



***Lidské B lymfocyty s autoreaktivním  
potenciálem objevené pomocí  
„single cell“ RT-PCR –  
od imunodeficitu k autoimunitě***

*Konference DNA analýza VIII  
Praha, 02/06/2011*

# B buňky a imunitní systém

## Jiné fyziologické procesy

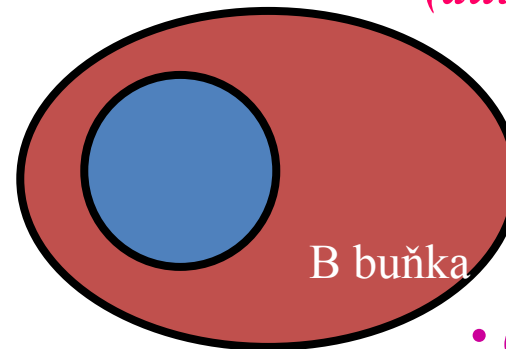
- tvorba osteoklastů



poruchy tvorby  
kostní hmoty

## Adaptivní/vrozená imunita

- (auto)Ab produkce



- „NK-like“ aktivita –  
produkce granzymu B



- (auto)Ag prezentace

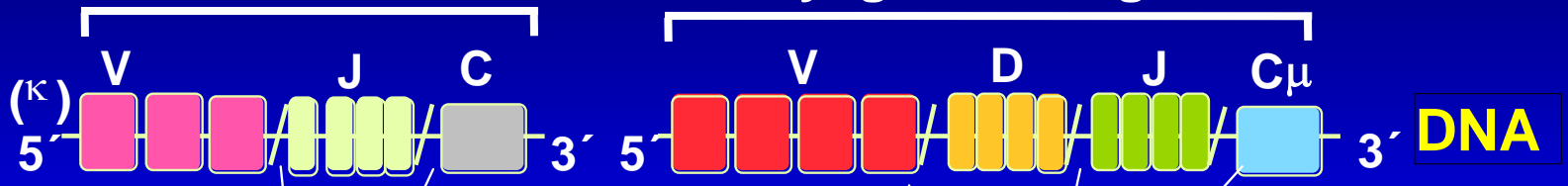
Autoimmunita

Ig deficiency

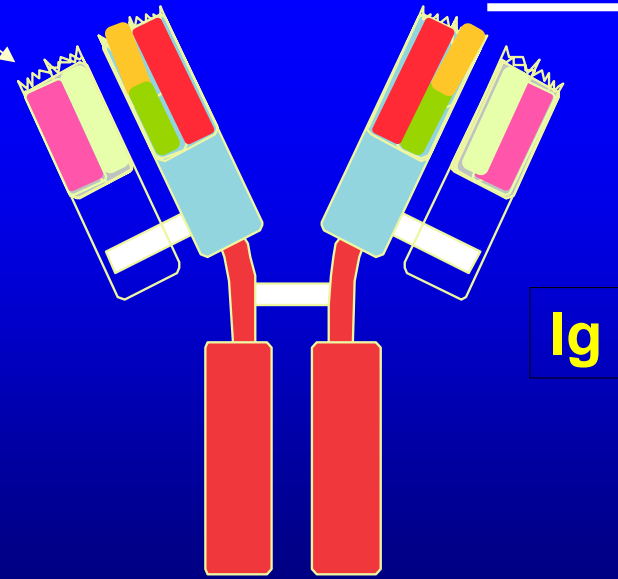
(Schlomchik J *Curr Opin Immunol* 2009; Hagn M et al. *J Immunol* 2009; Li Y et al. *Blood* 2007; Kawai T et al. *Am J Pathol* 2006)

Light Ig chain segments

Heavy Ig chain segments



Rearranged segments



Ig molecule

CDR1

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
VH4-34	GGT	GGG	TCC	TTC	AGT	GGT	TAC	TAC	TGG	AGC	TGG	ATC	CGC	CAG	CCC
	---	---	---	---	---	---	-t	---	---	-C-	---	---	---	-G-	---

CDR2

	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55
VH4-34	CCA	GGG	AAG	GGG	CTG	GAG	TGG	ATT	GGG	GAA	ATC	AAT	CAT	AGT	GGA
	--g	--a	G--	--a	---	---	---	---	---	---	---	---	---	G--	-A-

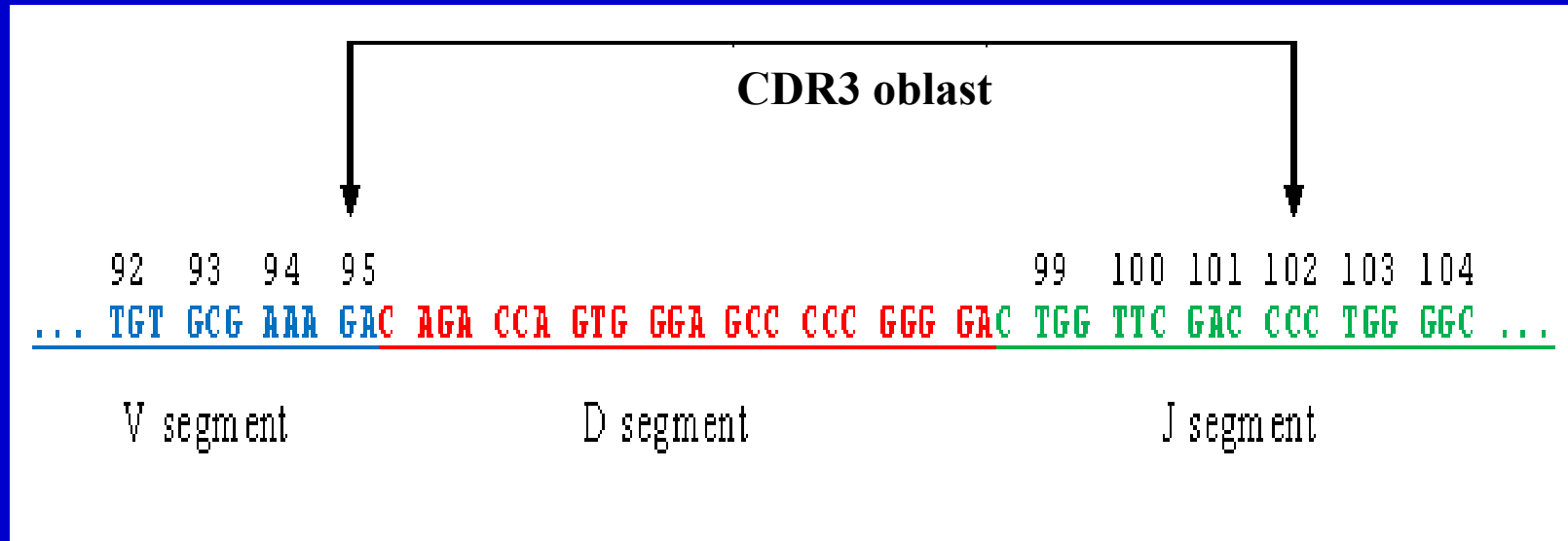
CDR2

	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
VH4-34	AGC	ACC	AAC	TAC	AAC	CCG	TCC	CTC	AAG	AGT	CGA	GTC	ACC	ATA	TCA
	-AT	---	---	---	---	---	---	---	---	---	---	---	---	---	---

	71	72	73	74	75	76	77	78	79	80	81	82	82a	82b	82c
VH4-34	GTA	GAC	ACG	TCC	AAG	AAC	CAG	TTC	TCC	CTG	AAG	CTG	AGC	TCT	GTG
	--g	---	---	---	---	---	---	---	---	---	--a	---	-C-	---	---

	83	84	85	86	87	88	89	90	91	92	93	94
VH4-34	ACC	GCC	GCG	GAC	ACG	GCT	GTG	TAT	TAC	TGT	GCG	AGA
	---	---	A--	---	---	---	--c	---	--t	---	---	---

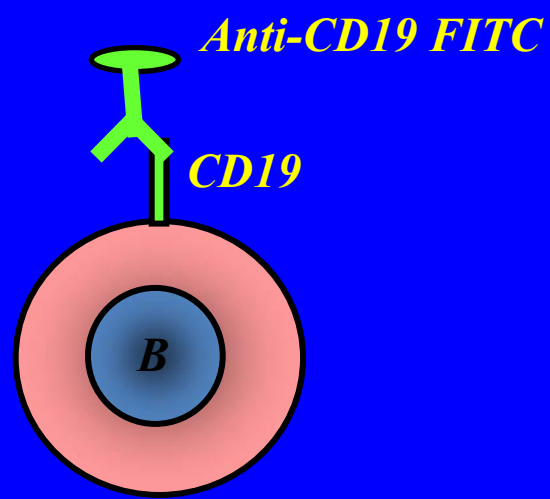
## Délka a sekvence CDR3 oblasti



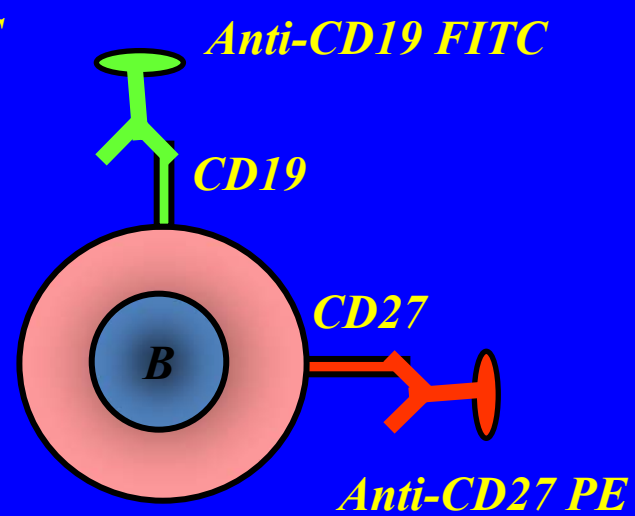
- informace o Ag řízené selekci/diferenciačním stádiu a klonální příbuznosti

*(Schroeder HW Dev Comp Immunol 2006,  
Zemlin M et al et al. Mol Immunol 2005)*

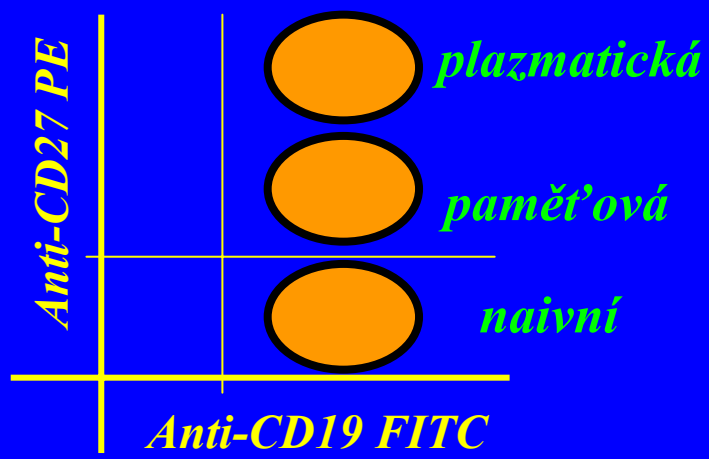
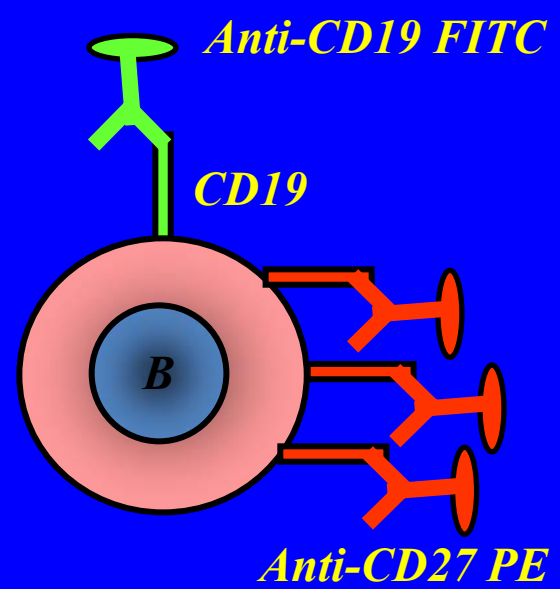
*NAIVNÍ B b.*  
*CD19<sup>+</sup>CD27<sup>-</sup>*



*PAMĚŤOVÁ B b.*  
*CD19<sup>+</sup>CD27<sup>+</sup>*



*PLAZMATICKÁ B b.*  
*CD19<sup>+</sup>CD27<sup>high</sup>*



# CD27 charakterizuje paměťové B lymfocyty

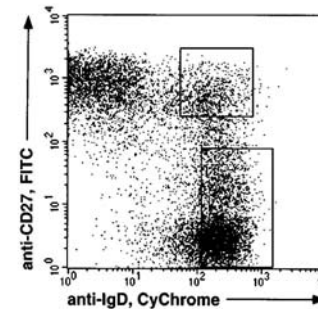
**Human Immunoglobulin (Ig)M<sup>+</sup>IgD<sup>+</sup> Peripheral Blood B Cells Expressing the CD27 Cell Surface Antigen Carry Somatically Mutated Variable Region Genes: CD27 as a General Marker for Somatically Mutated (Memory) B Cells**  
 By Ulf Klein, Klaus Rajewsky, and Ralf Küppers

*From the Institute for Genetics, University of Cologne, 50931 Cologne, Germany*

## Discussion

### IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B Cells Presumably Represent a Third Phenotypically Defined Memory B Cell Subset in Humans.

Almost all IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B cells analyzed carried somatically mutated V region genes, in contrast to IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>-</sup> B lymphocytes (Table 1). IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> cells phenotypically resemble both class-switched and IgM-only cells in that they are CD27<sup>+</sup>, CD23<sup>-</sup>, and CD5<sup>-</sup> (Fig. 1; reference 4), and generally appear to be larger than CD27<sup>-</sup> B cells (15, 16; our unpublished observations). Unlike IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>-</sup> cells, they express high levels of membrane IgM (Fig. 1), similar to IgM-only cells (4). That IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> cells may represent memory B cells is further supported by the following observations. (a) Upon stimulation in in vitro assays, both IgD<sup>-</sup>CD27<sup>+</sup> and IgD<sup>+</sup>CD27<sup>+</sup> cells—in contrast to IgD<sup>+</sup>CD27<sup>-</sup> cells—are quickly activated and secrete large amounts of Ig (15, 16).



**Figure 2.** Fluorescence analysis of B cells derived from the PB of a healthy adult. Anti-IgD/anti-CD27 two-color staining of CD19<sup>+</sup> PB B cells enriched by magnetic cell separation (>98% purity). Indicated are the gates set for sorting of single IgD<sup>+</sup>CD27<sup>+</sup> and IgD<sup>+</sup>CD27<sup>-</sup> cells.

**!!!1998 !!!**

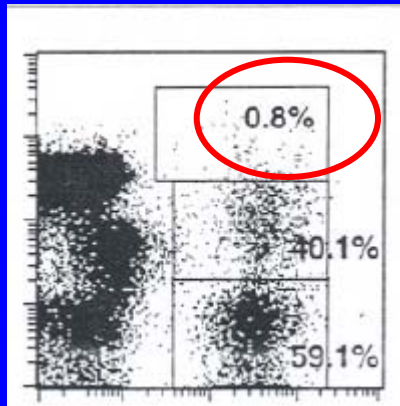
**Klein U, Rajewsky K, Küppers R**

***J Exp Med 188: 1679-1689***

**• paměťové IgM<sup>+</sup>IgD<sup>+</sup>CD27<sup>+</sup> B lymfocyty v PK se SHM - somatickými hypermutacemi**

**PK  
KONTROLA**

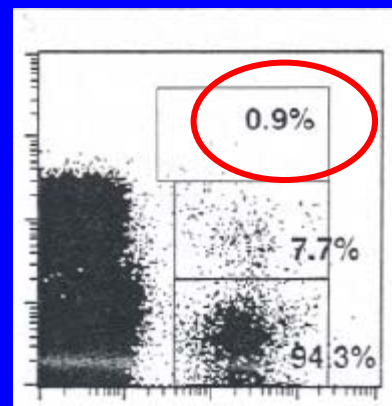
**CD27 PE**



**CD19 FITC**

**PK  
NEAKTIVNÍ SLE**

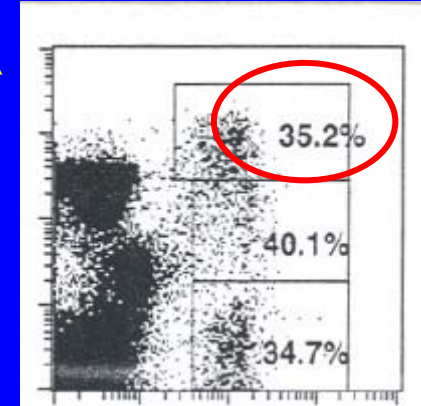
**CD27 PE**



**CD19 FITC**

**PK  
AKTIVNÍ RA**

**CD27 PE**

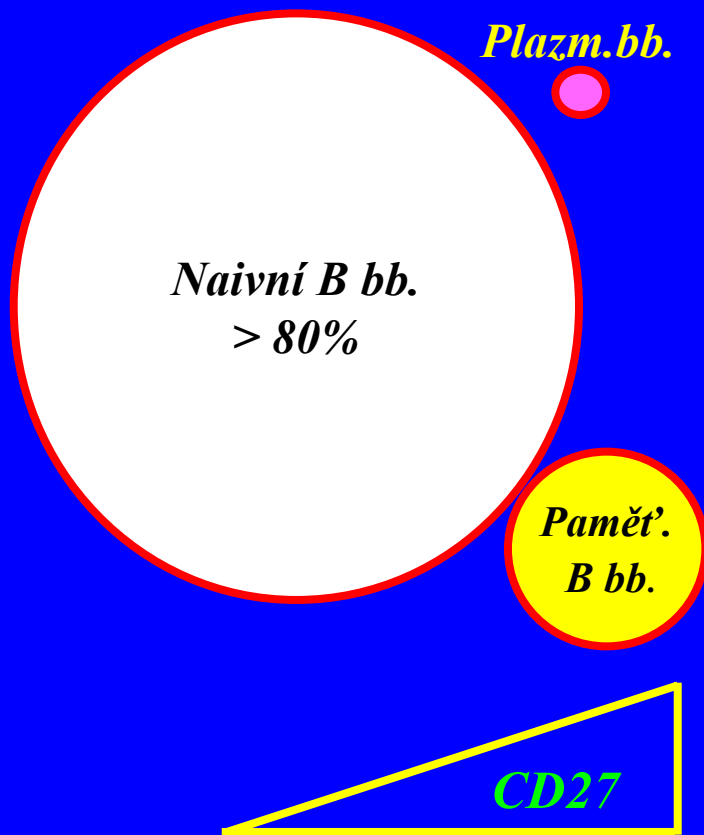


**CD19 FITC**

**!!! Frekvence CD19<sup>+</sup>CD27<sup>high</sup> plazmatických buněk  
souvisí s aktivitou choroby !!!**

*(Dörner T, Lipsky PE Arthritis Res 2002)*

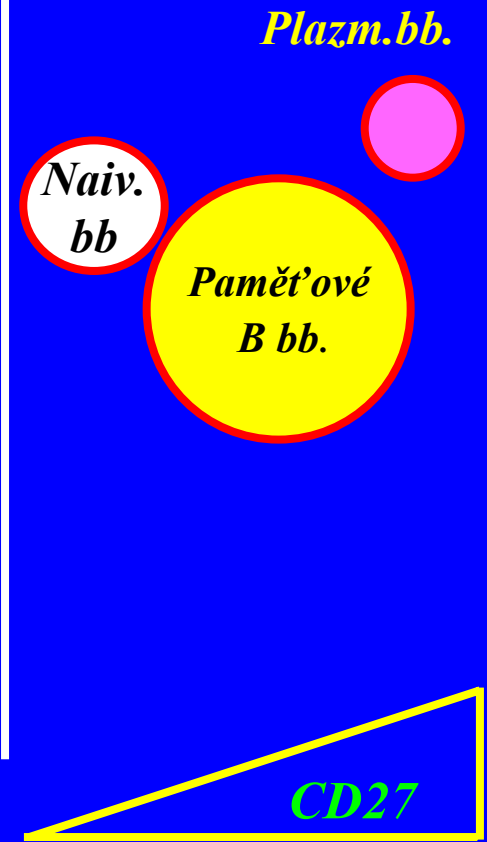
### Sjögrenův syndrom



### Zdravá populace



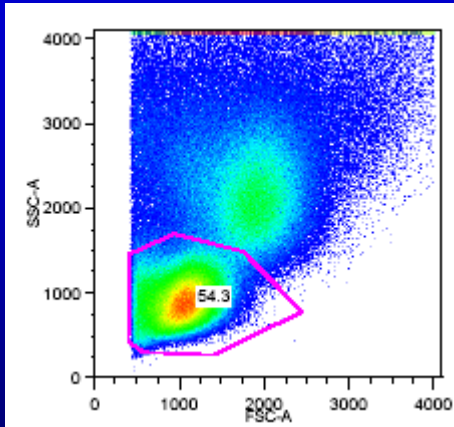
### SLE



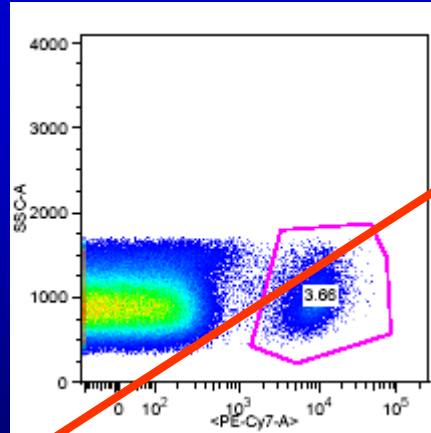
(Dörner T, Lipsky PE Arthritis Res 2002)

... nebo slouží jako predikční marker  
odpovědi na biologickou léčbu

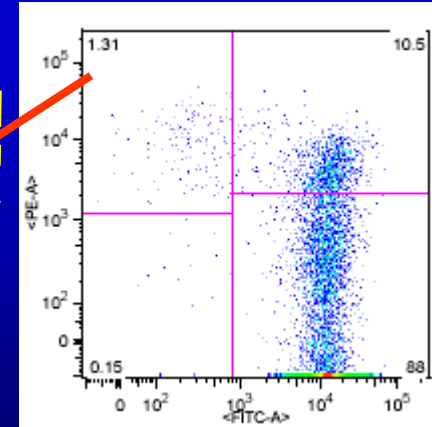
SSC



SSC



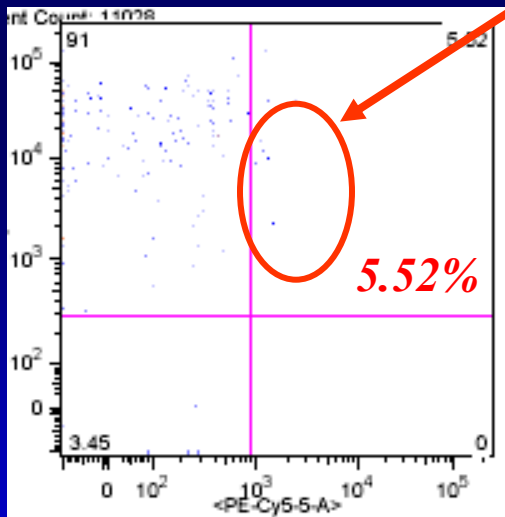
CD27 PE



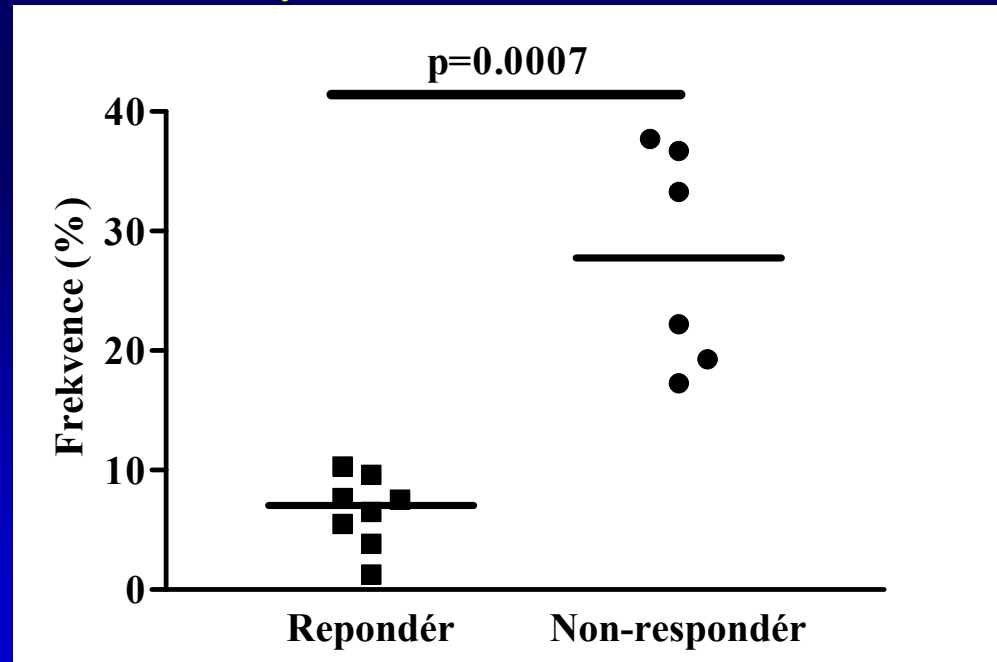
CD19 PE-Cy7 FITC

CD20 FITC

CD38 APC-Cy5.5



CD138 PE-Cy5.5



## Effect of Long-Term Belimumab Treatment on B Cells in Systemic Lupus Erythematosus

Extension of a Phase II, Double-Blind, Placebo-Controlled, Dose-Ranging Study

Annett M. Jacobi,<sup>1</sup> Weiqing Huang,<sup>1</sup> Tao Wang,<sup>1</sup> William Freimuth,<sup>2</sup> Inaki Sanz,<sup>3</sup> Richard Furie,<sup>4</sup> Meggan Mackay,<sup>1</sup> Cynthia Aranow,<sup>1</sup> Betty Diamond,<sup>1</sup> and Anne Davidson<sup>1</sup>

**Objective.** To understand the effects of long-term BlyS inhibition in human systemic lupus erythematosus (SLE).

**Methods.** Seventeen patients with SLE who were enrolled in a clinical trial of belimumab, a BlyS-specific inhibitor, plus standard of care therapy were studied. Phenotypic analysis of lymphocytes was performed using flow cytometry. Circulating antibody-secreting cells were enumerated using enzyme-linked immunospot assay. Serum was analyzed by enzyme-linked immunosor-

anti-double-stranded DNA antibody levels were available as part of the clinical trial analyses.

**Results.** Samples were collected on days 0, 84, 168, 365, and 532 and after day 730. The total number of B cells started to decrease from baseline between days 84 and 168. This was due to a decrease in naive and transitional B cells. CD27+IgD+ memory B cells and plasmablasts decreased only after 532 days, whereas CD27+IgD- memory B cells were not affected, and there were no changes in T cells. Serum IgM levels

*Jacobi AM et al,*

*Arthritis Rheum 2010*

• *signifikanční pokles*

*CD19<sup>+</sup>CD27<sup>+</sup>IgD<sup>+</sup>*

*paměťových B buněk*

*v PK*

*u pacientů se SLE*

*po anti-BlyS terapii*

# *ALE je to opravdu tak jednoznačné?*

*vlastní pozorování („single-cell“ RT PCR)*

*~ 5% PK [CD19<sup>+</sup>(CD20<sup>+</sup>)CD27<sup>-</sup>] naivních B lymfocytů*

*(KO, SLE, Sj. sy, RA)*

*vykazuje typ a rozložení SHM*

*charakteristických pro*

*klasické (konvenční) paměťové*

*B lymfocyty [CD19<sup>+</sup>(CD20<sup>+</sup>)CD27<sup>+</sup>]*

.....

**!!! Obvyklá „doporučená“ praxe !!!**

- *ignorování jejich přítomnosti*
  - *vyřazení z databáze*
  - *vyřazení z analýzy dat*

# *CD19<sup>+</sup>IgD<sup>-</sup>CD95<sup>+</sup>CD27<sup>-</sup> paměť'ové B lymfocyty v PK u SLE*

ARTHRITIS & RHEUMATISM  
Vol. 58, No. 6, June 2008, pp 1762-1773  
DOI 10.1002/art.23498  
© 2008, American College of Rheumatology

## Activated Memory B Cell Subsets Correlate With Disease Activity in Systemic Lupus Erythematosus

Delineation by Expression of CD27, IgD, and CD95

Annett M. Jacobi,<sup>1</sup> Karin Reiter,<sup>2</sup> Meggan Mackay,<sup>3</sup> Cynthia Aranow,<sup>3</sup> Falk Hiepe,<sup>4</sup>  
Andreas Radbruch,<sup>5</sup> Arne Hansen,<sup>2</sup> Gerd-R. Burmester,<sup>2</sup> Betty Diamond,<sup>3</sup>  
Peter E. Lipsky,<sup>6</sup> and Thomas Dörner<sup>4</sup>

*Objective.* Analysis of peripheral B cell subsets in patients with systemic lupus erythematosus (SLE) has provided evidence of specific alterations, such as an expansion of CD27<sup>++</sup> plasma cells/blasts and transitional B cells. However, memory B cells in lupus have not been thoroughly investigated, and only recently a CD27<sup>-</sup> memory B cell subset was identified in the peripheral blood of lupus patients. Focusing on CD27<sup>-</sup> B cells, this study aimed to identify abnormalities in peripheral B cell subsets in patients with SLE.

*Methods.* Three independent cohorts of lupus patients were used to characterize CD27<sup>-</sup> memory B cells, using multiparameter flow cytometry and single-cell reverse transcription-polymerase chain reaction of heavy-chain transcripts.

*Results.* We identified a homogeneous subset of

CD27<sup>-</sup>,IgD<sup>-</sup>,CD95<sup>+</sup> memory B cells with an activated phenotype that was increased in patients with disease flares and that correlated with disease activity and serologic abnormalities. In contrast, the entire subset of CD27<sup>-</sup>,IgD<sup>-</sup> B cells was found to be heterogeneous, did not correlate significantly with lupus activity, and was also increased in patients with bacterial infections.

*Conclusion.* We conclude that CD95 is a useful marker to identify CD27<sup>-</sup> memory B cells with an activated phenotype, which might serve as a biomarker for lupus activity and as a target of further investigations aiming to elucidate the pathogenic potential of these cells and the mechanisms involved in the generation as well as regulation of this CD27<sup>-</sup>,IgD<sup>-</sup>,CD95<sup>+</sup> memory B cell subset.

*Jacobi AM et al,*

*Arthritis Rheum 2008*

• *signifikantní korelace*

*s aktivitou choroby !*

• *SHM jako v konvenčních*

*paměť'ových B ly*

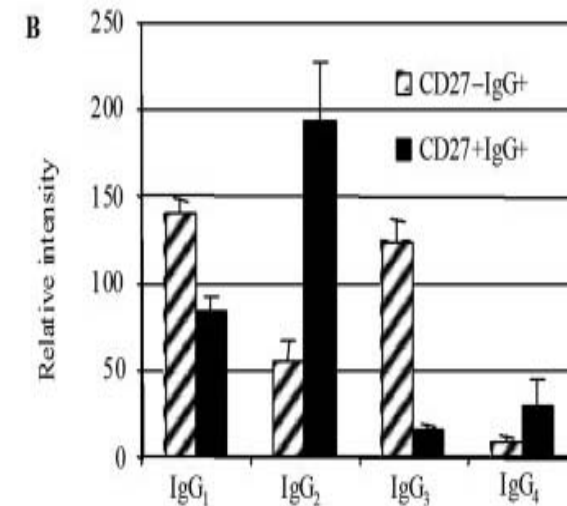
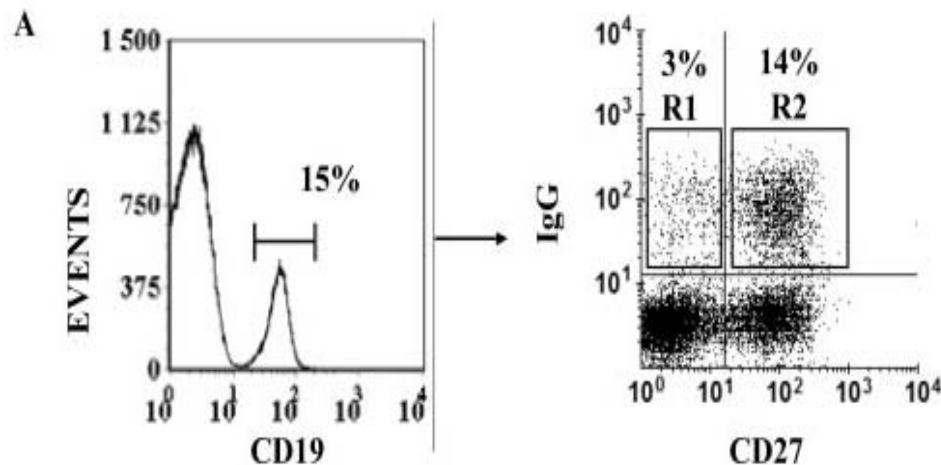
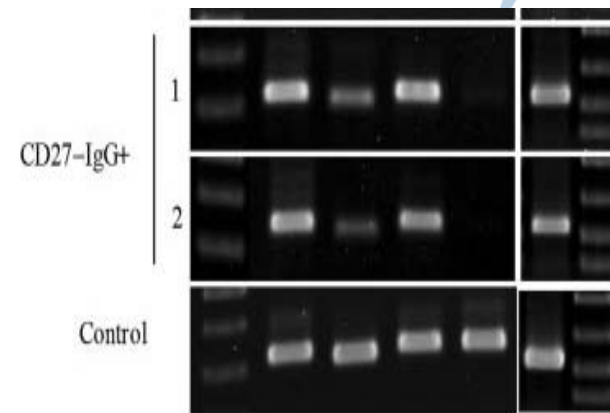
• *produkce IgG*

# *CD19<sup>+</sup>IgG<sup>+</sup>CD27<sup>-</sup> paměť'ové*

## *B lymfocyty v PK u KO*

A New Memory CD27<sup>-</sup>IgG<sup>+</sup> B Cell Population in Peripheral Blood Expressing V<sub>H</sub> Genes with Low Frequency of Somatic Mutation<sup>1</sup>

Jessie F. Fecteau, Geneviève Côté, and Sonia Néron<sup>2</sup>



*Fecteau JF et al, J Immunol 2006 – SHM a produkce IgG*

## *CD19<sup>+</sup>FCRL4<sup>+</sup> CD27<sup>-</sup> paměťové*

### *B lymfocyty v tonsilách KO*

*Erhardt GR et al, J Exp Med 2005*

- *Fc receptor-like 4 (FCRL4, FcRH4 pozitivní B lymfocyty)*

#### *CD27 negativní*

- *SHM jako v konvenčních paměťových B ly*
- *v TK odpovídají na stimulaci IL-2 a IL-10*


.....

## *CD19<sup>+</sup>IgD<sup>+</sup> IgM<sup>+</sup>CD27<sup>-</sup> paměťové B lymfocyty*

### *v PK u pacientů s leukémií*

*Weston-Bell N et al, Leukemia 2009*

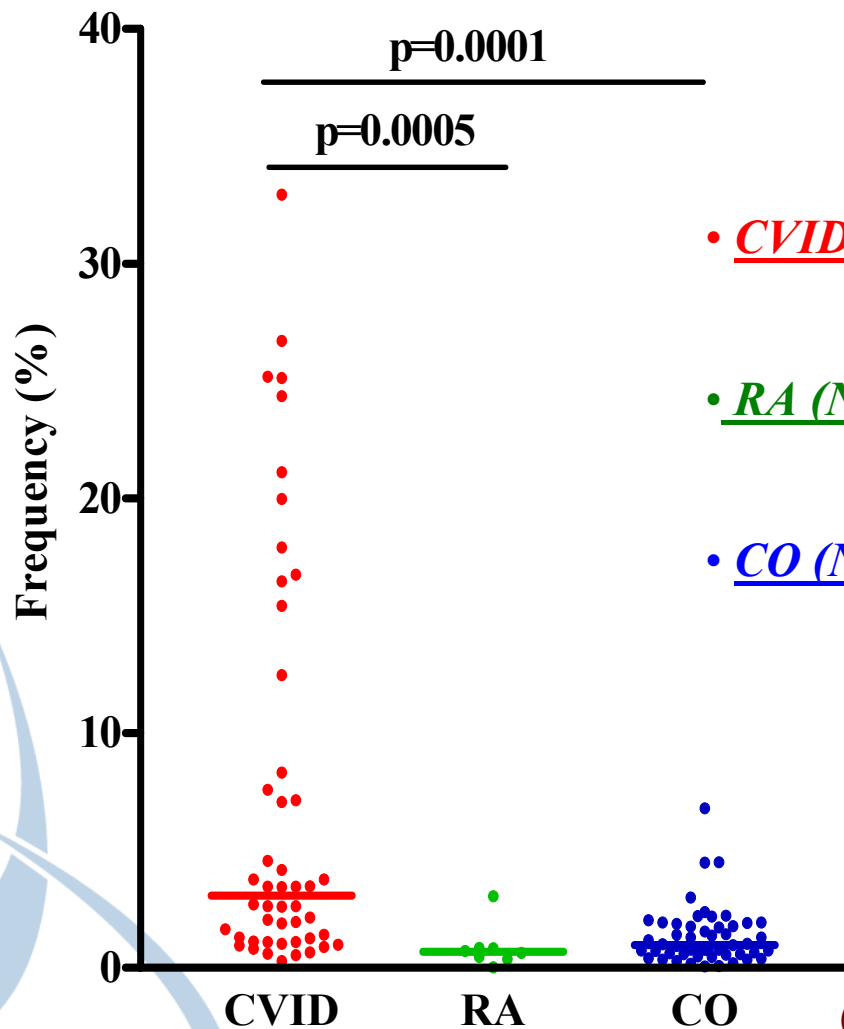
- *hairy cell leukemia*
- *SHM jako v konvenčních paměťových B ly*

A large, pink, jagged starburst shape that frames the central text.

... a co na to  
»přátelé B lymfocytů«  
v Praze?

## $CD19^+CD27^-CD21^{low}CD38^{low}CD24^{-/+}IgM^+$

### *B buňky pacientů s CVID, kontrol (KO) a pacientů s RA*



• CVID (N=48) 0.30-32.95%,

95% CI 4.72-9.83, median 3.08±1.27

• RA (N=8) 0.03-3.5%,

95% CI 0.10-1.64, median 0.68±0.33

• CO (N=51) 0.06-6.8%,

95% CI 1-1.70, median 0.98±0.17

*Významně zvýšená frekvence*

*v PK u pacientů s CVID oproti KO a RA*

*(minoritní; ze všech CD19<sup>+</sup> B lymfocytů;*

*Vlková M et al, J Immunol 2010)*

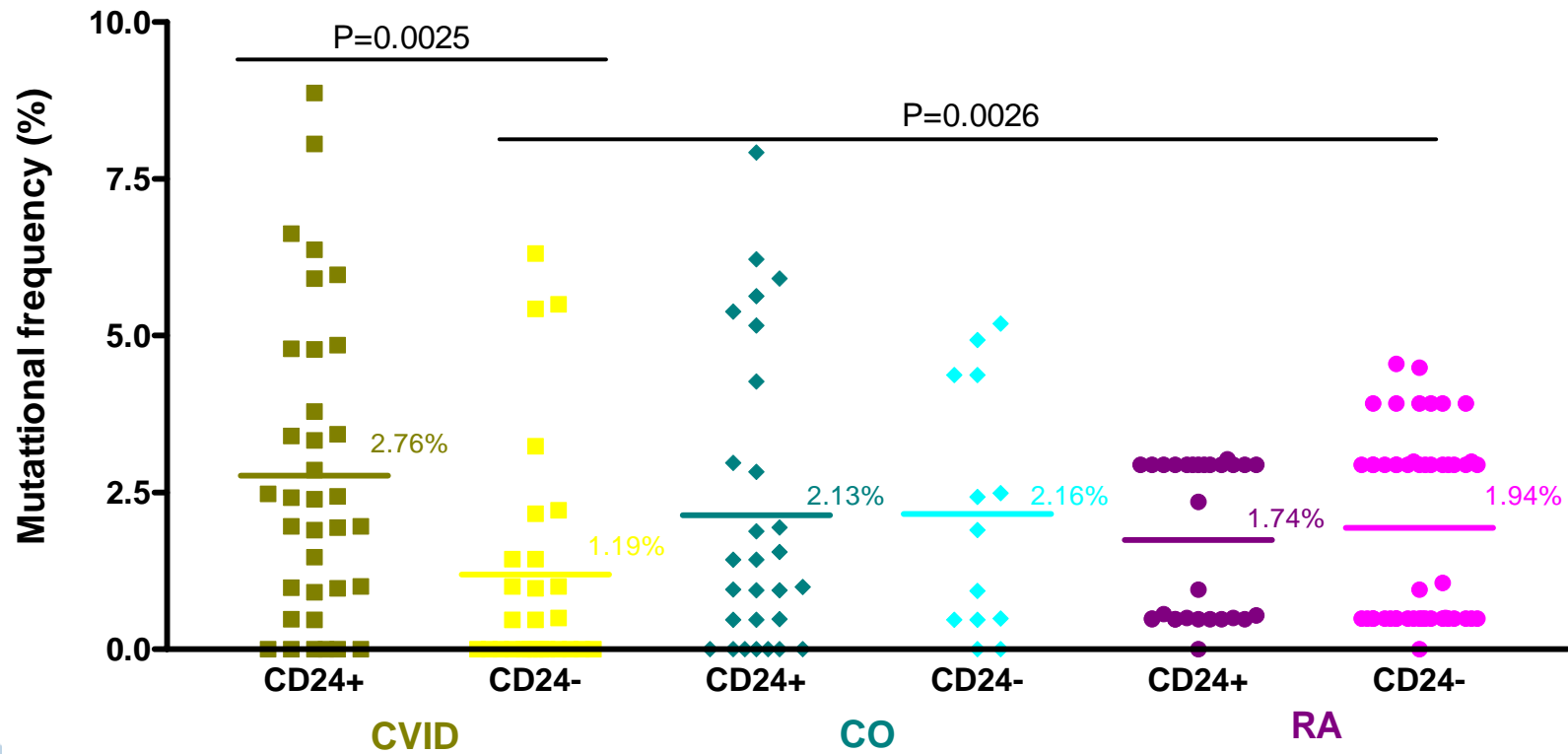
## Cíle:

- *analýza uvedených subpopulací pomocí „single-cell“ RT-PCR u pacientů s CVID (N=2), KO (N=2) a RA (N=2)*
- *mutační frekvence a rozložení, distribuce a typ aminokyselin, VH, DH, JH repertoár, délka a sekvence CDR3, přítomnost IgM/IgG mRNA*
- *CD27 negativita nasvědčuje pro naivní typ B lymfocytů*

*... „NUDA V PRAZE“?*

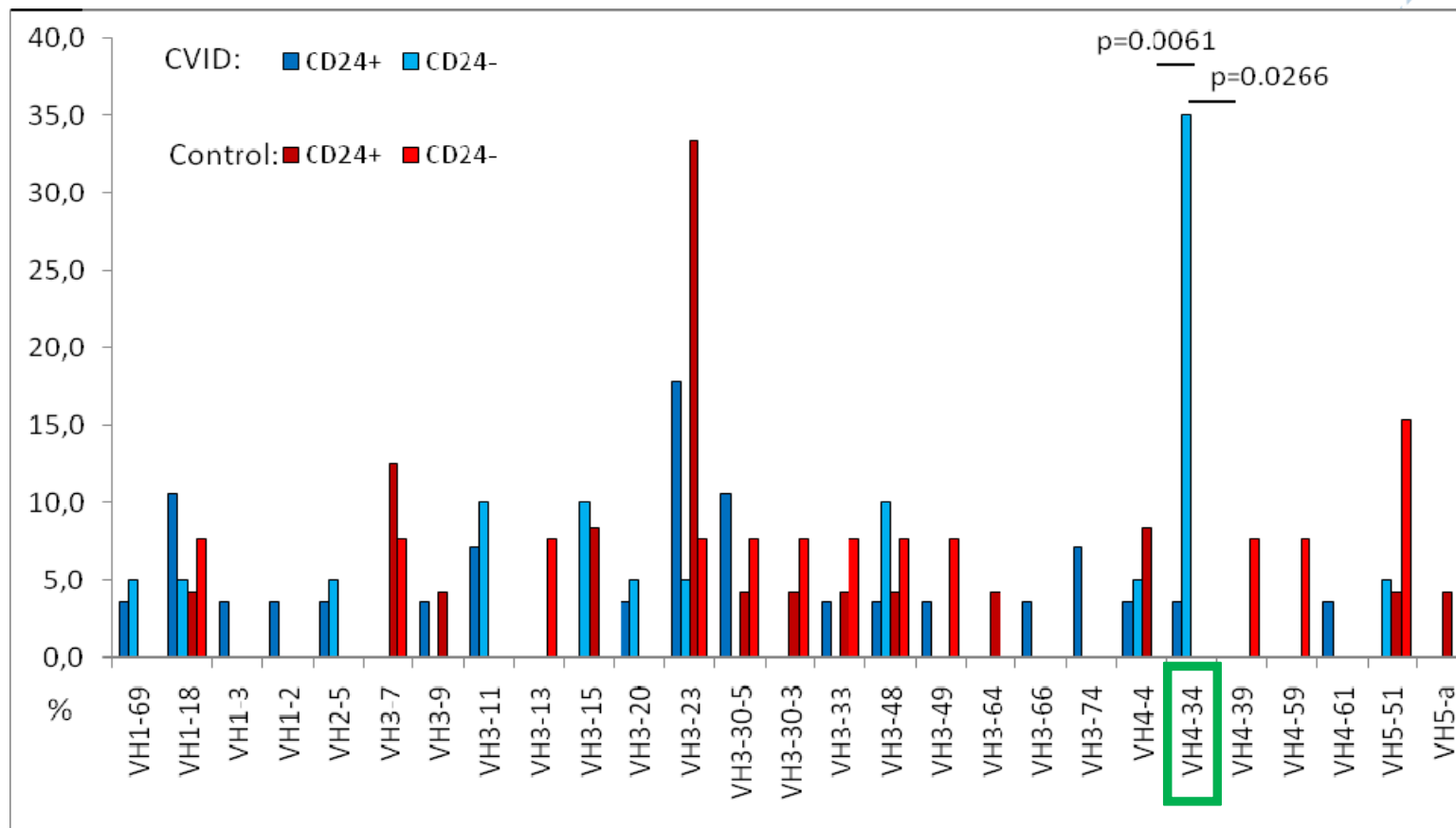


## *Mutační frekvence v IgM mRNA u CVID, KO a RA*



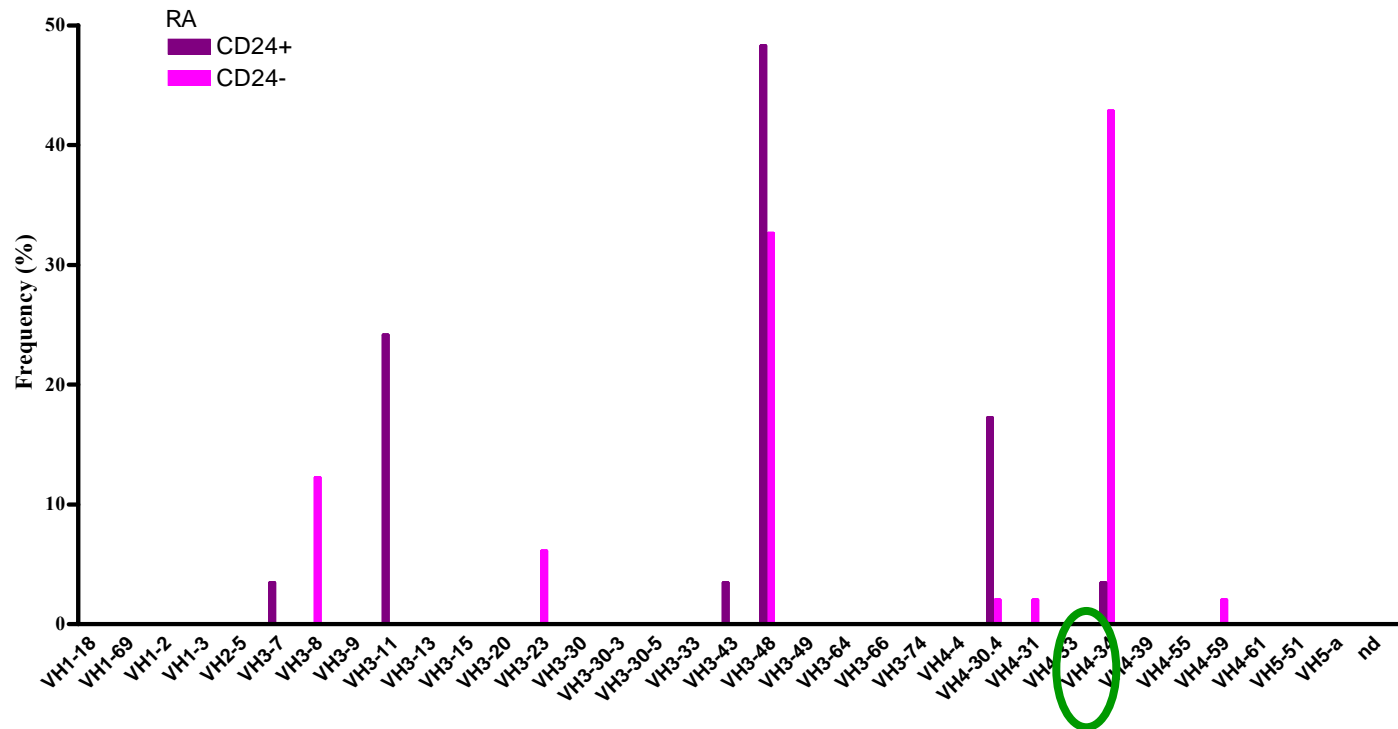
***Významná redukce mutační frekvence CD24<sup>-</sup> v B ly CVID a KO  
u RA pacientů mutační frekvence stejné***

## Frekvence VH genových segmentů I.



**• predominance VH4-34 segmentu u CVID  
a jeho significantní absence u KO; 35% vs. 0%; p=0,0026**

## Frekvence VH genových segmentů II.

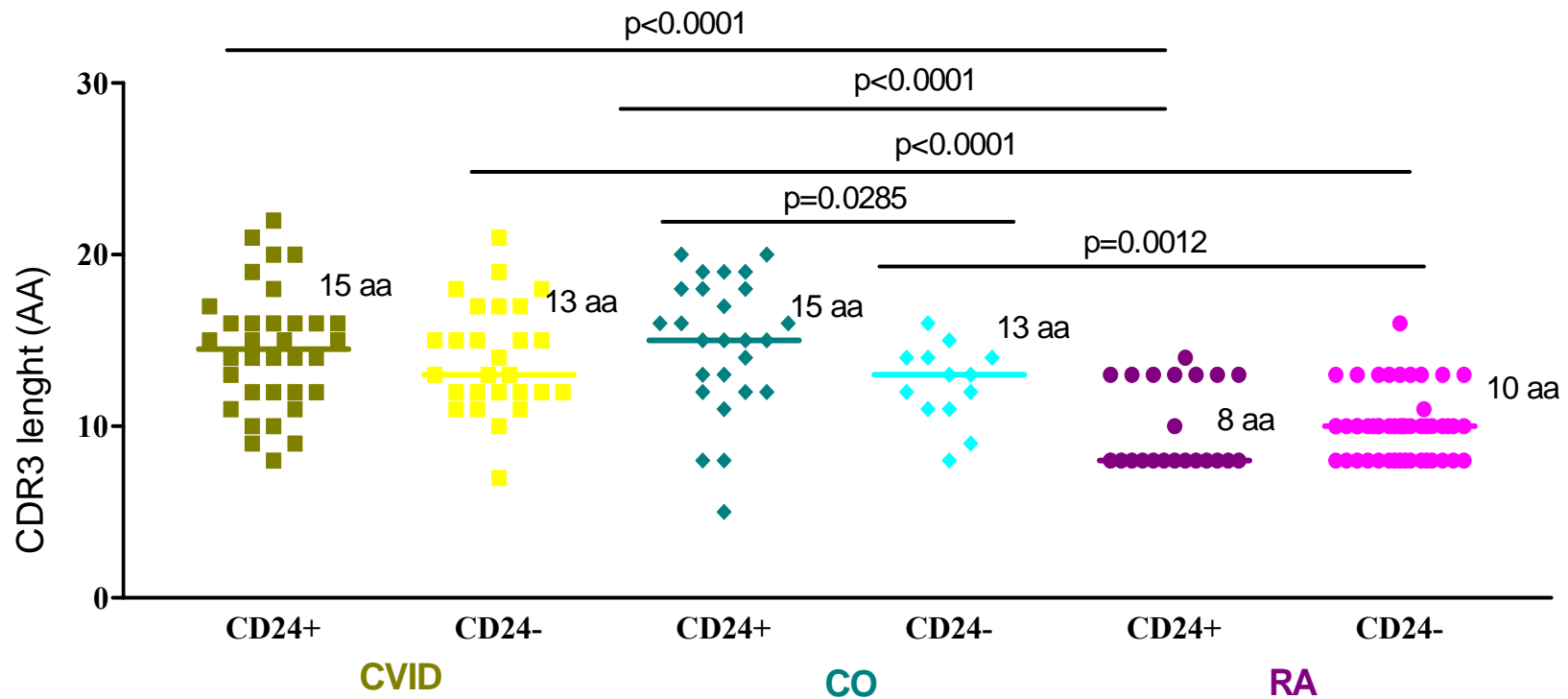


• *omezené využití VH genů (10 VH genů)*

• *predominance VH4-34 segmentu u RA*

*a jeho signifikantní absence u KO; (43%; vs. 0%;  $p=0,0092$ )*

## CDR3 délka (aminokyseliny)



- **většina CDR3s u CVID a KO obsahovala delší oblasti**
- **CD24<sup>-</sup> B ly u KO a CVID významně kratší CDR3 vs. jejich CD24<sup>+</sup> protějšky**
- **RA – významně zkrácená délka, srovnatelná u obou subpopulací**

# *Klonální příbuznost I. (amk. sekvence v CDR3)*

*3'-konec V<sub>H</sub> segmentu  
(VH 5-a)*

*D<sub>H</sub> segment  
(DH 5-12)*

*5'-konec J<sub>H</sub> segmentu  
(JH5)*

*S No. 7C:*

**TGT GCG AGA GAG TAT AGG GGC TAC GAT TCT TGG GGC CAG GGA ACC**

*S No. 7H:*

**TGT GCG AGA GAG TAT AGG GGC TAC GAT TCT TGG GGC CAG GGA ACC**

- pouze 2 klonálně příbuzné B bb. (IgG<sup>+</sup>, CD24<sup>-</sup> B ly u KO)*
- výrazná polyklonalita*

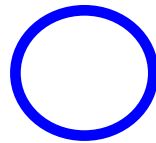
## *Klonální příbuznost II. (amk. sekvence v CDR3)*

**CVID**



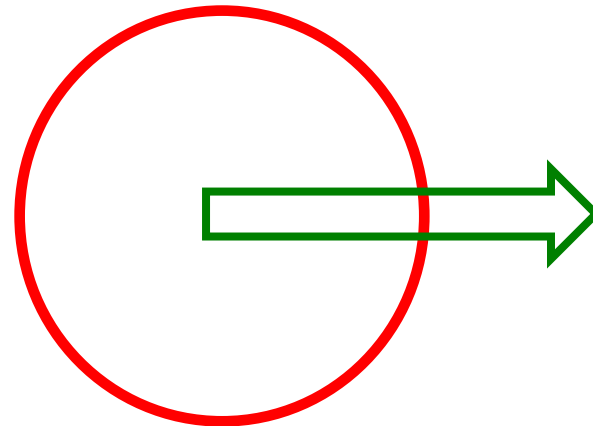
**0%**

**KO**



**4.5%**

**RA**



**76%**

**60% - VH4-34**

- u RA 76% všech B lymfocytů **KLONÁLNĚ PŘÍBUZNÉ**  
(více než 60% z nich jsou VH4-34 pozitivní)

## Závěr

- *ad analyzované CD27- B ly subpopulace*

- *Velmi heterogenní a polyklonální subpopulace u KO a CVID*
- *Tyto CD27- B bb. by mohly představovat nový typ diferencovaných „memory-like“ B ly/“Ag-experienced“ (u CVID méně diferencovaných)*
- *s pravděpodobnou aktivitou v ochraně organismu proti bakteriální a virové infekci*  
*(Rakhmanov M et al. PNAS 2009; Wenston-Bell N et al. Leukemia 2009; Erhardt GR et al. J Exp Med 2005)*

## *Závěr*

- *ad klonalita a VH4-34 genu RA*

- *téměř homogenní a oligoklonální subpopulace (všechny buňky celkem v 7 klonech, vysoké zastoupení VH4-34 genu)*
- *s pravděpodobnou schopností uniknout negativní selekci, s autoreaktivním potenciálem*

## Závěr

- *ad CD27 negativní paměťové B lymfocyty*
- *CD27<sup>-</sup> B bb. se znaky antigenem řízené selekce existují, heterogenní, minoritní, fyziologická funkce ?*
- *CD27 není výlučným znakem pro paměťové B ly*
- *imunofenotypizace NESTAČÍ*
- *paměťové B buňky:*  
*konvenční (klasické) vs. nekonvenční (neklasické)*  
*[CD19<sup>+</sup>CD27<sup>+</sup>] [CD19<sup>+</sup>CD27<sup>-</sup>]*
- *termín antigenně - zkušené B lymfocyty (Ag-experienced B cells)*

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