143	+	-		-	-	-	-	-	+	+	-	-	-
CD20	-	+	-	-	-	-	-	-	-	-	-	-	-
CD23	-	+	-	-	-	-	-	-	-	-	-	-	-
CD138	-	+*		-	-	-	-	-	-	-	-	-	-
CD79A	-	+		-	-	-	-	-	-	-	-	-	-
CD79B	-	+		-	-	-	-	-	-	+	+/-	-	-
CD72	-	+	+	-	+	-	-	-	-	-	-	-	-
CD73	+	+	+	-	+	-	-	-	+	+	-	-	-
CD25	+*	+	-	+	-	-	-	-	-	-	-	-	-
CD26	+	+	-	+	-	-	-	-	-	-	+	-	-
CD27	+	+	-	+	-	-	-	-	-	-	-	-	-
CD7	+	-		+	+	-	-	-	-	-	-	-	-
CD34	-	-	-	-	+	-	-	-	-	-	+	-	-
CD90	-	-		-	+	-	-	-	-	+	-	-	-
CD14	-	-		-		+	+	-	-	-	-	+	+
CD88	-	-	+	-	-	+	+	-	-	+	+	+	+
CD89	-	-		-	-	+	+	-	-	-	-	+	+
CD86	+*	+*	+	-	-	+	-	-	-	+	-	+	-
CD91	-	-		-	+	+	-	-	-	-	+	+	-
CD36	-	-	+	-	+	+	-	-	+	+	-	+	-
CD40	-	+	+	-	+	+	-	-	-	+	+	+	-
CD99	+	+		+		+	-	+	+	+	+	+	-
CD125	-	+*		-		-	+	-	-	-	-	-	+/-

+ (plus) within the "surface protein expression" columns were derived from the 7th International Workshop on Human Leukocyte Differentiation antigens and indicate that the proteins were detected via specific antibodies on the respective cell types;

- (minus) indicates absence of the respective gene/protein.

+ (plus) within the "gene expression" columns indicates that the Cy3 or Cy5 signal intensity for the respective gene is at least 2-fold of the mean signal of the negative controls (buffer only/herring sperm DNA);

+* indicates expression of the protein only in distinct activated cell populations;

+/- indicates gene expression in some of the analyzed samples.

many putative differentially expressed genes. Those genes, however, might just represent the result of a difference in relative cell numbers rather than indicating disease-specific regulations. Therefore, gene expression profiling of highly purified cell subpopulations represents a major advantage over whole blood cell samples.

With our experimental design, cell-type specific gene transcripts could be detected. A good correlation of the expression of these genes and their translated proteins as specific cell surface markers was found.

Gene expression profiles of the different donor-derived CD14⁺ cells revealed inter-individual variations, as expected. However, cluster analysis made it possible to separate most of the AS patients from control individuals. Additionally, we were able to identify genes differentially regulated between patients and healthy individuals. These genes could be mainly grouped into a) induction of pro-inflammatory cytokines and chemokines and b) up-regulation of anti-apoptotic signaling molecules. Our data indicate that gene expression analysis of highly purified cells is feasible. Clearly, the number of AS patients and controls has to be enlarged to fulfill the statistical requirements that are necessary for the classification of disease-specific expression profiles.

References

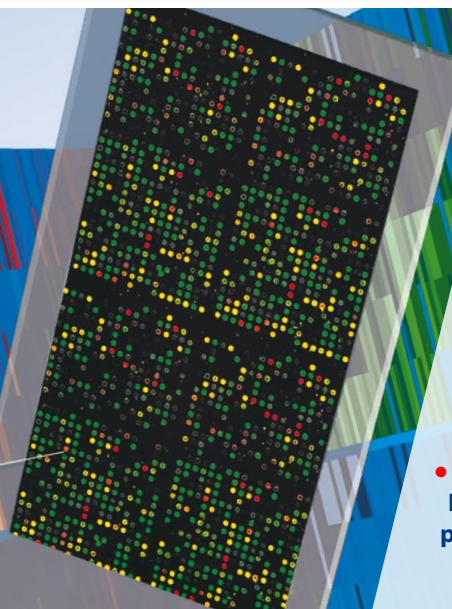
1. Sieper, J. et al. (2002) Ann. Rheum. Dis. 61: Suppl. 3, iii8-18.
2. Tomiuk, S. et al. (2001) Brief Bioinform. 2: 329-40.
3. Bosio, A. et al. (2002) Carcinogenesis 23:741-8.

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Tracking Epstein-Barr-virus-associated diseases

Transcription factor purification via DNA binding from isolated CD19⁺ B cells.

Toni Portis and Richard Longnecker: Epstein-Barr virus LMP2A interferes with global transcription factor regulation when expressed during B-lymphocyte development J. Virol. (2003) 77: 105–114.

Epstein-Barr virus (EBV) is a human herpes virus which, whilst found as a widespread asymptomatic infection, is also associated with certain tumors of lymphoid and epithelial origin including Burkitt's lymphoma, Hodgkin's Disease, nasopharyngeal carcinoma, and various lymphoproliferate disorders. In the development of EBV related malignancies the viral latent membrane protein 2A (LMP2A) is thought to play an important role by allowing the virus to persist in latently infected B lymphocytes. It is discussed that LMP2A prevents B-cell activation through altered B-cell receptor signal transduction and thereby inhibits lytic EBV replication and subsequent immune recognition.

In this study LMP2A expressing transgenic mice were used as a mouse model for EBV associated disease development. Toni Portis and Richard Longnecker compared gene and protein expression of transgenic and wildtype B cells from bone marrow and spleen.

B cells were isolated from mice spleen with

CD19 MicroBeads to >95% purity before RNA or protein was purified.

In DNA microarray analysis transgenic mice showed a differential expression of several B-cell development genes and their transcription factors compared to wildtype mice. In particular, a downregulation of the transcription factor E2A and an upregulation of two E2A inhibitors was revealed. E2A regulates the expression of genes involved in normal B-cell development, e.g. Ig- α , RAG-1, TdT.

To investigate if not only RNA levels but also E2A protein amount and DNA-binding activity was reduced, nuclear extracts of bone marrow and splenic B cells were prepared, and the DNA-binding E2A protein was specifically isolated.

The authors therefore incubated the protein lysates with a 5'-biotinylated 23-bp double-stranded oligonucleotide comprising the sequence of the κ E2 Ig kappa-chain enhancer for 20 min, added the μ MACS™ Streptavidin MicroBeads and 15 min later loaded the samples on the μ Columns in the magnet. After four times washing, the E2A

protein was eluted with high-salt buffer. SDS-PAGE followed by silver staining and specific western blots confirmed the reduced DNA-binding activity of E2A in the LMP2A-expressing B cells.

The results indicate that the viral LMP2A protein interferes with normal transcription factor expression and activity when expressed during B lymphopoiesis and thereby alters B cell development and survival. In this process, the decreased E2A activity likely results in the inhibition of Ig heavy-chain rearrangement and B-cell receptor expression. Future studies should give further insights how LMP2A maintains latency in EBV-infected B lymphocytes. (ko) ■

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in the magnetic field on the column. Alternatively, interacting proteins can be eluted via pH shift or increased salt concentrations, while the tagged protein stays on the column.

2 Incorrect. The kits contain lysis, wash and elution buffers optimized for protein isolation from mammalian cells. However, these buffers can also be used for bacteria or other eukaryotic cells like yeast or insect cells. For native protein elution there is no buffer supplied, but the user manual gives recommendations for its composition.

3 Correct. As the lysis and wash buffers only contain small amounts of non-ionic detergents and even lower concentrations of anionic detergents, the purification can generally be considered as non-denaturing. Accordingly, the target proteins, which are labeled with MicroBeads, are retained in their native conformation on the column. By applying enzymes, buffer and further components, enzymatic reactions can be performed on the column. After washing off the reagents, the pure, processed protein can be eluted. In case an enzyme is bound on the column, substrates can be added, incubated, and eluted to analyze enzyme activity.



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