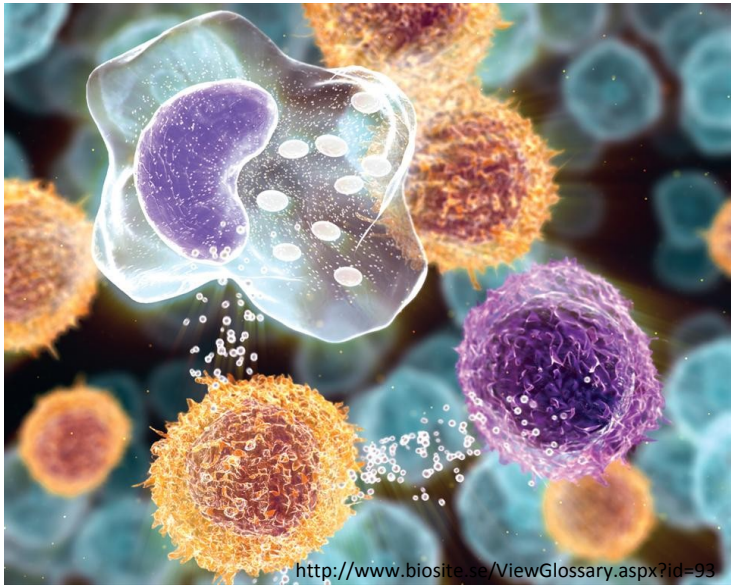
Intracelulární detekce cytokinů.

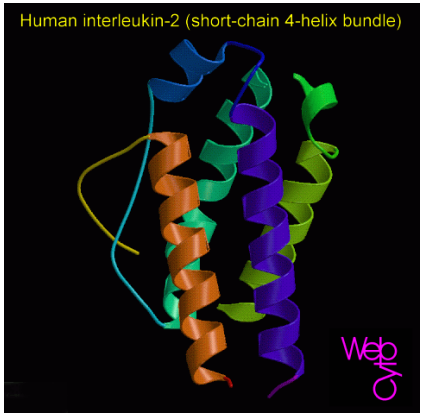


http://www.biosite.se/ViewGlossary.aspx?id=93

Markéta Rodová

Analytická cytometrie 2011

Cytokiny



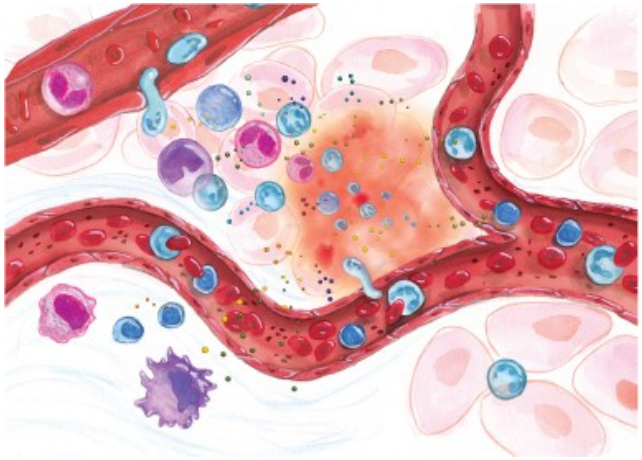
http://www.genscript.com/protein/Z00368-Interleukin_2_IL_2_human.html

- molekuly zapojené do procesu buněčné signalizace
- základní regulátory IS
- klíčová role v apoptóze, angiogenezi, diferenciaci, růstu buněk
- podílí se na morfogenezi a udržování homeostázy celého organismu
- působí prostřednictvím receptorů cílových buněk

- pleiotropní účinky
- působí v kaskádě
- cytokinový systém – redundantní

- působení
  - autokrinní
  - parakrinní
  - endokrinní

- proteiny, glykoproteiny, peptidy



http://www.beltina.org/health-dictionary/cytokines-definition.html

Proč analyzovat intracelulární produkci cytokinů ?

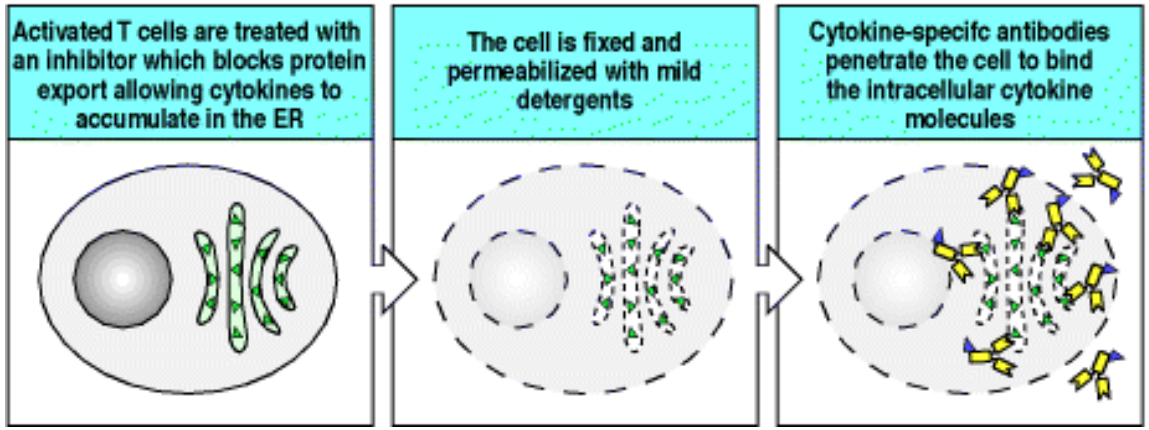
Cytokine	Cell source	Target	Actions
Proinflammatory Cytokines			
IL-1	Macrophage Dendritic cell	Lymphocytes Endothelial cell CNS Liver	Enhances responses Activates Fever, sickness behavior Synthesis and release of acute-phase proteins
IL-6	Macrophage Dendritic cell Endothelium Th2 cell	Liver B cell	Synthesis and release of acute-phase proteins Proliferation
TNF-alpha	Macrophage Dendritic cell Th1 cell	Endothelial cell Neutrophil Hypothalamus Liver	Activates vascular endothelium – increased permeability and stimulates adhesion molecules Activates Fever Synthesis and release of acute-phase proteins
Anti-inflammatory Cytokines			
IL-10	Macrophage Th2	Macrophage Dendritic cell	Inhibits IL-12 production Inhibits pro-inflammatory cytokine synthesis
Il-12	Macrophage Dendritic cell	CD4+T helper cell NK cell	Th1 differentiation IFN-gamma synthesis
Cytokines Involved in the Acquired Immune Response			
IL-2	T cell	T cell NK Cell B cell	Proliferation Activation and proliferation Proliferation
IL-4	Th2 cell Mast cell	T cell B cell Macrophage	Th2 cell development/proliferation Isotype switch to IgE Inhibit IFN-gamma activation
IFN-gamma	Th1 cell Cytotoxic T cell NK cell	T cell B cell Macrophage	Th1 cell development Isotype switch to IgG Activation

http://www.pmbcii.psy.cmu.edu

- Charakterizace různých subpopulací leukocytů, které lze rozlišit na základě rozdílné produkce cytokinů.
- Určení stupně aktivace/suprese lymfocytů.
- Charakterizace funkčních vlastností buněk.
- Identifikace klíčových faktorů onemocnění.
- Identifikace molekulárních mechanismů účinků léčiv + vývoj nových.
- Lze sledovat experimentálně ovlivněnou odpověď konkrétní populace na stimulaci.
- …

http://www.bdbiosciences.com/research/tcell/tools/intrace_cyto.jsp

Jak detekovat intracelulární cytokiny



Immunobiology, Janeway, 2004

TAKE-HOME MESSAGE

Problém – buňky uvolňují cytokiny do okolí = ztrácíme informaci o tom, která buňka daný cytokin produkuje.

- Inhibice exportu proteinů z buňky (akumulace ve vezikulárním aparátu)
- Fixace ⟶ permeabilizace ⟶ průnik protilátek do kompartmentů

Cytokine assays

http://www.virology-online.com

- ELISAs
- Radioimmunoassays

\+ citlivé, kvantitativní metody

\- pouze průměrné množství cytokinů

\- neposkytují „single-cell level“ analýzy

- **Flow cytometry**

\+ determinace antigenně specifických buněk produkujících cytokiny

\+ poskytuje „single-cell level“ analýzy, fenotypizace

\+ analýza aktivačních markerů, přítomnosti jednotlivých receptorů, dalších antigenů

\+ vícebarevná analýza

(-) limitace – množství detekovatelných fluorescenčních parametrů

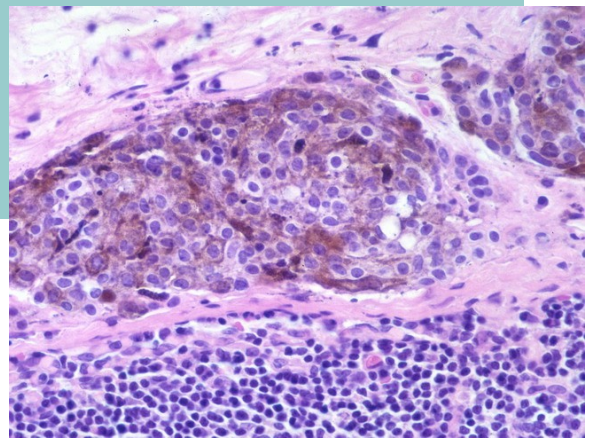

http://www.biotech.iastate.edu

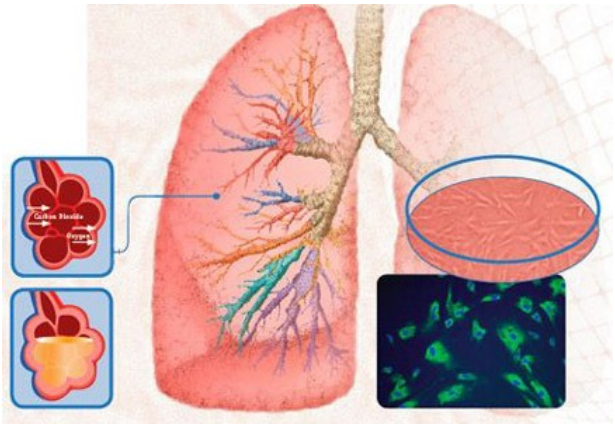
Princip

- Stimulace
- Aktivace
- Značení povrchových molekul (fenotypizace)
- Fixace
- Lyzace
- Permeabilizace
- Značení intracelulárních cytokinů
- Analýza průtokovým cytometrem

Jaké buňky lze analyzovat…?

- PBMC (peripheral blood mononuclear cells)
- whole blood
- BAL (bronchoalveolar lavage cells)
- mouse splenocytes
- lymph node cells
- thymocytes, etc.



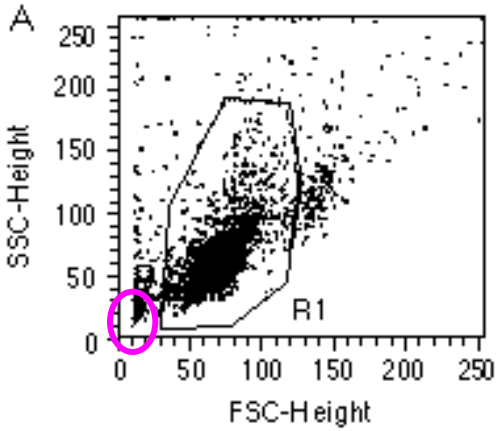


Kvalita buněk

- leukocyty treatované imunosupresivy jsou velmi citlivé na lyzační reagencie
- mohou mít pozměněnou morfologii, na pohled vypadají poškozeně
- percentage of cytokine-positive cells - low
- background staining – high

- FSC/SSC – increase of debris area

- zmírnění – zkrácení doby lyzace a permeabilizace buněk

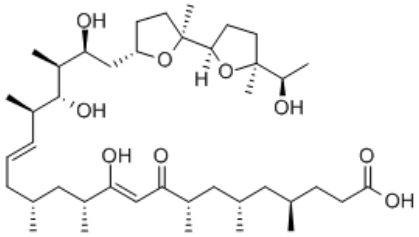


http://www.currentprotocols.com/protocol/mb1413

PROTOKOL

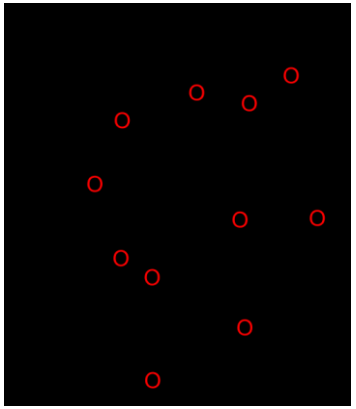
- Stimulace
  - Ionomycin (*Streptomyces conglobatus*)
  - PMA (phorbol myristate acetate)
  - -> zvýšení produkce cytokinů
- Aktivace
  - monensin (*Streptomyces cinnamonensis*)
  - brefeldin A
  - zabraňuje vyplavení cytokinů z Golgiho komplexu

IONOMYCIN



http://www.chemicalbook.com/ProductChemicalPropertiesCB5380046_EN.htm

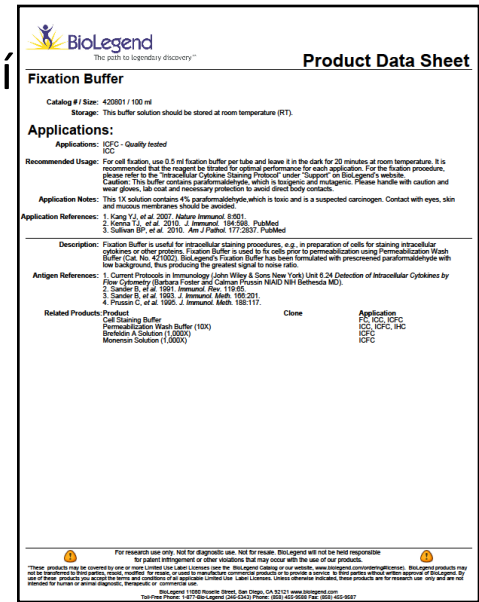
MONENSIN



http://en.wikipedia.org/wiki/File:Monensin_A.svg

PROTOKOL

- Fenotypizace
  - značení povrchových antigenů
  - vždy ***před*** fixací – zabránění destrukce antigenů fixačním médiem
- Fixace
  - fixační pufr
  - obsahuje paraformaldehyd (1 nebo 4%)
  - před permeabilizací - důležité pro zabránění ztráty cytokinů
  - doba fixace – důležitý parametr
    - pokud jsou buňky „přefixované“, cytokiny nemusí reagovat s protilátkami



BioLegend
The path to legendary discovery™

Product Data Sheet

Fixation Buffer

Catalog # / Size: 420801 / 100 ml
Storage: This buffer solution should be stored at room temperature (RT).

Applications:

Applications: ICFC - *Quality tested*
IOC

Recommended Usage: For cell fixation, use 0.5 ml fixation buffer per tube and leave it in the dark for 20 minutes at room temperature. It is recommended that the reagent be titrated for optimal performance for each application. For the fixation procedure, please refer to the "Intracellular Cytokine Staining Protocol" under "Support" on BioLegend's website.
Caution: This buffer contains paraformaldehyde, which is toxigenic and mutagenic. Please handle with caution and wear gloves, lab coat and necessary protection to avoid direct body contacts.

Application Notes: This 1X solution contains 4% paraformaldehyde,which is toxic and is a suspected carcinogen. Contact with eyes, skin and mucous membranes should be avoided.

Application References: 1. Kang YJ, *et al.* 2007. *Nature Immunol.* 8:601.
2. Kemna TJ., *et al.* 2010. *J. Immunol.* 184:598. PubMed
3. Sullivan BP, *et al.* 2010. *Am J Pathol.* 177:2837. PubMed

Description: Fixation Buffer is useful for intracellular staining procedures, *e.g.*, in preparation of cells for staining intracellular cytokines or other proteins. Fixation Buffer is used to fix cells prior to permeabilization using Permeabilization Wash Buffer (Cat. No. 421002). BioLegend's Fixation Buffer has been formulated with prescreened paraformaldehyde with low background, thus producing the greatest signal to noise ratio.

Antigen References: 1. Current Protocols in Immunology (John Wiley & Sons New York) Unit 6.24 *Detection of Intracellular Cytokines by Flow Cytometry* (Barbara Foster and Camran Prussin NIAID NIH Bethesda MD).
2. Sander B, *et al.* 1991. *Immunol. Rev.* 119:65.
3. Sander B, *et al.* 1993. *J. Immunol. Meth.* 166:201.
4. Prussin C, *et al.* 1995. *J. Immunol. Meth.* 188:117.

Related Products:

Product	Clone	Application
Cell Staining Buffer		FC, IOC, ICFC
Permeabilization Wash Buffer (10X)		IOC, ICFC, IHC
Brefeldin A Solution (1,000X)		ICFC
Monensin Solution (1,000X)		ICFC

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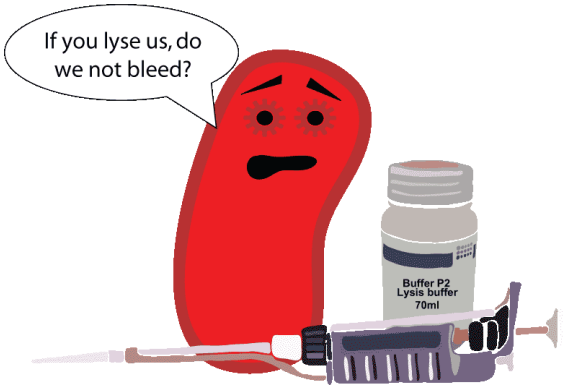
PROTOKOL



http://parts.mit.edu/igem07/index.php/BerkiGEM2007Present1

- Lyzace erytrocytů
  - RBC Lysis Buffer BioLegend
- Permeabilizace
  - permeabilizační pufr
- Fixace a permeabilizace v jednom kroku

  BD Cytofix/Cytoperm™ BD

NOTE 2: Because saponin-mediated cell permeabilization is a reversible process, it is important to keep the cells in the presence of **saponin** during intracellular cytokine staining.

Kity pro stanovení IC

- **Human**: IL-1, IL-2, IL-3, IL-4, IL-6, IL-8, IL-10, IL-11, IL-12, IL-13, IFN, TNF, GM-CSF, GRO, IP-10, MCP-1, MCP-3, MIG, MIP-1, RANTES
- **Mouse**: IL-2, IL-3, IL-4, IL-6, IL-10, IFN, TNF, GM-CSF, MCP-1
- **Rat**: IL-4, IL-10, GM-CSF

R&D SYSTEMS

Fit for Science BIOZOL

BioLegend

full spectrum cell analysis eBioscience

BD

Molecular Probes

Fluorochromy

Kompenzace fluorescence

Překryv spekter (spectral overlap)

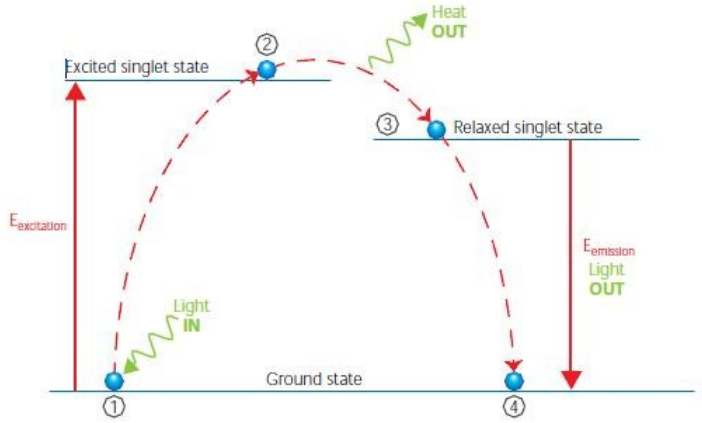
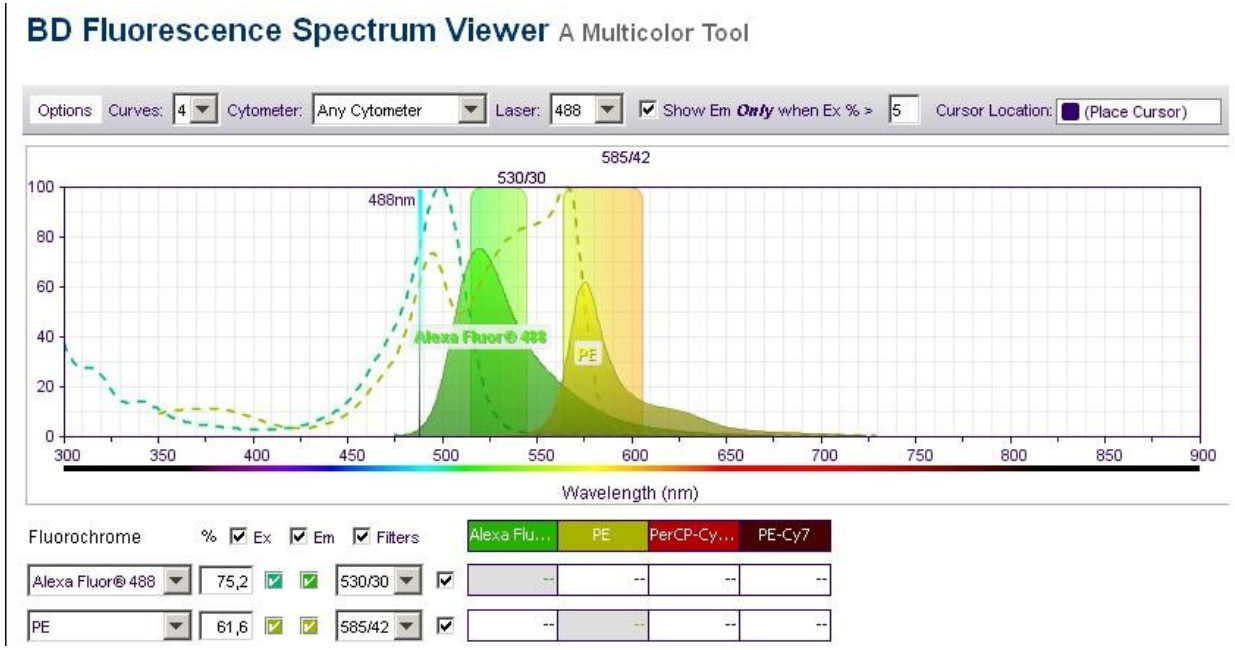


FIGURE 6 Stokes Shift — Introduction to Flow Cytometry, Rahman, 2006)

- *Alexa Fluor 488*
- *Alexa Fluor 647*
- *FITC*
- *PE*
- *PerCP*
- *Cy5*
- *PE-Cy5*
- *PE-Cy5.5*



Excitační a emisní spektra FITC a PE

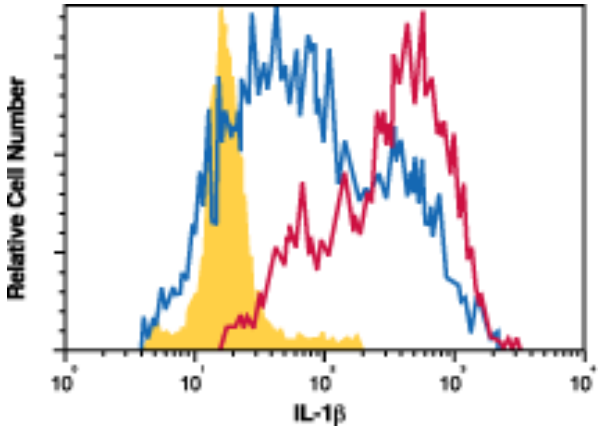
http://www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp

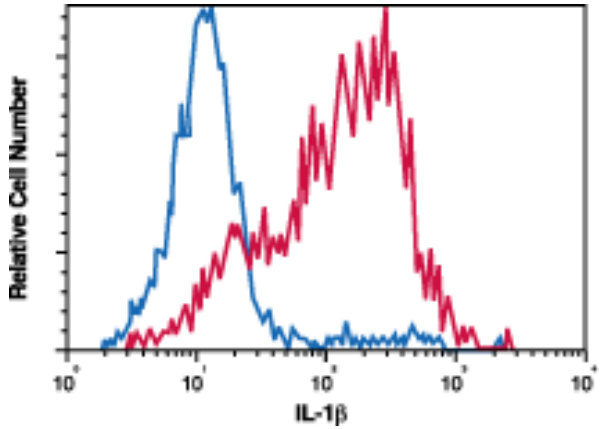
Izotypová kontrola

- odhalení nespecifických vazeb

Cells stained for intracellular IL-1 beta.

Human IL-1 beta/IL-1F2 Fluorescein MAb (Clone 8516), Mouse IgG1





Yellow - isotype control alone

Blue - non-permeabilized cells

Red - permeabilized cells

Blue - surface-blocked, non-permeabilized cells

Red – surface blocked, permeabilized cells

http://www.rndsystems.com/cb_detail_objectname_SP99_TN_IntCytokineStaining.aspx

MEASUREMENT OF INTRACELLULAR INTERFERON GAMMA AND INTERLEUKIN 4 IN T LYMPHOCYTES

Histograms

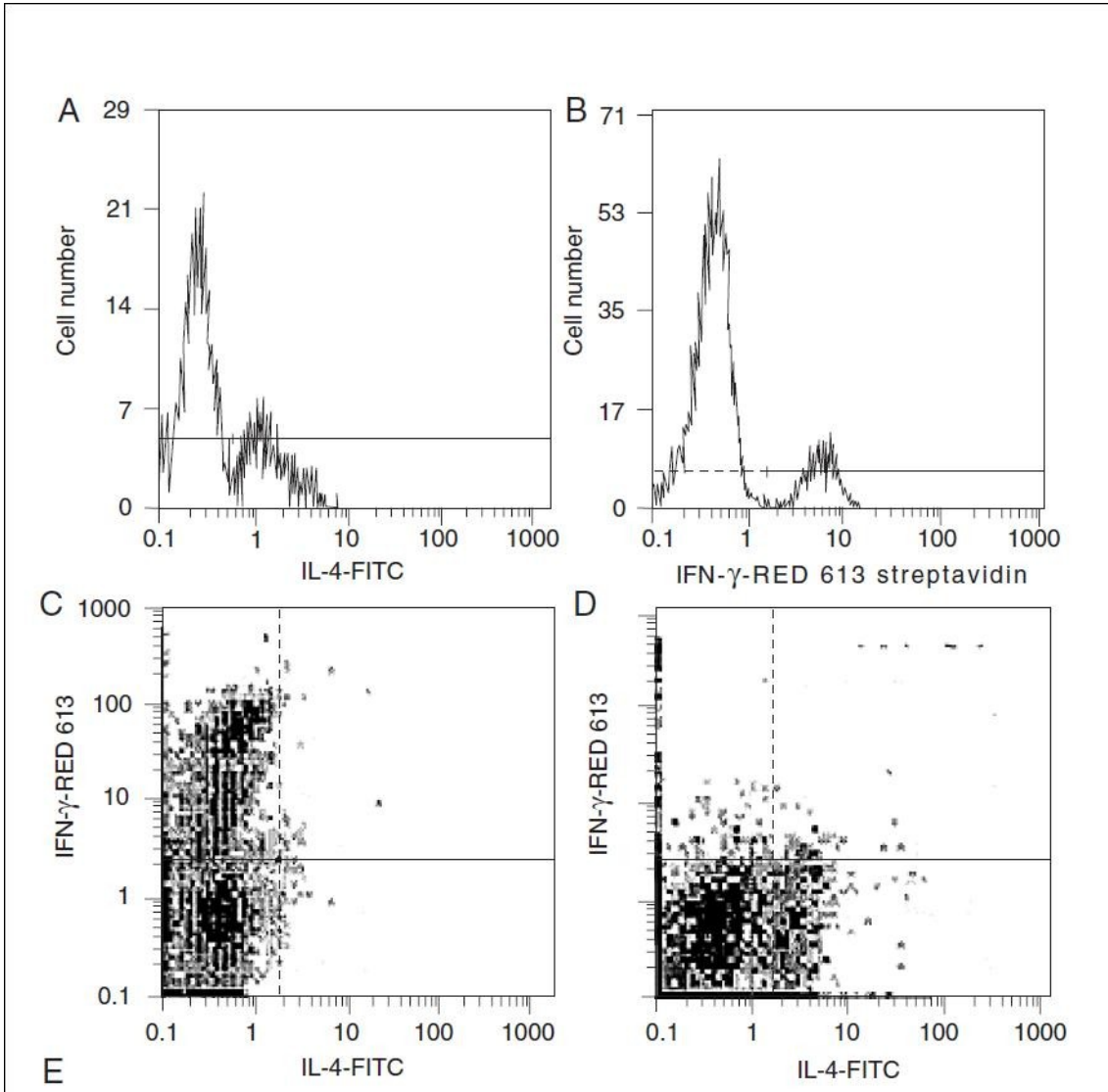
IL-4-stained cells **(A)**

IFN-γ-stained cells **(B)**

Dot plots

population of cells producing IFN-γ and almost no IL-4 **(C)**

population of cells producing mostly IL-4 and little IFN-γ **(D)**



Current Protocols in Cytometry (2004)

TGFβ1 inhibuje diferenciaci Th2 inhibicí exprese GATA-3.

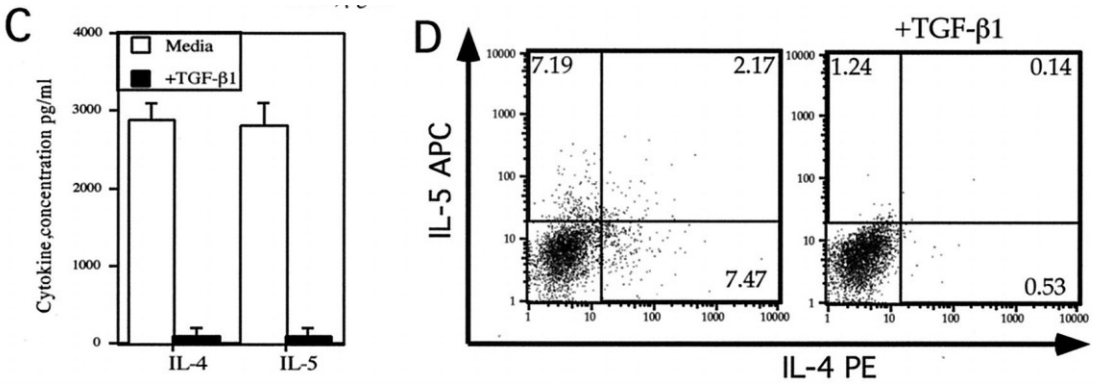
Leonid Gorelik, Patrick E. Fields, Richard A. Flavell. *The Journal of Immunology,* 2000, 165: 4773–4777.

GATA-3 podporuje sekreci IL-4 a IL-5 v Th2

TGFβ1 inhibuje množství buněk diferencovaných do Th2 fenotypu

C – ELISA

D - FACS



CD4⁺ T cells (transgenic mice)

Literatura

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- Schuerwegh AJ, De Clerck LS, Bridts CH, Stevens WJ. 2003.Comparison of Intracellular Cytokine Production With
- Extracellular Cytokine Levels Using Two Flow Cytometric Techniques. Cytometry Part B (Clinical Cytometry) 55B:52–58
- Flow cytometry educational guide (http://www.upci.upmc.edu/cf/pdf/DAKO_flow_cytometry_educational_guide.pdf)

- http://www.bd.com
- http://www.biolegend.com
- http://www.ebioscience.com

- Obrázky

- http://www.abcam.com/index.html?pageconfig=resource&rid=11448
- http://www.rndsystems.com/cb_detail_objectname_SP99_TN_IntCytokineStaining.aspx
- http://www.bdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp
- http://parts.mit.edu/igem07/index.php/BerkiGEM2007Present1
- http://www.currentprotocols.com/protocol/mb1413
- http://www.virology-online.com
- http://www.biotech.iastate.edu
- http://www.pmbcii.psy.cmu.edu
- http://www.bdbiosciences.com/research/tcell/tools/intrace_cyto.jsp
- http://www.beltina.org/health-dictionary/cytokines-definition.html
- http://www.genscript.com/protein/Z00368-Interleukin_2_IL_2_human.html