# Innovative Diagnostics of Alloimmune Thrombocytopenia

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Conference
Safety on Platelet Transfusion
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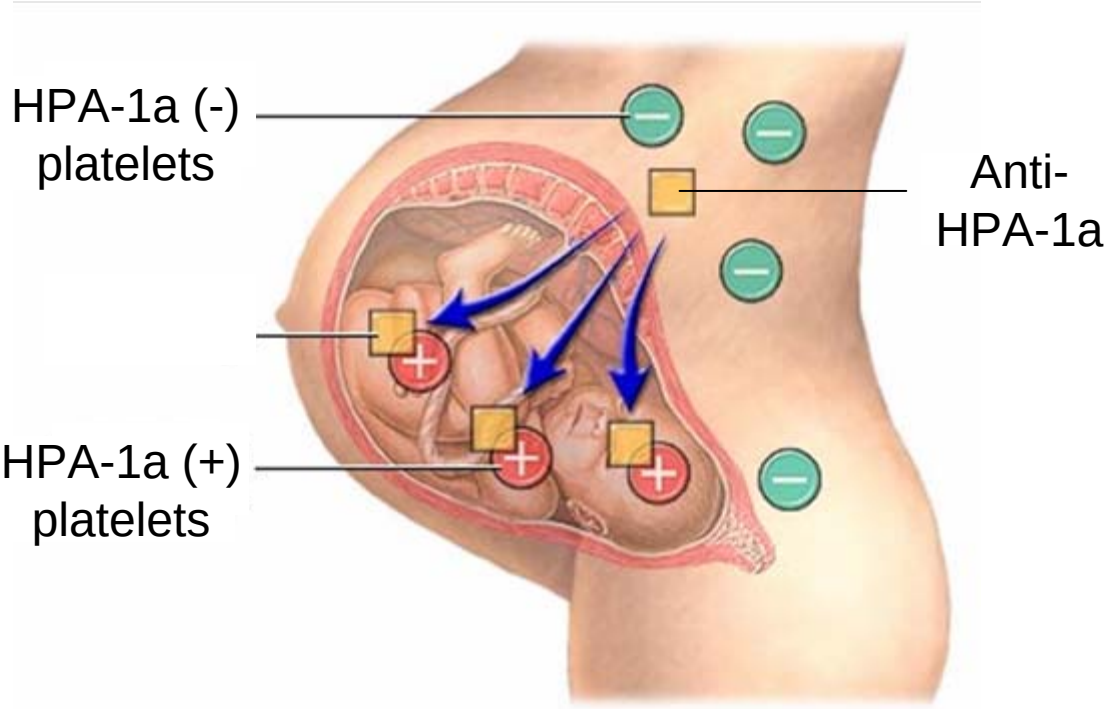
# Alloimmune Thrombocytopenic Syndromes

- **Fetal/Neonatal Alloimmune Thrombocytopenia (FNAIT)**
- Platelet Transfusion Refractoriness
- Post Transfusion Purpura
- Passive Alloimmune Thrombocytopenia
- Transplantation-Associated Thrombocytopenia

# Pathomechanism of FNAIT



# Human Platelet Alloantigens

| Antigen | Glycoprotein | Chromosome | Nucleotide Change | Mature Protein |
|---|---|---|---|---|
| HPA-1 | GPIIIa | 17 | 176T>C | L33P |
| HPA-2 | GPIbα | 17 | 482C>T | T145M |
| HPA-3 | GPIIb | 17 | 2621T>G | I843S |
| HPA-4 | GPIIIa | 17 | 506G>A | R143Q |
| HPA-5 | GPIa | 5 | 1600G>A | E505K |
| HPA-6w | GPIIIa | 17 | 1544G>A | R489Q |
| HPA-7w | GPIIIa | 17 | 1297C>G | P407A |
| HPA-8w | GPIIIa | 17 | 1984C>T | R636C |
| HPA-9w | GPIIb | 17 | 2602G>A | V837M |
| HPA-10w | GPIIIa | 17 | 263G>A | R62Q |
| HPA-11w | GPIIIa | 17 | 1976G>A | R633H |
| HPA-12w | GPIbβ | 22 | 119G>A | G15E |
| HPA-13w | GPIa | 5 | 2483C>T | T799M |
| HPA-14w | GPIIIa | 17 | 1909_1911del AAG | K611del |
| HPA-15 | CD109 | 6 | 2108C>A | S703Y |

# New Human Platelet Alloantigens

| Antigen | Glycoprotein | Chromosome | Nucleotide Change | Mature Protein |
|---|---|---|---|---|
| HPA-16w | GPIIIa | 17 | 497C>T | T140I |
| HPA-17w | GPIIIa | 17 | 622C>T | T195M |
| Sta | GPIIIa | 17 | AAG>CAG | K137Q |
| Bec | GPIIIa | 17 | AAG>GAG | K646E |
| Sol | GPIIIa | 17 | GAA>AAA | E628K |
| Kno | GPIIb | 17 | ACG>GCC | T619M |
| Rou | GPIIb | 17 | ACC>GCC | T9A |
| | | | | |
| | | | | |

# HPAs among Chinese population

TABLE 3. Comparison of HPA allelic frequencies (%) among Chinese population from different geographic regions

| | Geographic region | | | | |
|---|---|---|---|---|---|
| PLT antigen | Xiamen (n = 150) | Hong Kong (n = 100) | Taiwan (n = 566) | Minnan (n = 208) | Total (n = 1000) |
| HPA-1a | 99.00 | 99.50 | 99.83 | 99.80 | 99.40 |
| HPA-1b | 1.00 | 0.50 | 0.17 | 0.20 | 0.60 |
| HPA-2a | 93.33 | 97.50 | 96.10 | 95.70 | 95.15 |
| HPA-2b | 6.67 | 2.50 | 3.90 | 4.30 | 4.85 |
| HPA-3a | 57.67 | 52.50 | 59.50 | 55.00 | 59.45 |
| HPA-3b | 42.33 | 47.50 | 40.50 | 45.00 | 40.55 |
| HPA-4a | 100.00 | 100.00 | 99.83 | 99.50 | 99.55 |
| HPA-4b | 0.00 | 0.00 | 0.17 | 0.50 | 0.45 |
| HPA-5a | 99.00 | 96.50 | 98.14 | 99.00 | 98.60 |
| HPA-5b | 1.00 | 3.50 | 1.86 | 1.00 | 1.40 |
| HPA-15a | 59.67 | NA | 46.20 | NA | 53.20 |
| HPA-15b | 40.33 | NA | 53.80 | NA | 46.80 |
| Reference | This study | 16 | 17 | 18 | 19 |

*Ruan et al, Transfusion 2007*

# HPAs among Chinese population

Table 2. Distribution of HPA in the Chinese Han

| System | South Chinese ($n = 500$) | | | North Chinese ($n = 500$) | | | Combined ($n = 1000$) | | | | | Gene frequency | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | aa | ab | bb | aa | ab | bb | aa (%) | ab (%) | bb (%) | $\chi^2$ | $P_{(1)}$ | a | b | MP* (%) |
| HPA-1 | 494 | 6 | 0 | 494 | 6 | 0 | 988 (98·9) | 12 (1·2) | 0 (0) | 0·0364 | >0·75 | 0·9940 | 0·0060 | 1·19 |
| HPA-2 | 452 | 48 | 0 | 452 | 47 | 1 | 904 (90·4) | 95 (9·5) | 1 (0·1) | 0·8571 | >0·25 | 0·9515 | 0·0485 | 8·80 |
| HPA-3 | 177 | 233 | 90 | 181 | 240 | 79 | 358 (35·8) | 473 (47·3) | 169 (16·9) | 0·2050 | >0·50 | 0·5945 | 0·4055 | 36·59 |
| HPA-4 | 498 | 2 | 0 | 493 | 7 | 0 | 991 (99·1) | 9 (0·9) | 0 (0) | 0·0205 | >0·75 | 0·9955 | 0·0045 | 0·89 |
| HPA-5 | 487 | 13 | 0 | 486 | 13 | 1 | 973 (97·3) | 26 (2·6) | 1 (0·1) | 3·2980 | >0·05 | 0·9860 | 0·0140 | 2·72 |
| HPA-6 | 490 | 10 | 0 | 483 | 17 | 0 | 973 (97·3) | 27 (2·7) | 0 (0) | 0·1873 | >0·50 | 0·9865 | 0·0135 | 2·63 |
| HPA-7 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |
| HPA-8 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |
| HPA-9 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |
| HPA-10 | 499 | 1 | 0 | 500 | 0 | 0 | 999 (99·9) | 1 (0·1) | 0 (0) | 0·0003 | >0·97 | 0·9995 | 0·0005 | 0·10 |
| HPA-11 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |
| HPA-12 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |
| HPA-13 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |
| HPA-14 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |
| HPA-15 | 136 | 252 | 112 | 145 | 250 | 105 | 281 (28.1) | 502 (50·2) | 217 (21·7) | 0·0660 | >0·75 | 0·5320 | 0·4680 | 37·40 |
| HPA-16 | 500 | 0 | 0 | 500 | 0 | 0 | 1000 (100) | 0 (0) | 0 (0) | NA | | 1·0000 | 0·0000 | 0 |

NA, not appreciable. The $\chi^2$ test was used to test HW equilibrium.
*MP, calculated mismatch probability in random transfusion $= a^2(1 - a^2) + b^2(1 - b^2) = 2ab(1 - ab)$.

*Feng et al, Trans Med 2006*

# Diagnostics of Alloimmune Thrombocytopenia

- **Antigen Determination:**
  Phenotyping (*PIFT, MAIPA*)
  Genotyping (*PCR-RFLP, PCR-SSP, TaqMan*)

  limited amount of specific sera, platelets and DNA of rare HPA phenotypes!

- **Antibody Determination:**
  Binding Assays (*PIFT, ELISA, Flowcytometry*)
  Glycoprotein Specific Immunoassay (*MAIPA*)

  new generation of platelet antibody testing?

# Question

**How we can improve the diagnostics of alloimmune thrombocytopenia?**

# Production of stable cell lines as source for DNA and platelet alloantigens

- Generation of B-LCL cell panel from HPA phenotyped donors for genotyping analysis

- Production of stable transfected CHO Cells expressing HPAs for antibody characterization

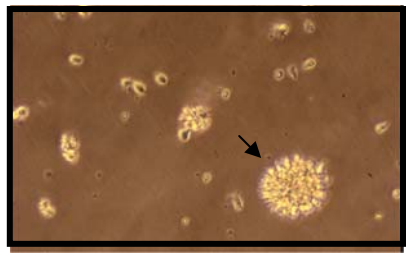
# Development of B-Cell Lines as Source for Standard DNA

B-Cells from HPA phenotyped Donors

EBV Transformation

B-Lymphoblastoid Cell Lines (B-LCL)

DNA



GIBS Cell Lines with known HPA genotypes

*Carl et al, Transfusion 2000*

*GIBS: Giessen Biological Standard*

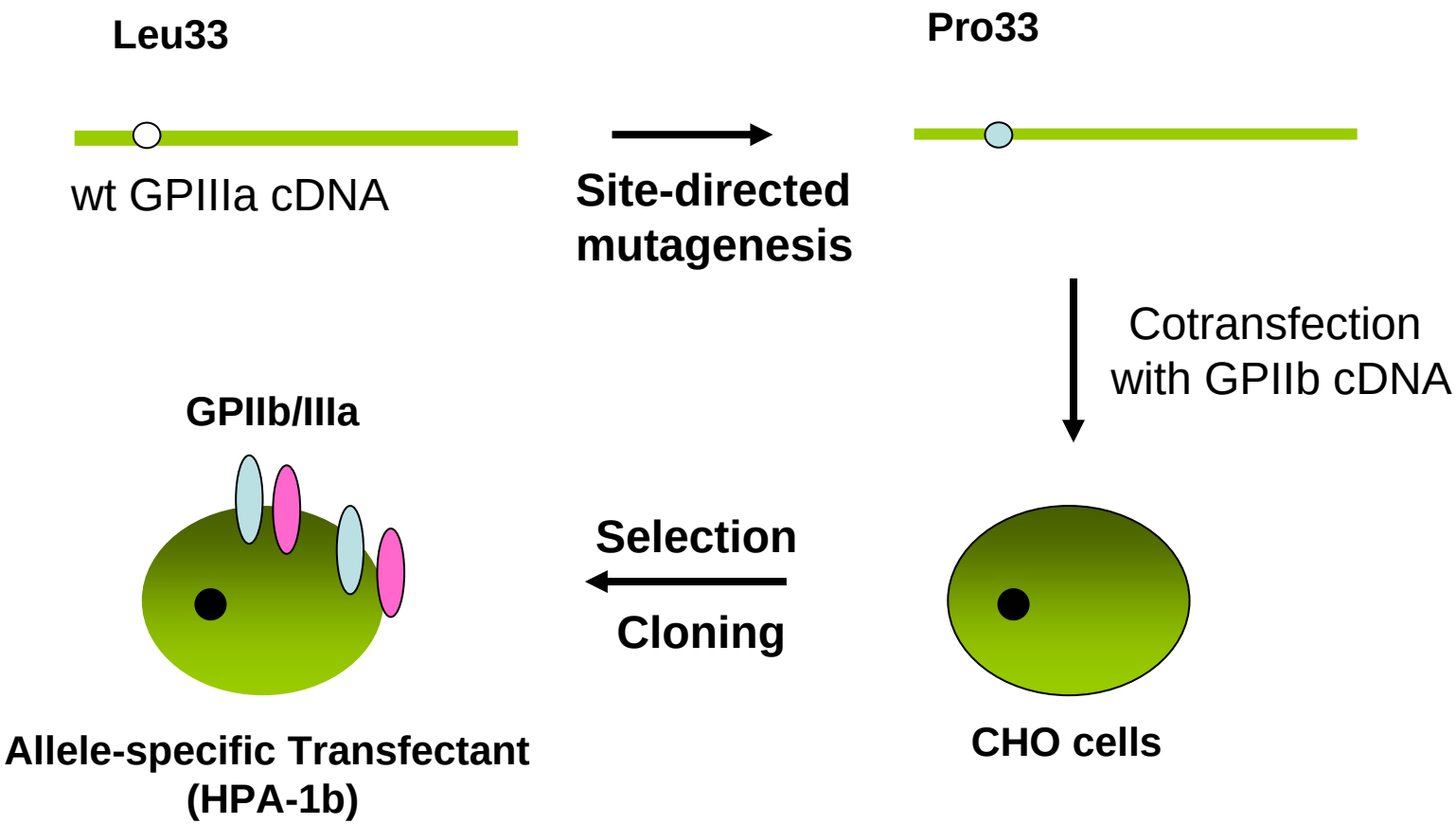
# Panels of B-LCL for HPA Genotyping

Giessen (Repository and Reference Laboratory of ISTH and ISBT)

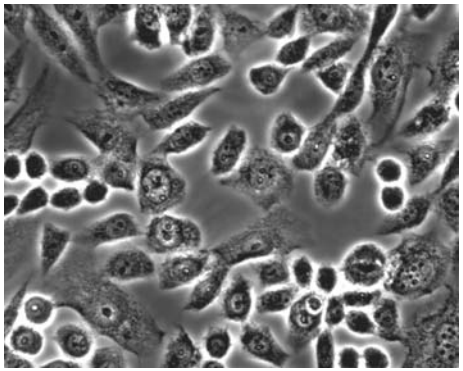
| Antigen | aa | bb | ab |
|---|---|---|---|
| HPA-1 | #02 | #01 | #05 |
| HPA-2 | #01 | #02 | #03 |
| HPA-3 | #02 | #03 | #05 |
| HPA-4 | #01 | #04 | #04.1 |
| HPA-5 | #02 | #05 | #01 |
| HPA-6w | #01 | #06 | #06.1 |
| HPA-7w | #01 | - | #07 |
| HPA-8w | #01 | - | #08 |
| HPA-9w | #01 | - | #09 |
| HPA-10w | #01 | - | Missing* |
| HPA-11w | #01 | - | #11 |
| HPA-12w | #01 | - | #12 |
| HPA-13w | #01 | - | #13 |
| HPA-14w | #01 | - | Missing* |
| HPA-15 | #15 | #16 | #17 |
| HPA-16w | #01 | - | Missing* |
| Swi(a) | #01 | - | #19 |
| Tu(a) | #01 | - | missing* |

*exist as DNA fragment produced by site-directed mutagenesis

# Generation of allele-specific stable cell lines expressing HPAs



# Panels of stable CHO transfected cells expressing HPAs



| Antigen | Cell line | Antigen | Cell line |
|---|---|---|---|
| HPA-1a | + | HPA-6bw | + |
| HPA-1b | + | HPA-7bw | + |
| HPA-2a | - | HPA-8bw | + |
| HPA-2b | - | HPA-9bw | + |
| HPA-3a | + | HPA-10bw | + |
| HPA-3b | + | HPA-11bw | + |
| HPA-4b | + | HPA-12bw | - |
| | | HPA-13bw | + |
| HPA-5a | + | HPA-14bw | + |
| HPA-5b | + | HPA-16bw | + |
| HPA-15a | - | HPA- | |
| HPA-15b | - | HPA- | |

- : missing

# Immunization against low-frequency human platelet alloantigen In fetal alloimmune thrombocytopenia is not a single event: characterization by the combined use of reference DNA and novel allele-specific cell lines expressing recombinant antigens

Hartmut Kroll, Julie Yates and Sentot Santoso

*Transfusion 2005;45:353-358*

Identification of alloimmunization against low-frequency HPA-8bw, -11bw and -13bw

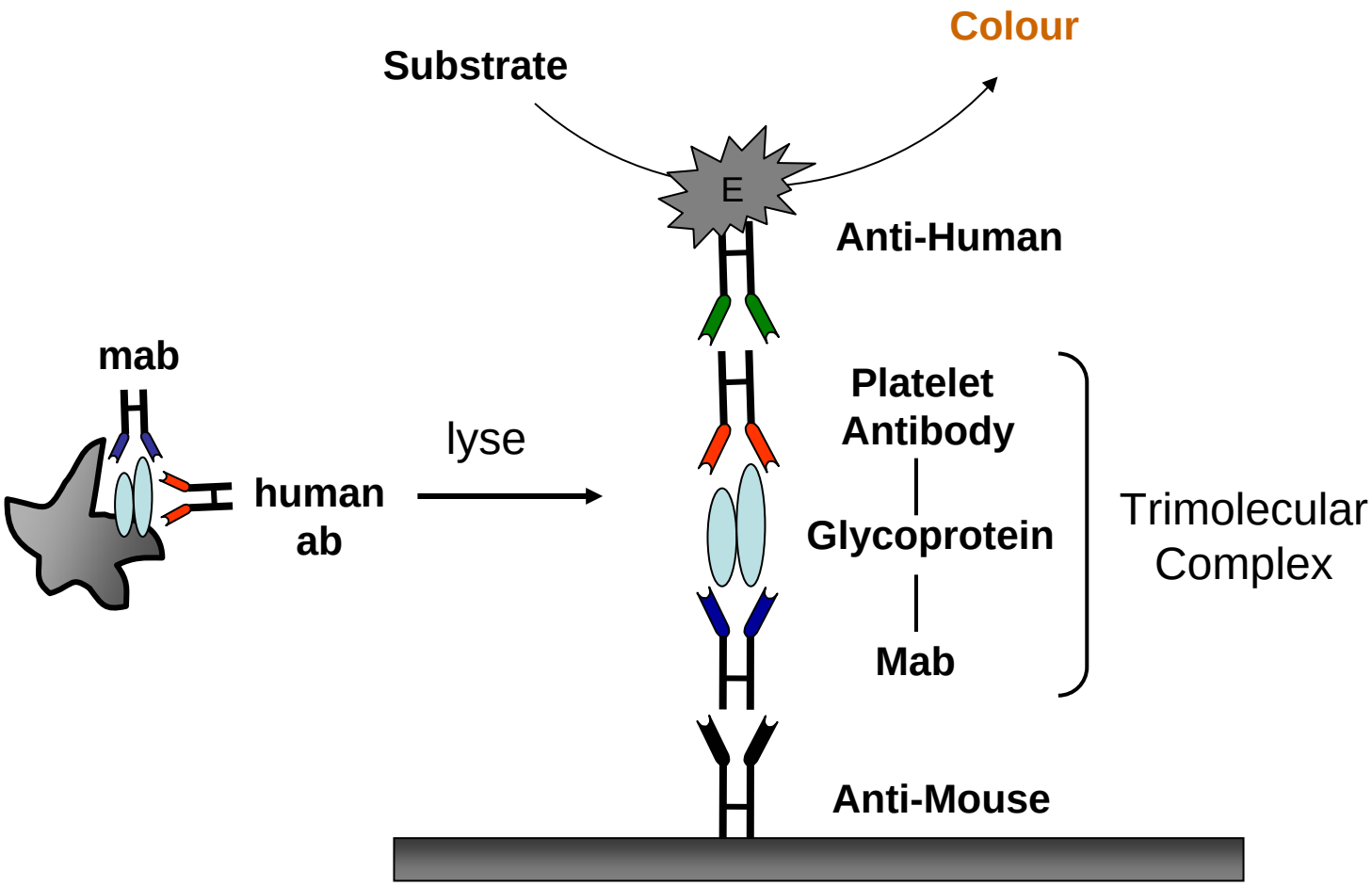
# Question

**Further improvement of platelet antibody testing?**

# Development of Platelet Antibody Assays

| Assay | Principle | Assays |
|---|---|---|
| I. Generation | Platelet function endpoint | Platelet aggregation<br>Complement fixation<br>Platelet factor 3 release<br>$^{14}$C Serotonin release |
| II. Generation | Measurement of surface platelet associated IgG | Radioimmunoassay<br>Immunofluorescence test<br>ELISA<br>Solid phase red cell adherence |
| III. Generation | Use of platelet glycoproteins as targets | Immunobead assay<br>Monoclonal Antibody Immobilization of Platelet Antigens (MAIPA, MACE)<br>Modified MAIPA (SASPA, ASPA) |
| IV. Generation | Use of recombinant antigens? | ? |

# MAIPA
## 20 Years

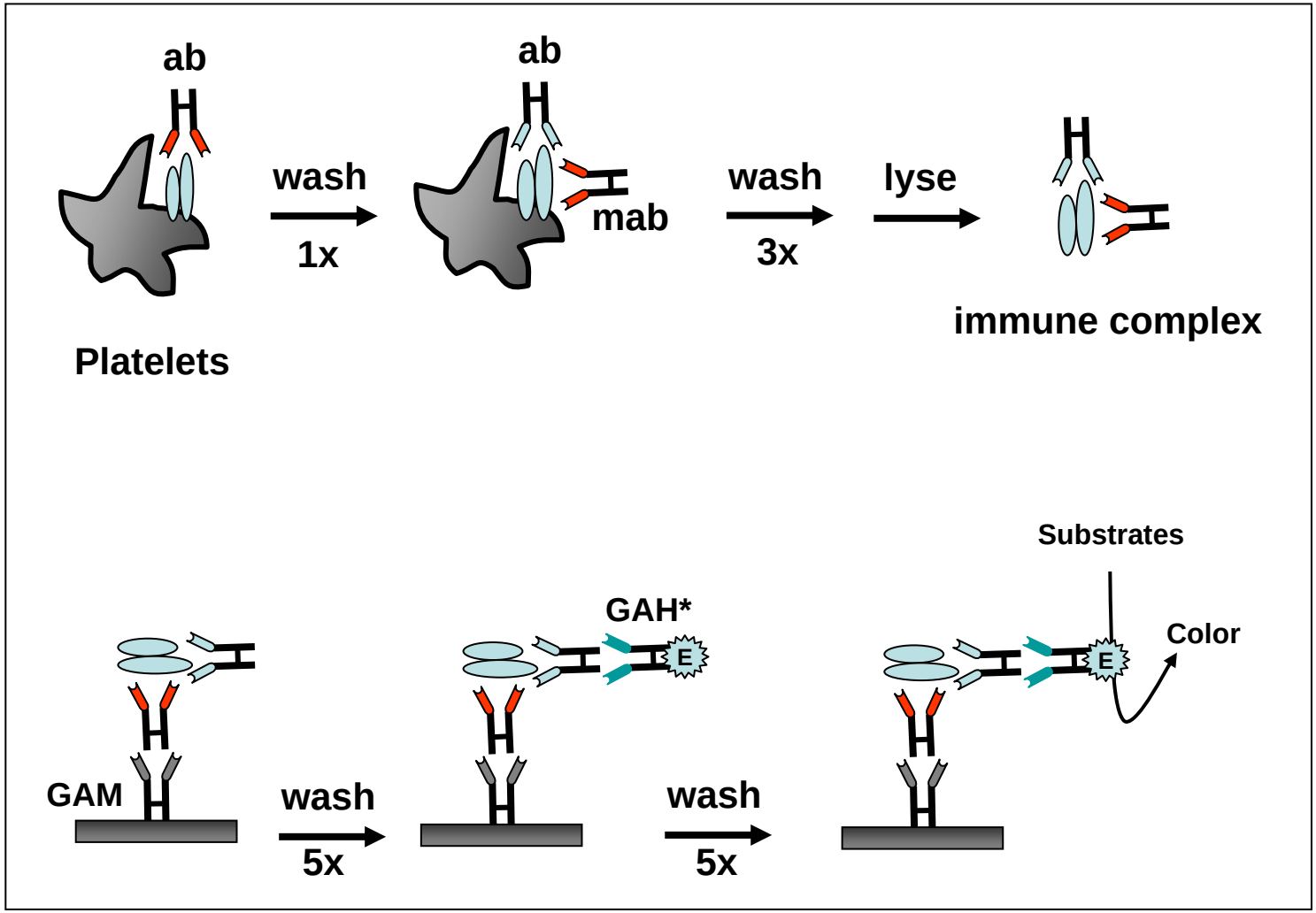



*Kiefel et al, Blood 1987*

# Procedure of MAIPA Assay

1. Stage

2. Stage




*Kiefel et al, Blood 1987*

# Advantages and Disadvantages of Antigen Capture Assays

| ☺☺ | ☹☹ |
|---|---|
| highly specific | washing steps |
| wide spectrum of GPs | need of mabs |
| robust | need of platelets |
| objective | anti mouse |
| standardized | time consuming |
| accepted | experienced technologist |

# New Platelet Antibody Testing ? (1)

w/o the use of mab
w/o the use of whole platelets
w/o any washing step

Rapid, sensitive, specific and reliable

# New Platelet Antibody Testing? (2)

**There is some evidence that some platelet alloantibodies are not detactable by the current technique**

# Suspected FNAIT with fetomaternal incompatibility without detectable anti-HPA-1a

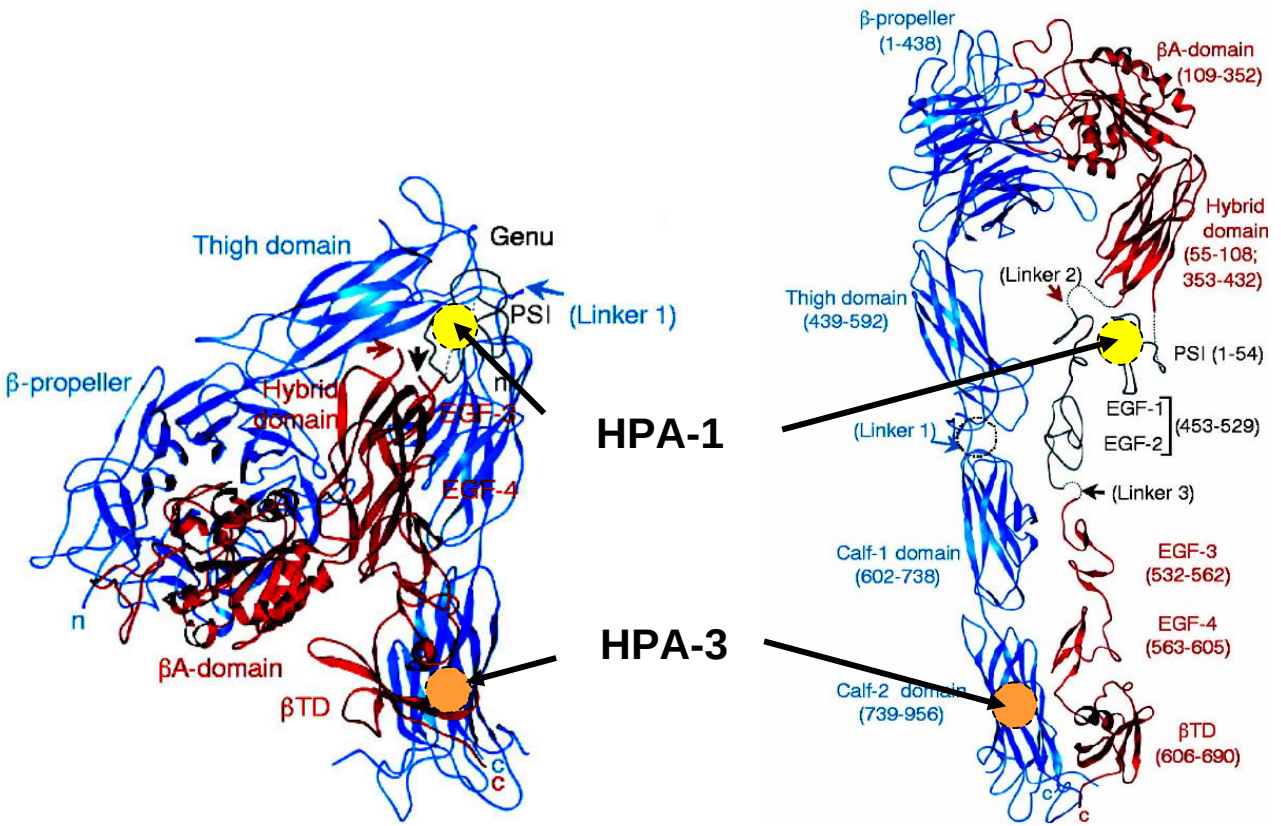
| FNAIT Cases* | Test | Number | % |
|---|---|---|---|
| HPA-1a Incompatibility | | 569 | 100 |
| No anti-HPA-1a | MAIPA and PIFT | 99 | 17.3 |
| Anti-HPA-1a | MAIPA or PIFT | 470 | 82.7 |
| | MAIPA and PIFT | 438 | 77.1 |
| | PIFT | 32 | 5.6 |

* Giessen 1988-2004

# Factors Influenced Antibody Testing

- **Platelet Antigens**
  *3D-Structure, Density, Epitopes*
- **Platelet Antibodies**
  *Concentration, Avidity, Heterogeneity*
- **Other factors** (e.g ligands)?

# Conformations of Platelet GPIIb/IIIa




**bent conformer**

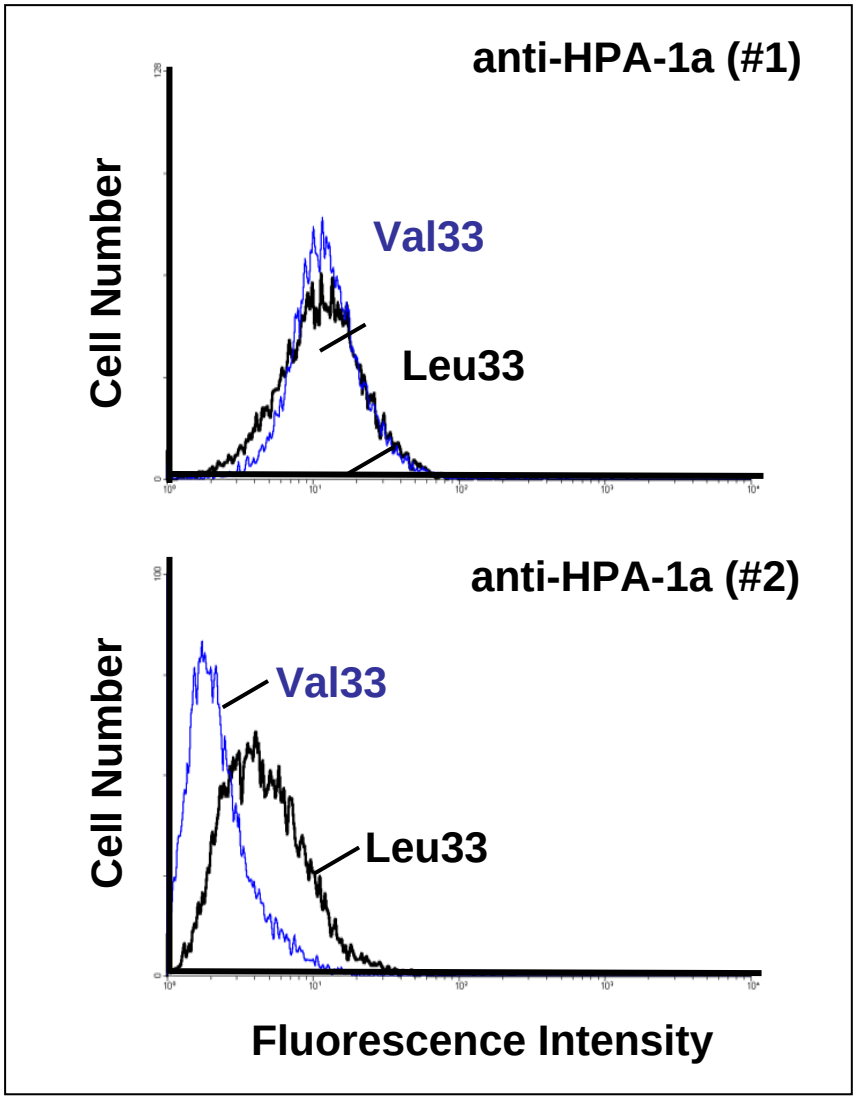
**„inactive state“**

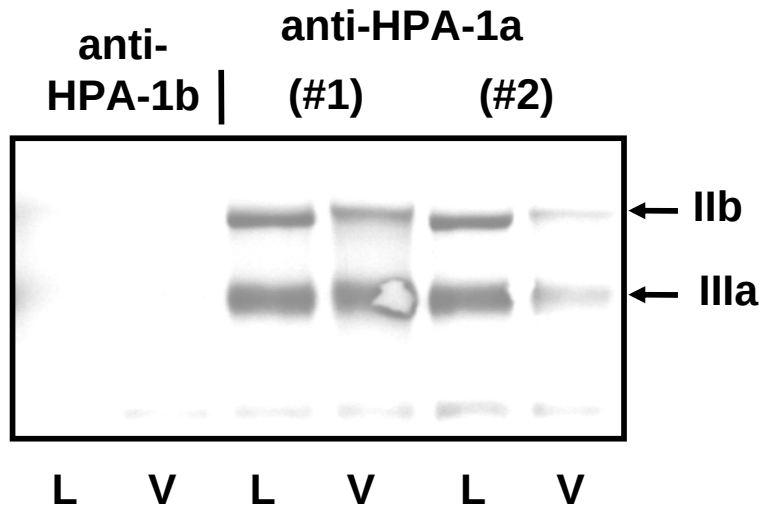
**extended conformer**

**„active state“**

*Xiong et al, Science 2002*

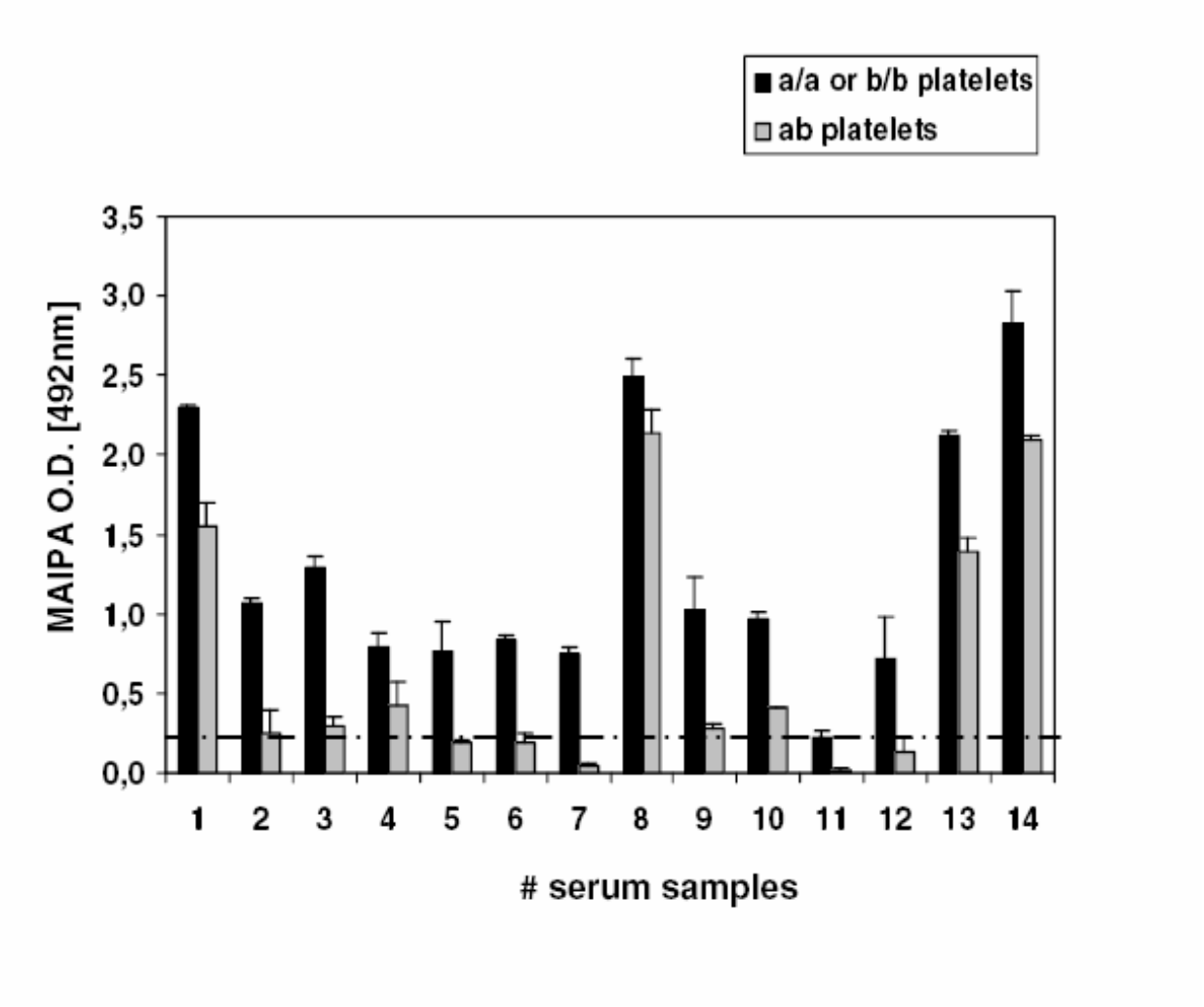
# Two Types of HPA-1a Alloantibodies




*Santoso et al, Transfusion, 2006*

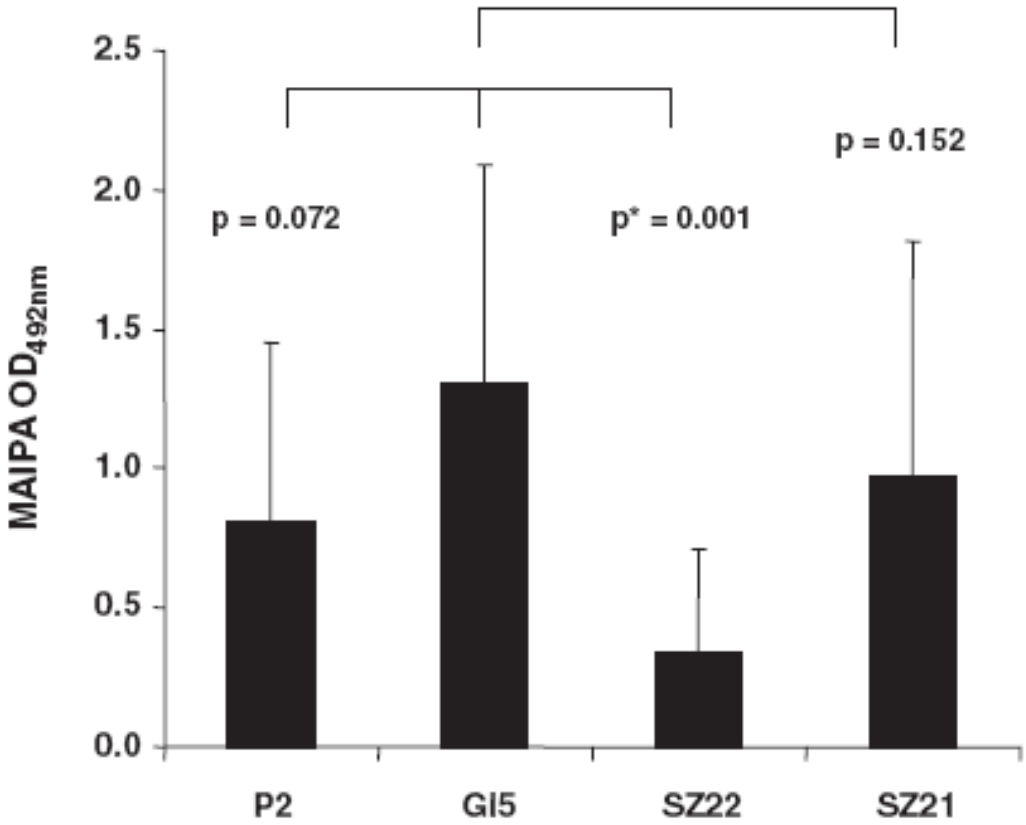
# Antigen Density




Different HPA-3 sera

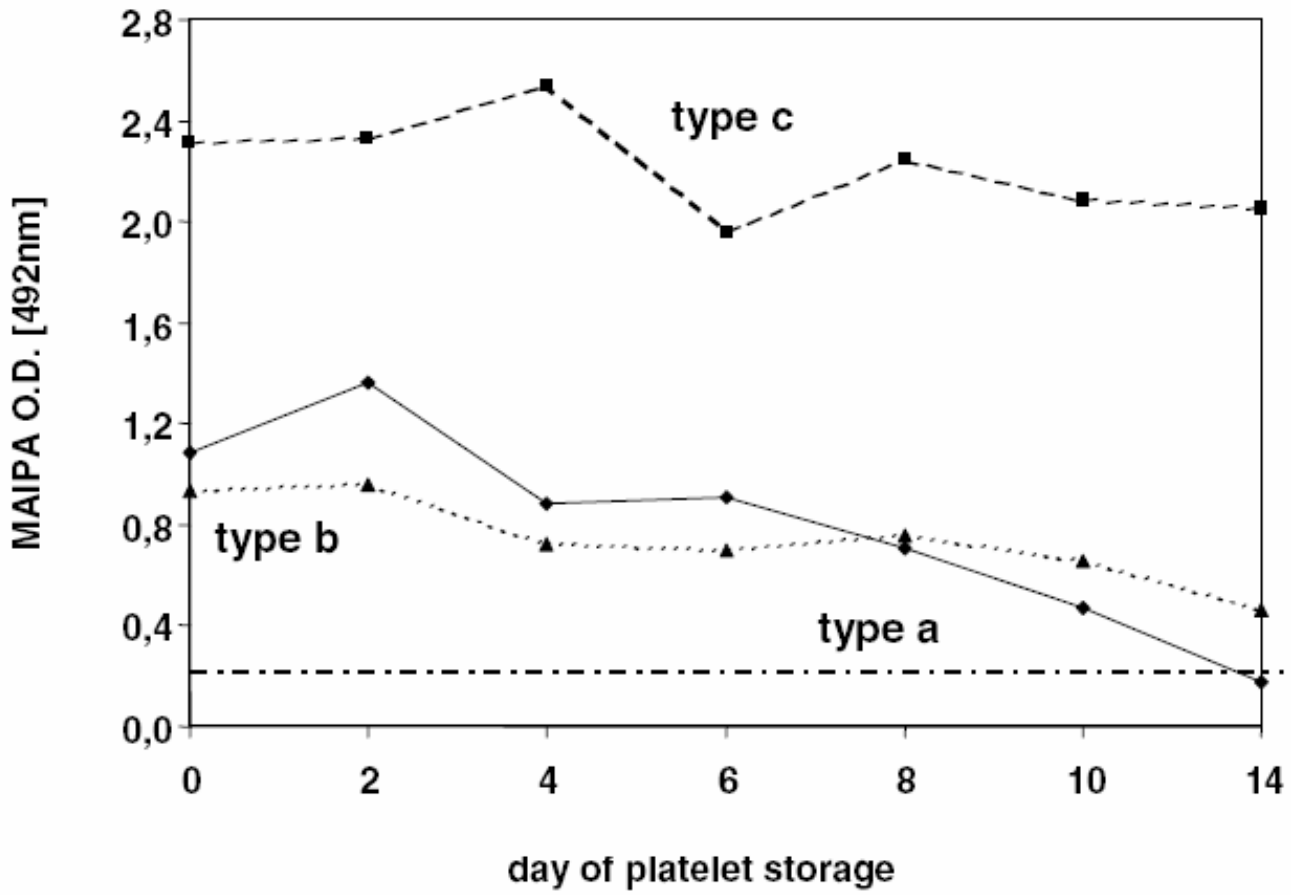
*Socher et al, Transfusion 2008*

# Heterogenous Epitopes: Monoclonal Antibody




*Socher et al, Transfusion 2008*

# Heterogenous Epitopes: Stability

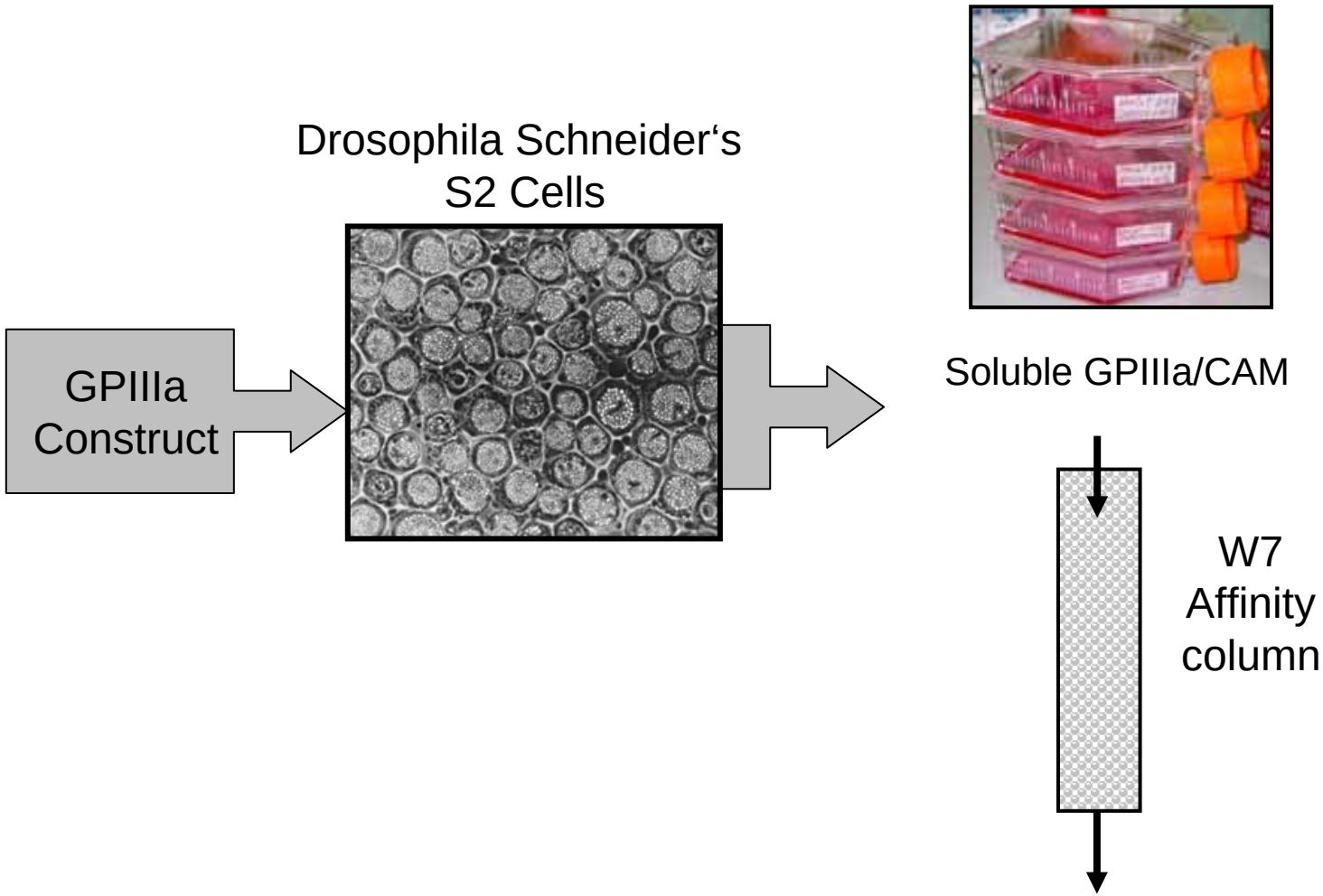



*Socher et al, Transfusion 2008*

# New Platelet Antibody Testing

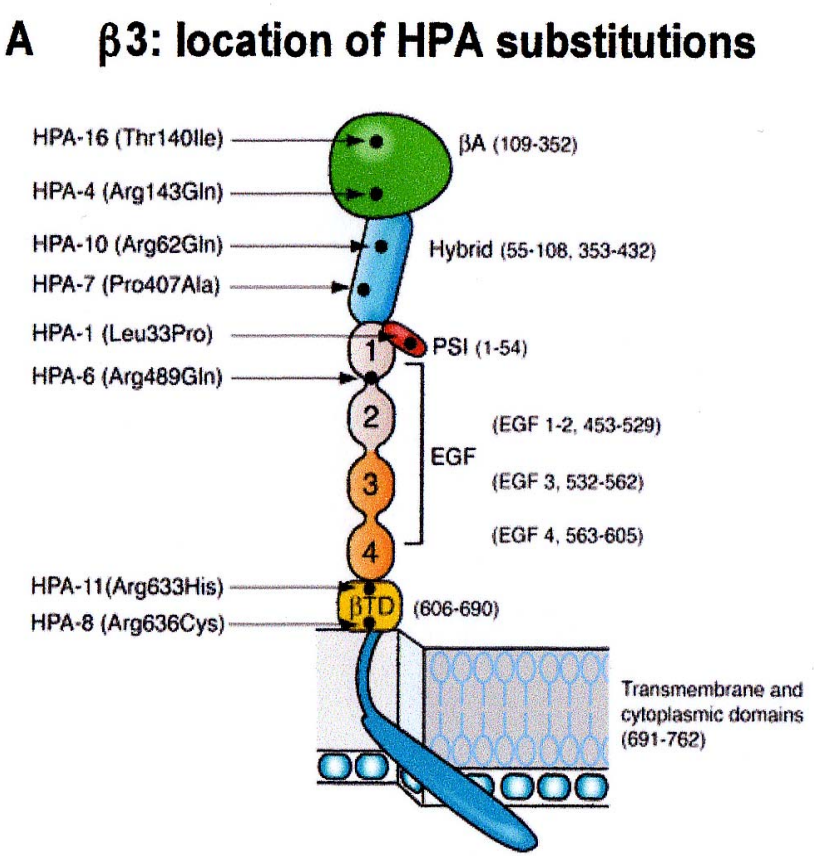
## The use of recombinant platelet antigens in ELISA System

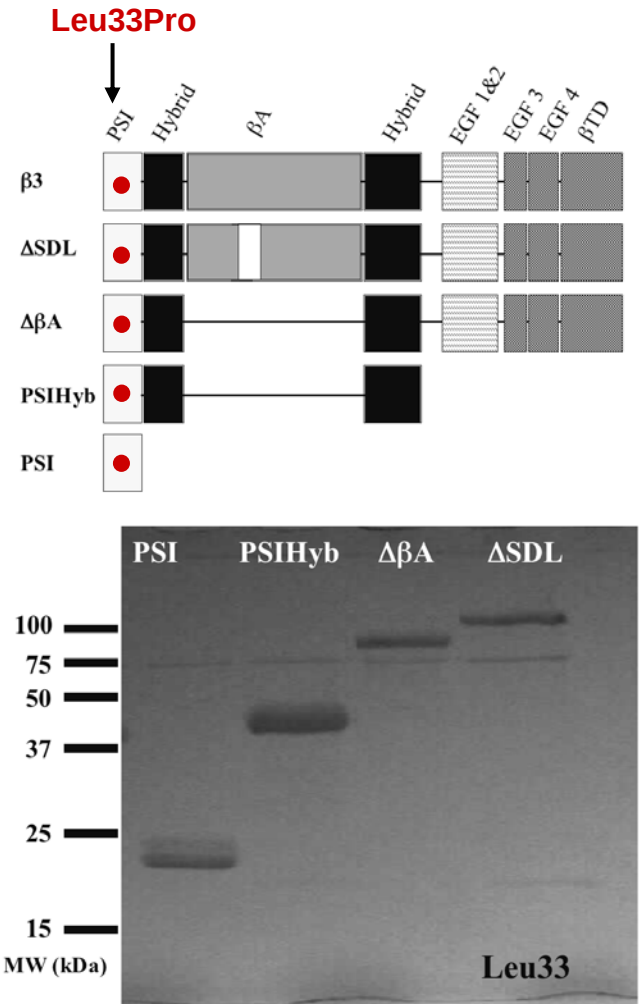
# Production of soluble recombinant human platelet antigen in insect cells




CAM: Calmodulin

# Soluble recombinant HPA-1a and -1b

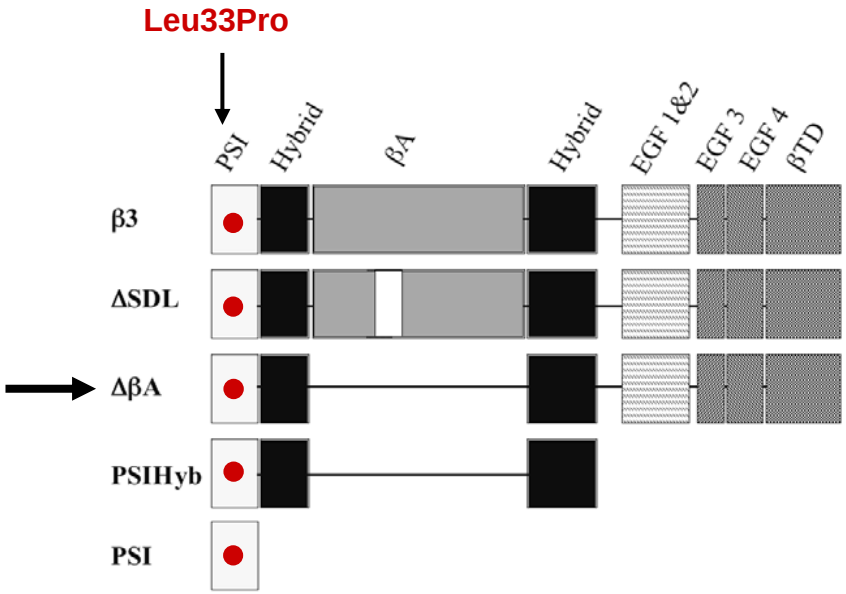



*Stafford et al, JTH 2008 (a)*

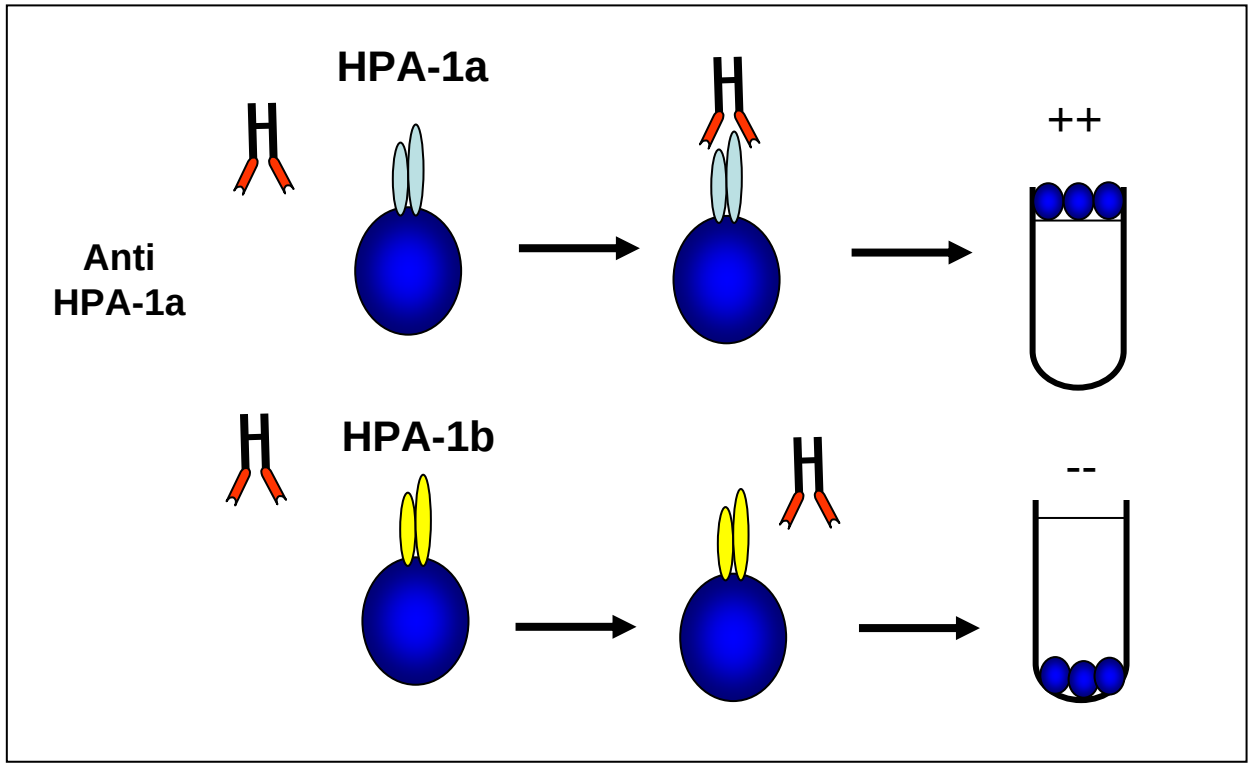
# Detection of anti-HPA-1a with rGPIIIa by ELISA

| Peptide | GPVI-D1D2 | ΔSDL Pro33 | ΔSDL Leu33 | PSIHyb Leu33 | PSI Leu33 | ΔβA Leu33 |
|---|---|---|---|---|---|---|
| ≥ average ± 4 SD | 0 (0%) | 30 (24%) | 110 (87%) | 79 (63%) | 86 (68%) | 116 (92%) |




*Stafford et al, JTH 2008 (a)*

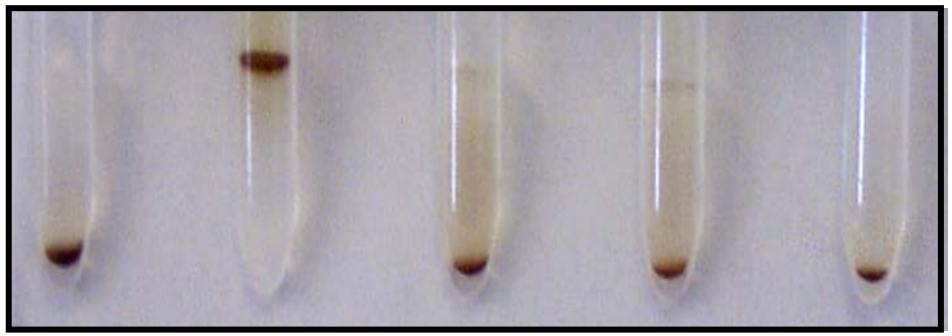
# Gel Antigen Specific Assay (GASA)




*Bakchoul et al, Transfusion (2007)*

# Platelet Antibody Testing: MAIPA vs. GASA

HPA-1a Microbeads



HPA-1b HPA-1a HPA-5b HLA AB

3/36 anti-HPA-1a, which showed moderate reactivity
In MAIPA, reacted strongly in GASA

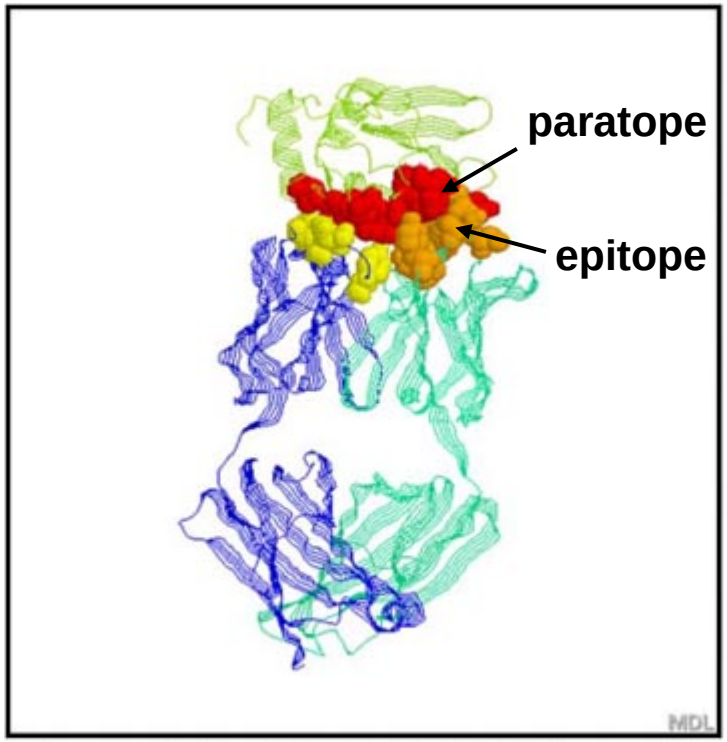
*Bakchoul et al, Transfusion (2007)*

# Antigen – Antibody
# Lock and Key Concept

non-covalent bonds

↓

reversible




Source: Li, Y., Li, H., Smith-Gill, S. J., Mariuzza, R. A., Biochemistry 39, 6296, 2000

affinity

↓

avidity

$$Ag + Ab \rightleftarrows Ag\text{-}Ab$$

Applying the Law of Mass Action:

$$K_{eq} = \frac{[Ag\text{-}Ab]}{[Ag] \times [Ab]}$$

# New Platelet Antibody Testing

**The use of purified platelet antigens or recombinant platelet antigens in SPR technology**

~~w/o the use of mab~~

~~w/o any washing step~~

~~w/o the use of whole platelets~~

# Real-time Analysis of Antigen-Antibody Interaction by SPR Technology

1. Rapid quantitation of immunoglobulin G antibodies specific for blood group antigens A and B by surface plasmon resonance *(Kimura et al, Transfusion 2005)*

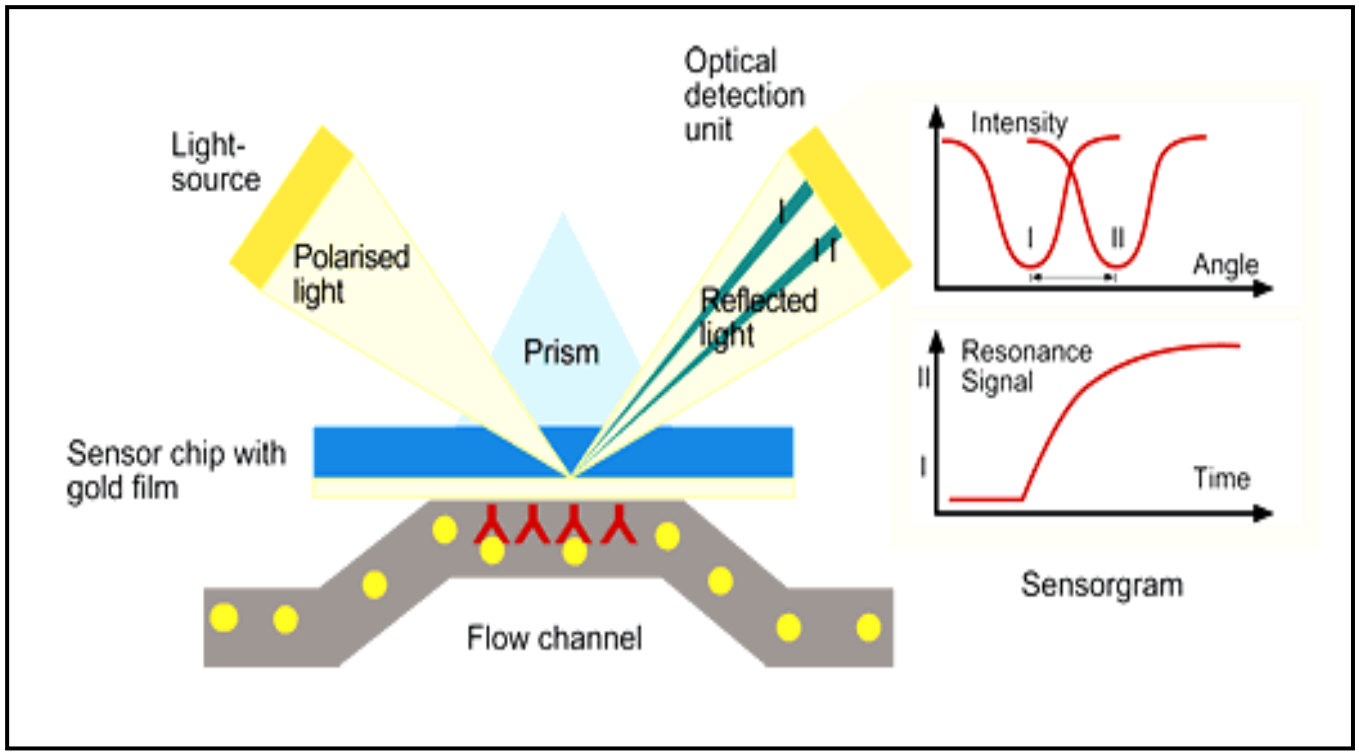
2. Development of non-agglutination microarray blood grouping *(Robbs et al, Transfus Med 2006)*

3. Qualification and application of a surface plasmon resonance-based assay for monitoring potential HAHA responses induced after passive administration of a humanized anti Lewis-Y antibody *(Szolar et al, J Pharma Biomed Anal 2006)*

# Surface Plasmon Resonance (SPR)
# Real Time Protein-Protein Interaction

**Biacore 3000**

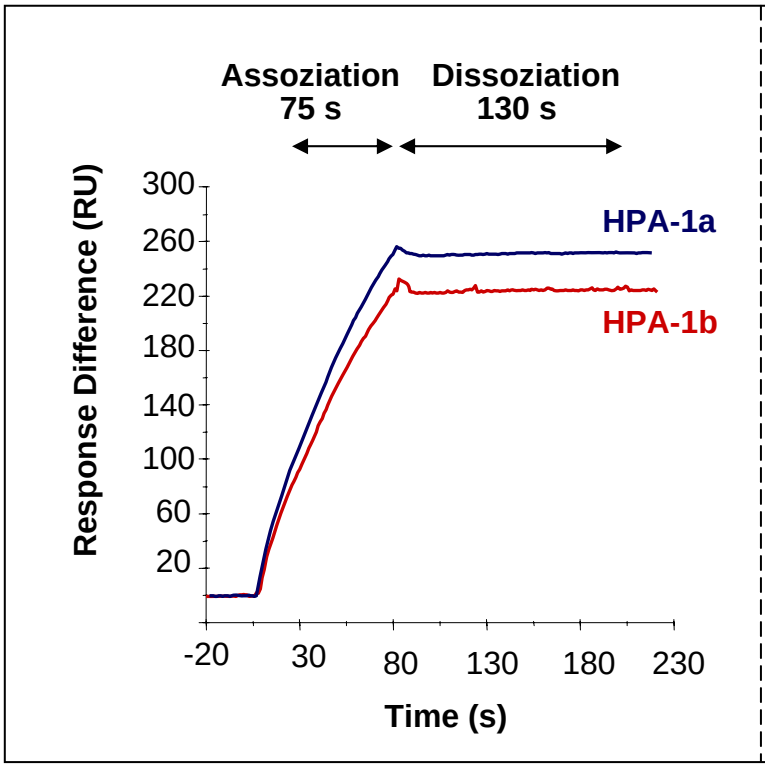
# Principle of SPR Technology



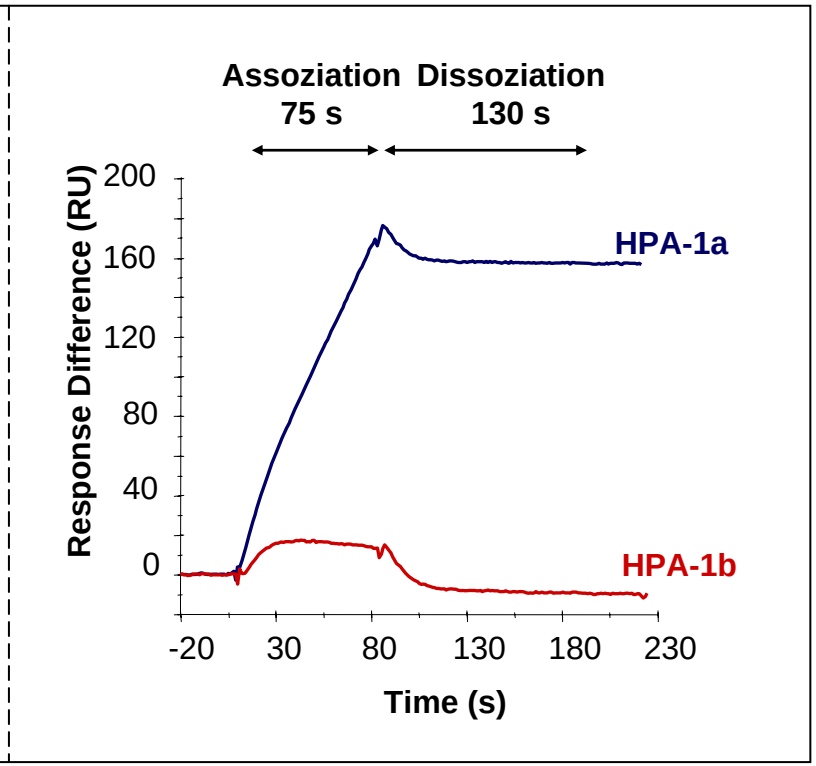

SurfaceBinding.wmv

# Reactivity of immobilized HPA-1a and HPA-1b antigens with Mabs in SPR

**Anti GPIIb/IIIa**
**(Mab MBC 132.1)**




**Anti HPA-1a**
**(Mab CamTran007)**



# Mab SZ21: HPA-1a „Specific“

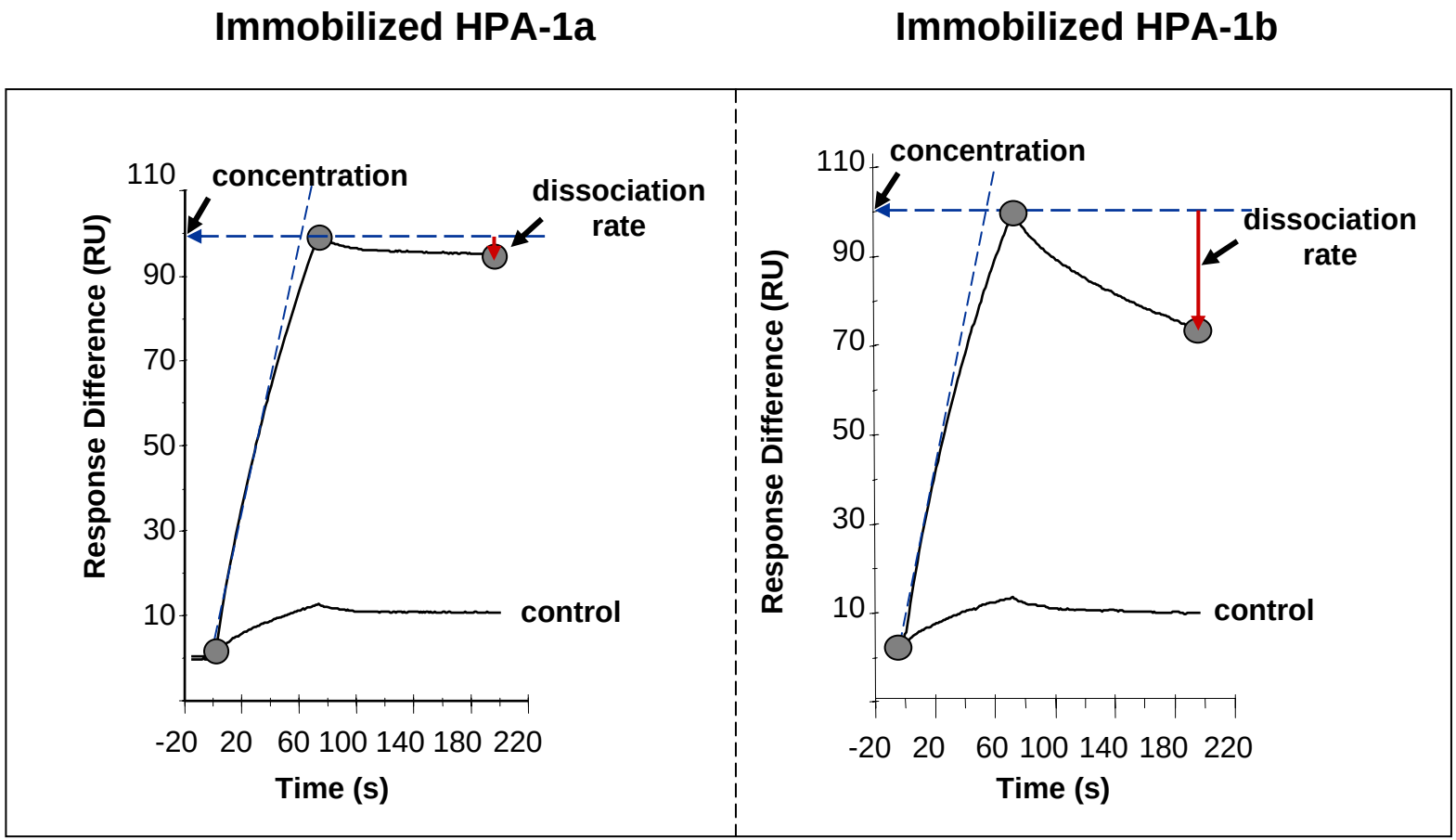
## Competitive binding of a monoclonal antibody SZ-21 with anti-PlA1 antibodies and its potential for clinical application

*Xi X*, *Zhao Y*, *Jiang H*, *Wu Q*, *Li P*, *Ruan C*.

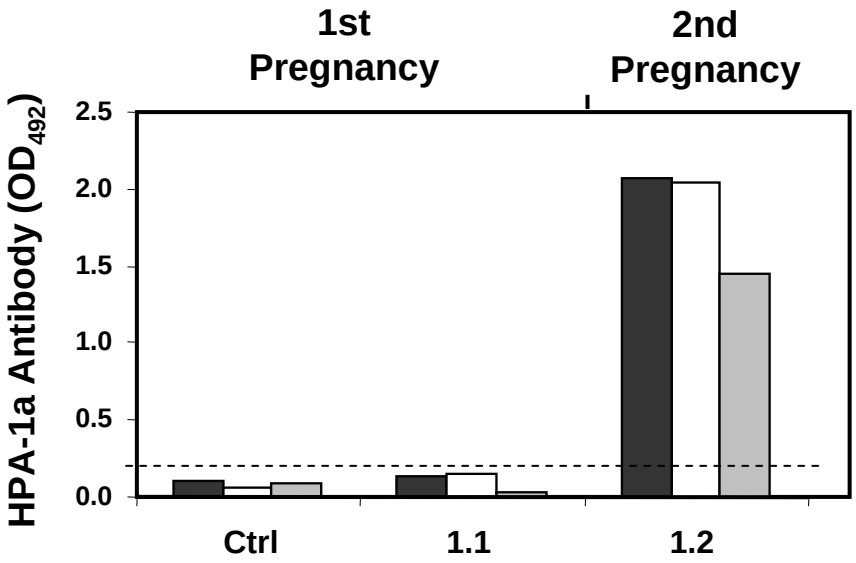
**Jiangsu Institute of Hematology, Suzhou Medical College, People's Republic of China.**

*Nouv Rev Fr Hematol. 1992;34(3):239-42*

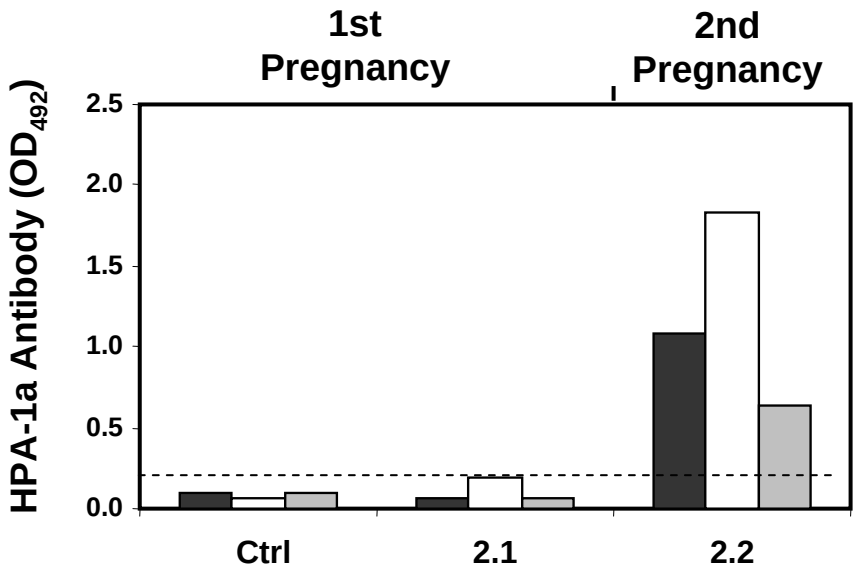
# Reactivity of Mab SZ21 (HPA-1a like) with immobilized HPA-1a and HPA-1b antigens in SPR



# Low-Affinity HPA-1a Alloantibodies Associated with Severe NAIT Case (1)



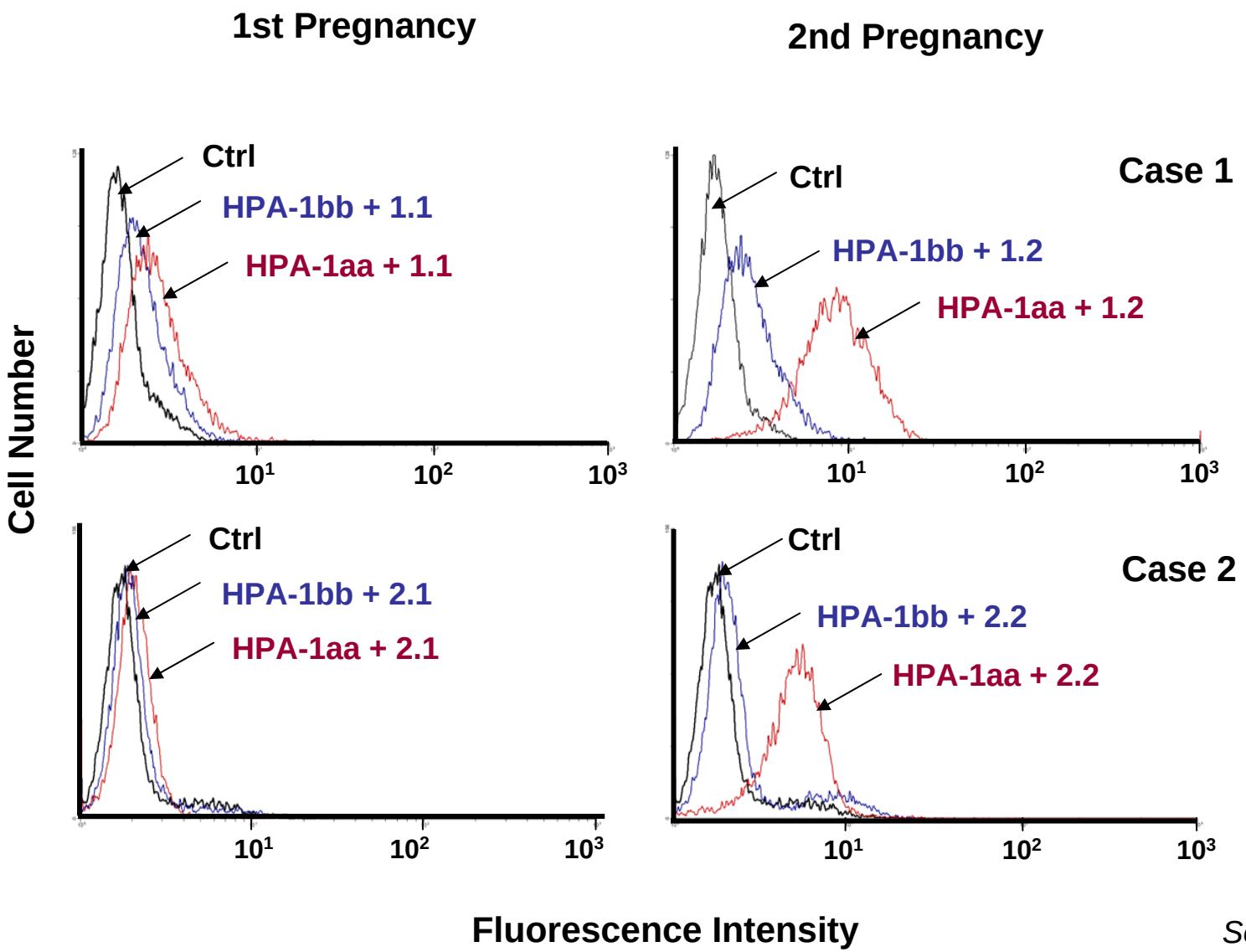

Case 1




Case 2

*Socher et al*

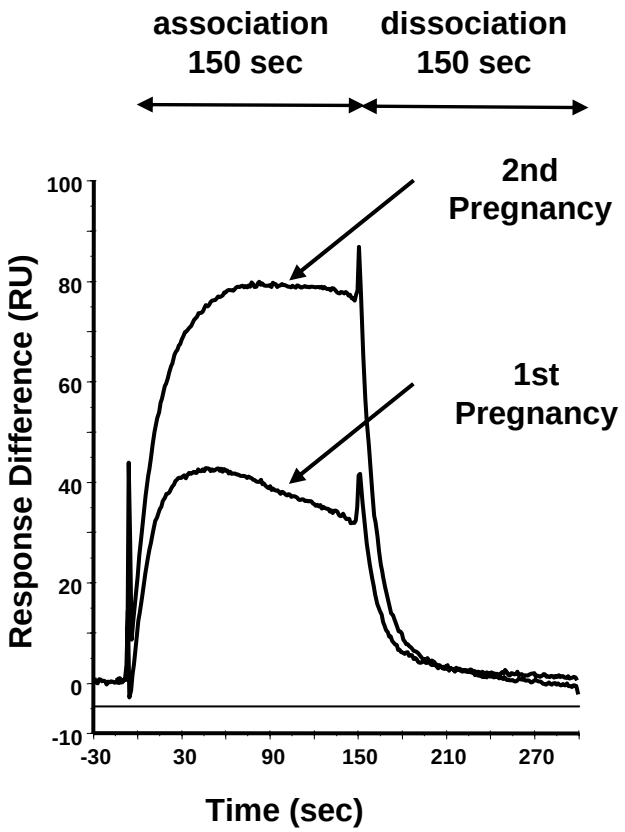
# Low-Affinity HPA-1a Alloantibodies Associated with Severe NAIT Case (2)



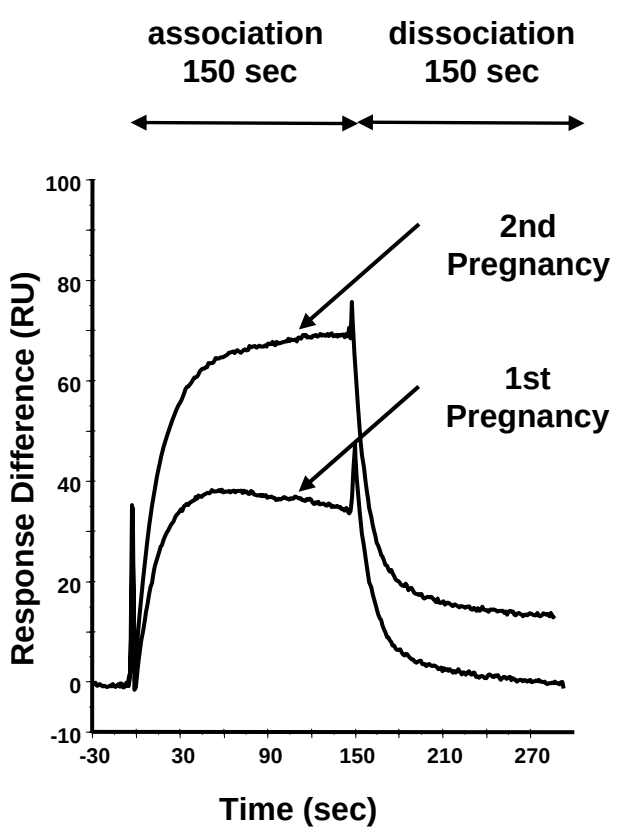

*Socher et al*

# Low-Affinity HPA-1a Alloantibodies Associated with Severe NAIT Case (3)

## Case 1




## Case 2




*Socher et al*

# Analysis of Human Platelet Antibodies

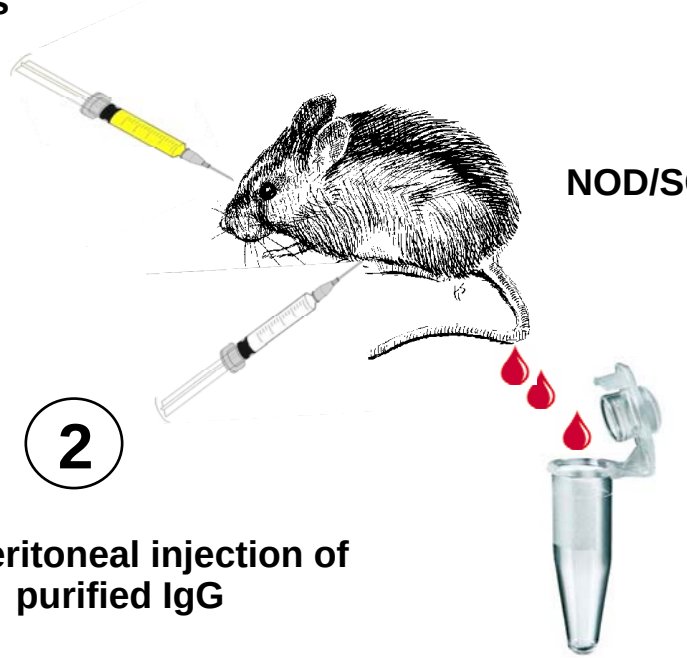
***Animal Model?***

**Testing antibody mediated platelet clearance and related inhibitors**

# NOD/SCID Mouse as a Tool for Analysis of Human Platelet Antibodies

**Introduce ~200 μl "concentrated" human PRP into the retroorbital plexus**

**1**



**NOD/SCID Mouse**

**2**

**Intraperitoneal injection of purified IgG**

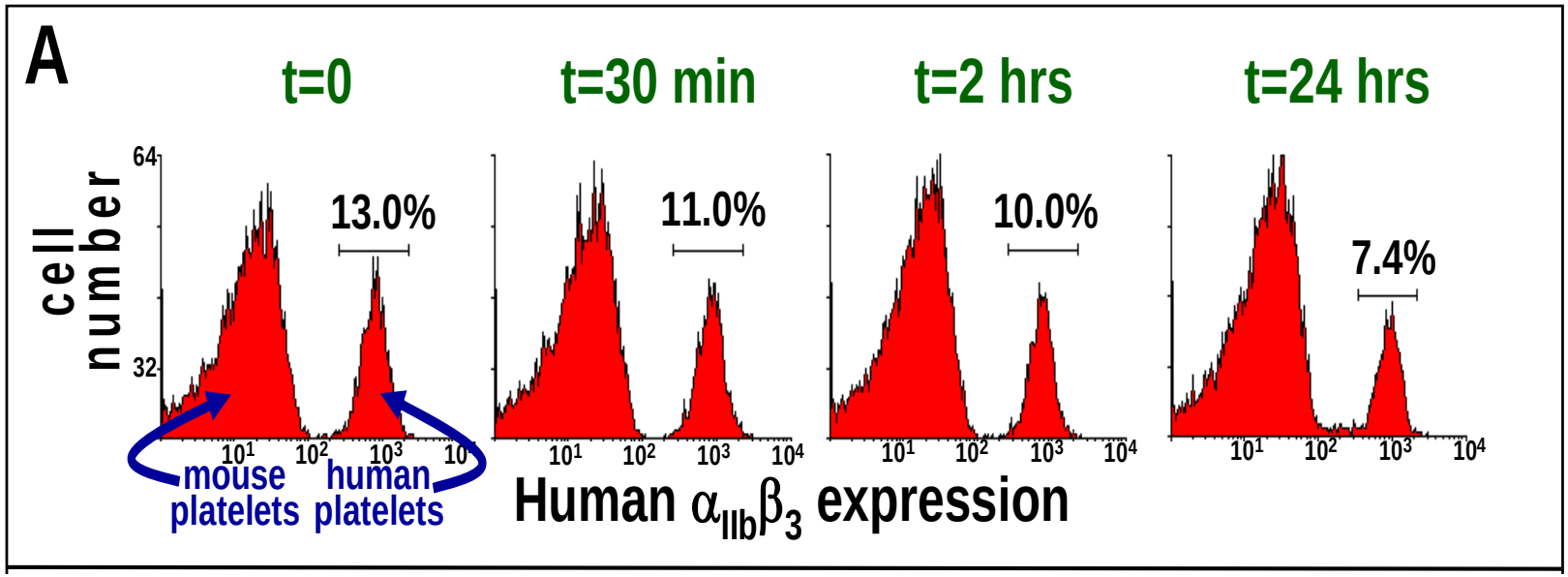
**3** **Periodically collect blood samples**

**4**

**<u>Flow Cytometric Analysis for</u>:**
**- Platelet number**
**- Platelet function**

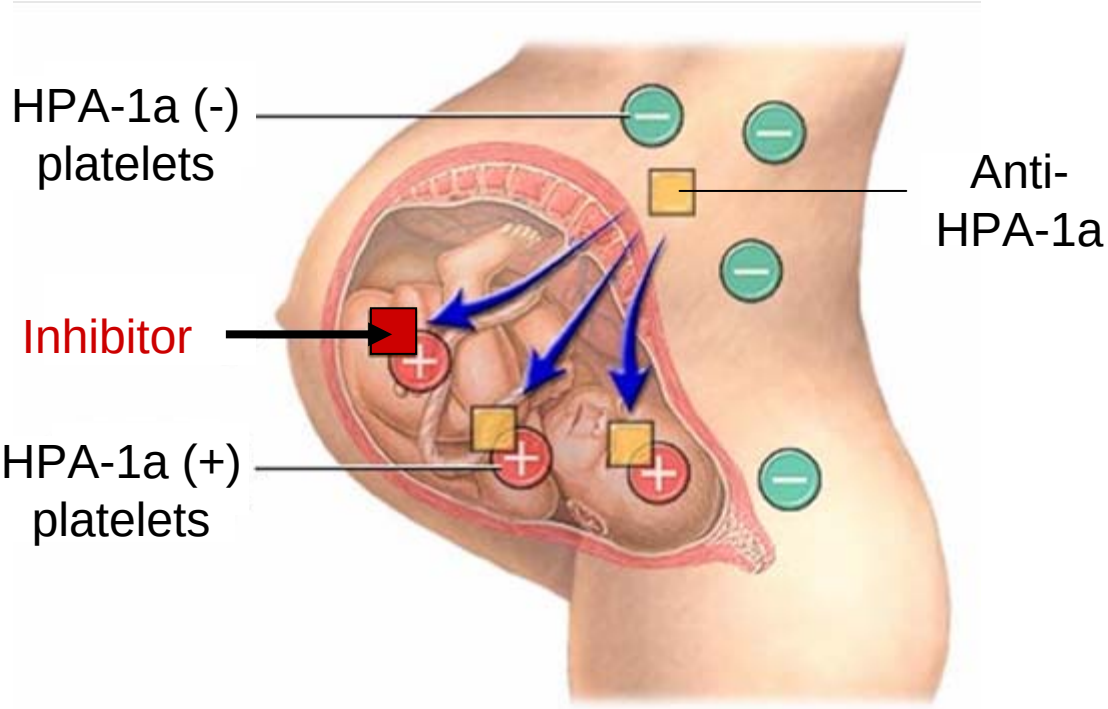
@PJ Newman

# Kinetics of Human Platelet Survival During Circulation in NOD/SCID Mice

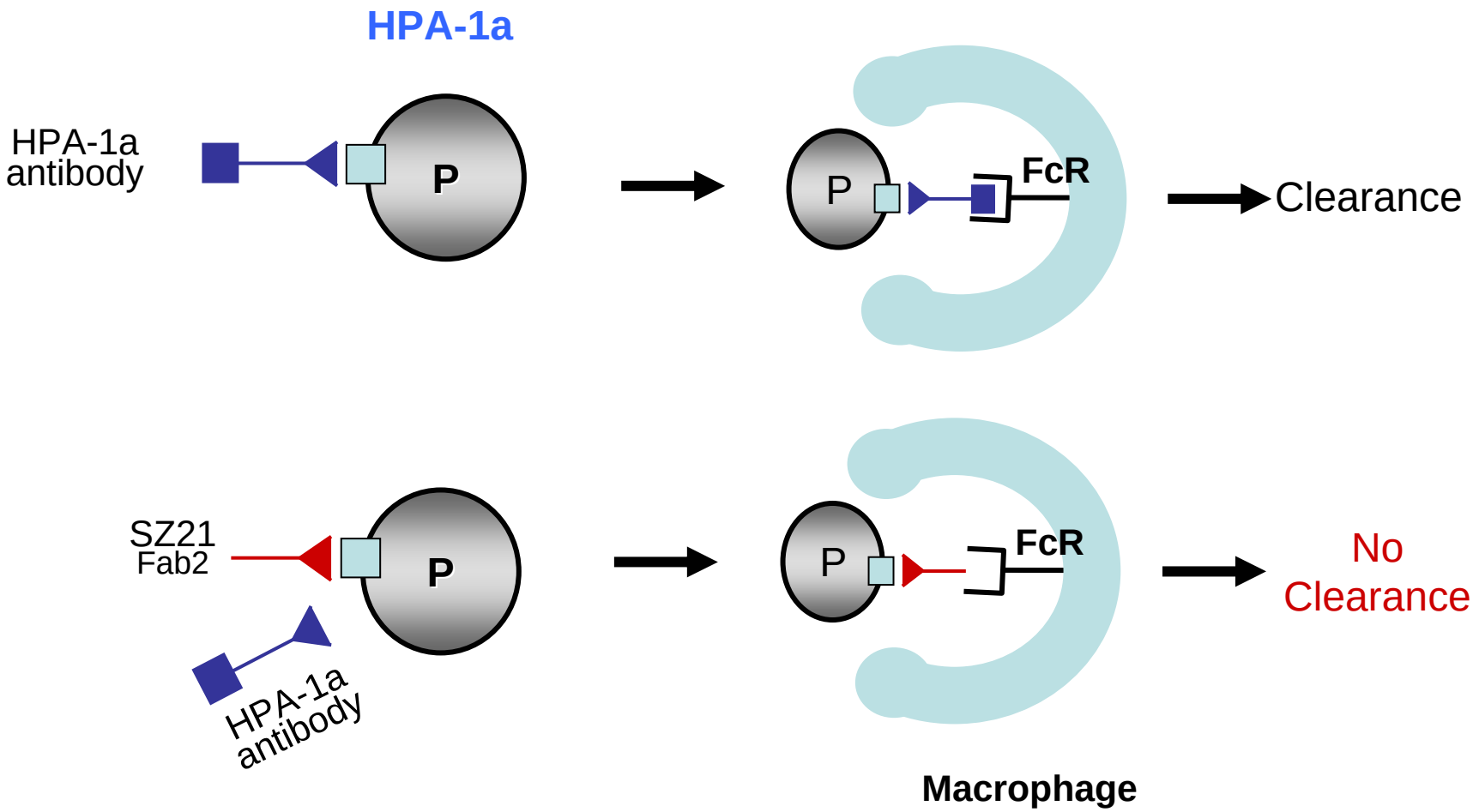



*Boylan et al, Blood 2006*

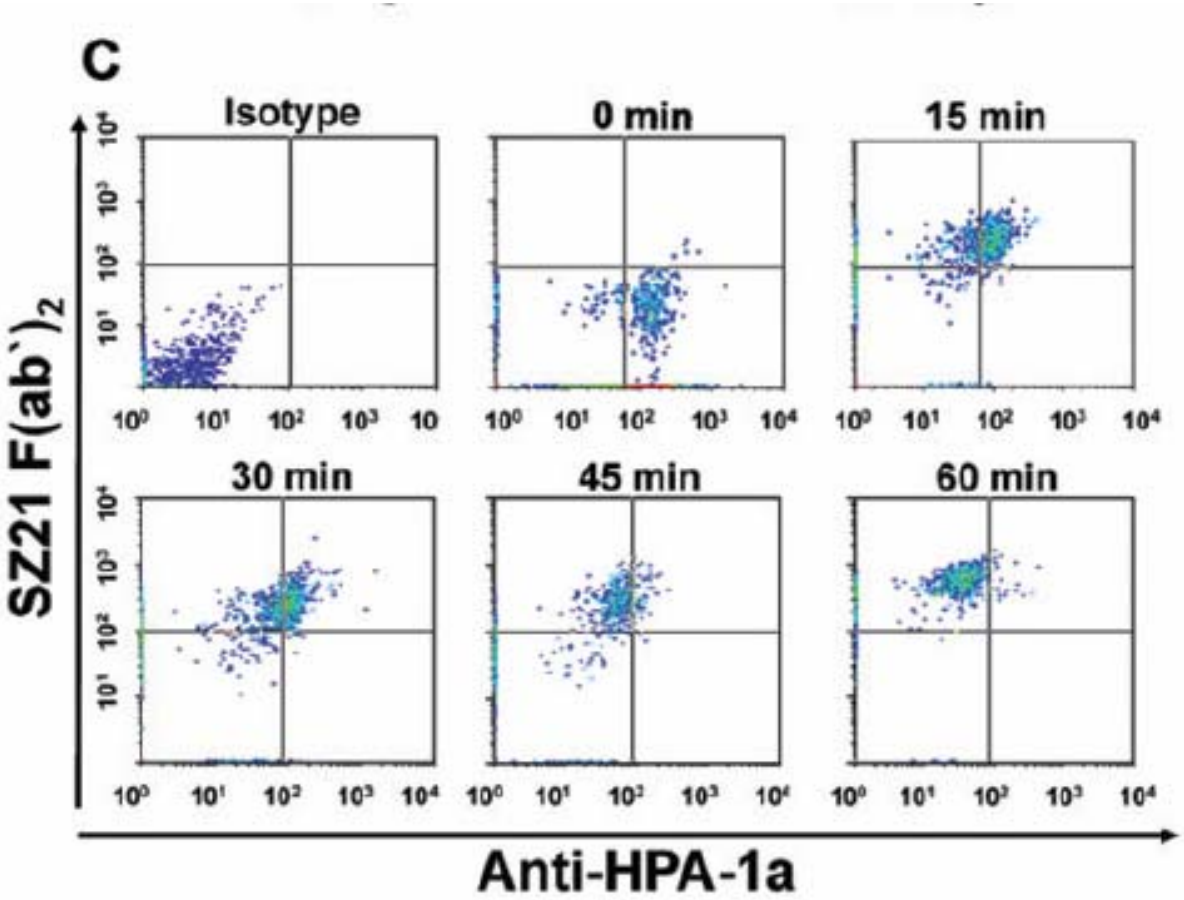
# Pathomechanism of FNAIT



# Does Mab SZ21 inhibits HPA-1a alloantibody mediated platelet clearance in vivo?

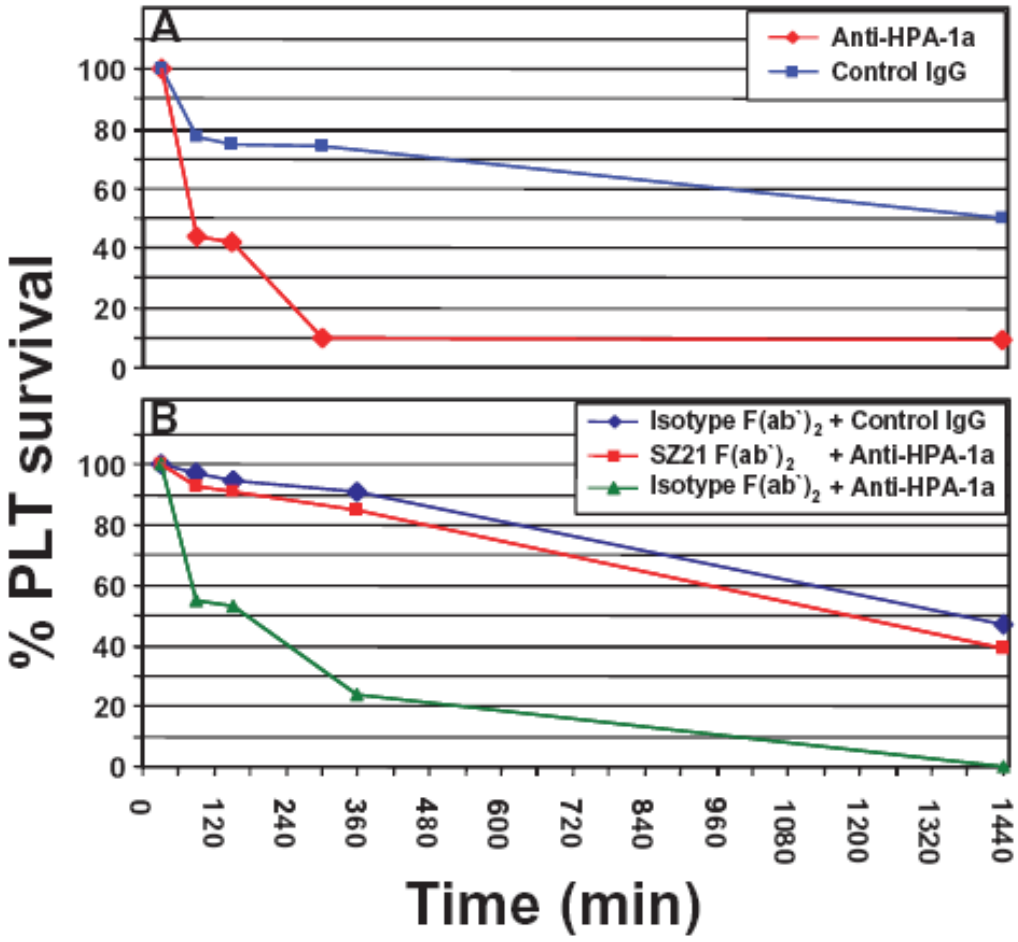


# Mab SZ21 inhibits HPA-1a alloantibody binding to human platelet *in vitro*




*Bakchoul et al, Transfusion 2008*

# Mab SZ21 inhibits HPA-1a alloantibody mediated platelet clearance *in vivo*




*Bakchoul et al, Transfusion 2008*

# Conclusions

- Based on the molecular bases of HPA the fourth generation of genotyping technique could be developed, which allows rapid genotyping of HPA. Reference DNA derived from immortalized cell lines are now available for the quality control of genotyping

- The detection of alloantibody, however, is still time consuming, cumbersome and unreliable.Thus, new generation of pletelet antibody testing should be established (e.g SPR technology)

- For the development of new generation of platelet antibody testing, however, additional informations about the nature of antigens, complexity of epitopes and heterogeneity of antibodies are of importance.

- In addition, *in vivo* studies of may help us to define „pathogenic“ antibodies and our understanding about the pathomechanism of immune mediated thrombocytopenia. Animal model can be useful tool for testing of „drug“

# New Generation of Platelet Antibody Testing?

**On maturation process…**

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# Relationship between anti-HPA-1a level and the severity of NAIT

| Authors | Method | Assoziation |
|---|---|---|
| Williamson et al, 1998 | MAIPA | Yes |
| Jaegtrik et al, 2000 | MAIPA | Yes |
| Killie et al, 2007 | MAIPA | Yes |
| Bessos et al, 2005 | ELISA | No |
| Turner et al, 2005 | ELISA | No |
| Ghevaert et al, 2007 | MAIPA | No |