Assays to evaluate cell-mediated immunity

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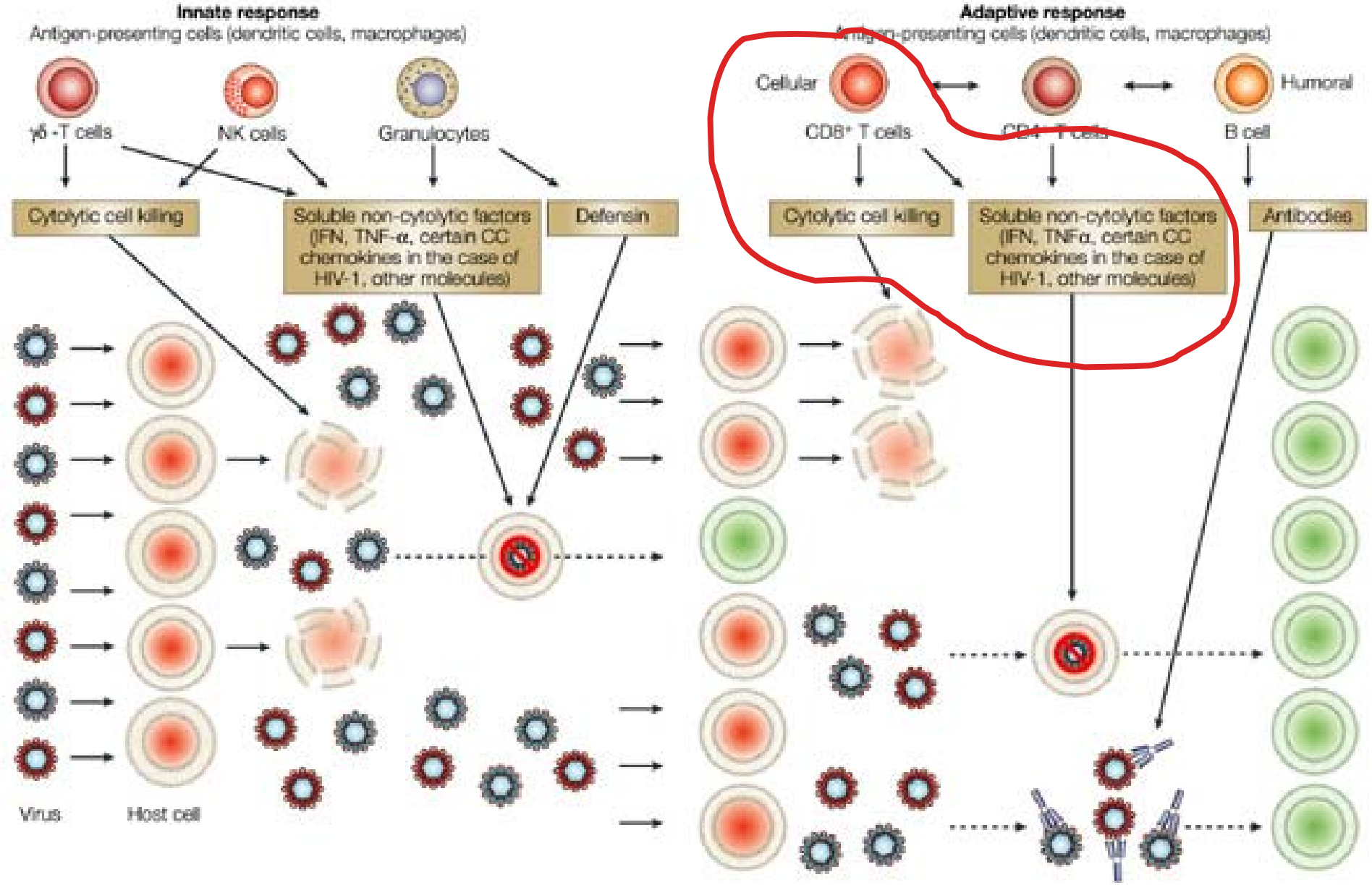
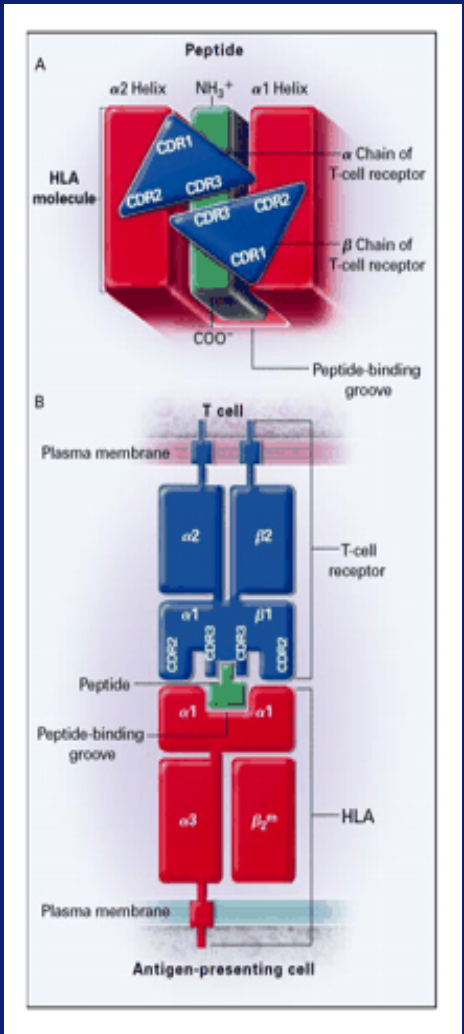
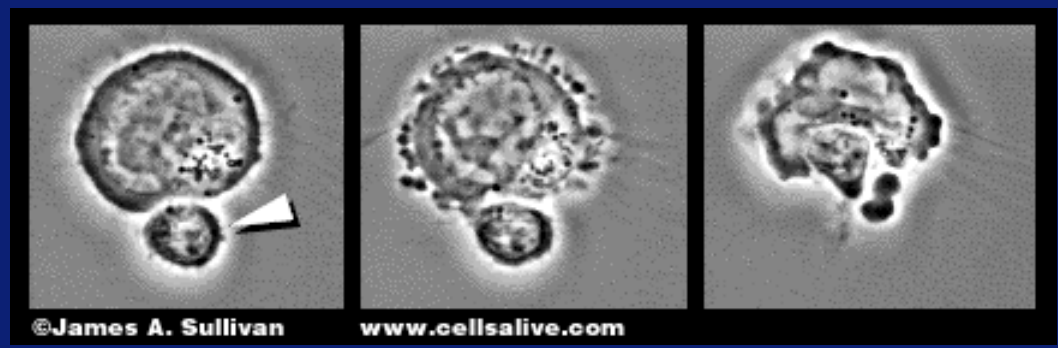


Antiviral immune responses

CTL function



- Elimination of infected cells
  by lysis and induction of apoptosis through release of perforin, granzym and FasL-expression
- Release of cytokines, e.g. IFN-γ and TNF-α



- Recognition by CTL is MHC class I restricted

Virus specific CTL: a correlate of protection

-humans-

Protection by CTL in humans: *McMichael et al, N. Eng. J. Med. 1983, 309:13-17*

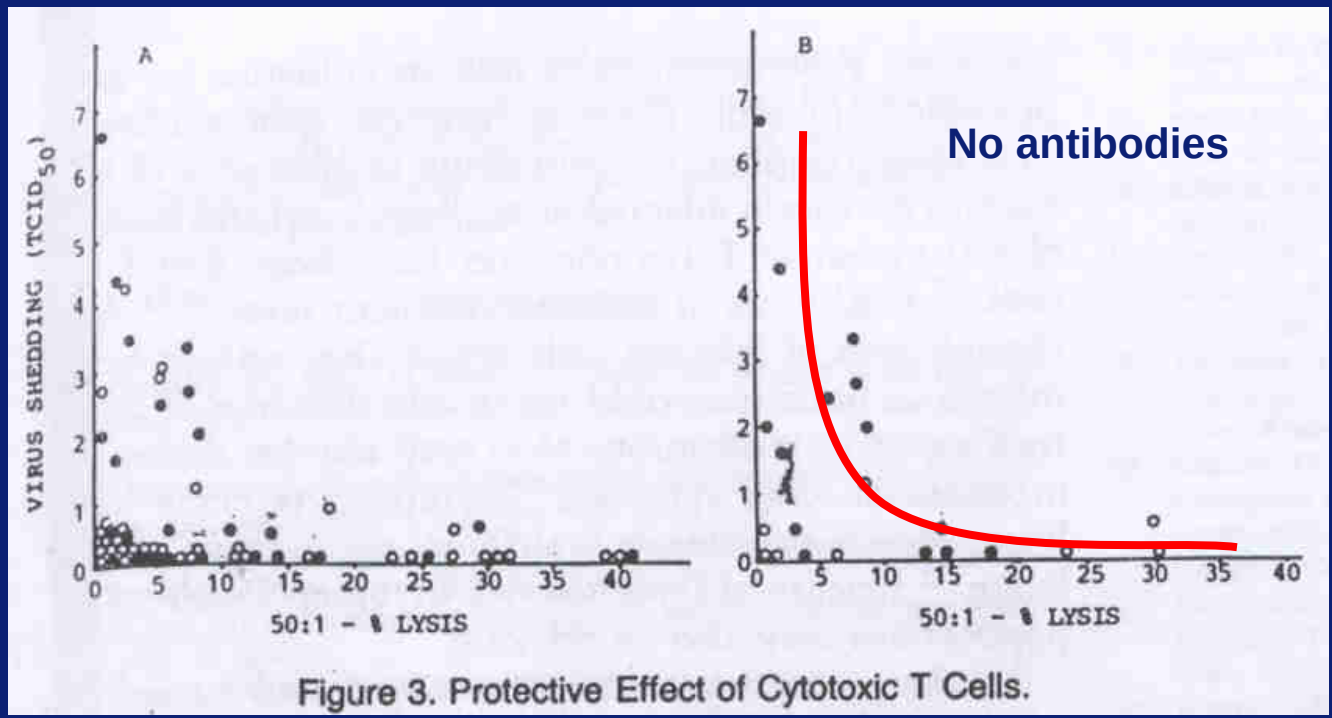


Figure 3. Protective Effect of Cytotoxic T Cells.

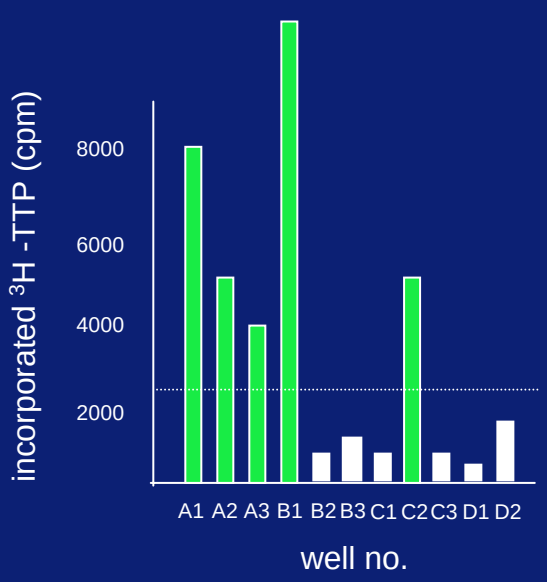
Detection of virus specific T lymphocytes

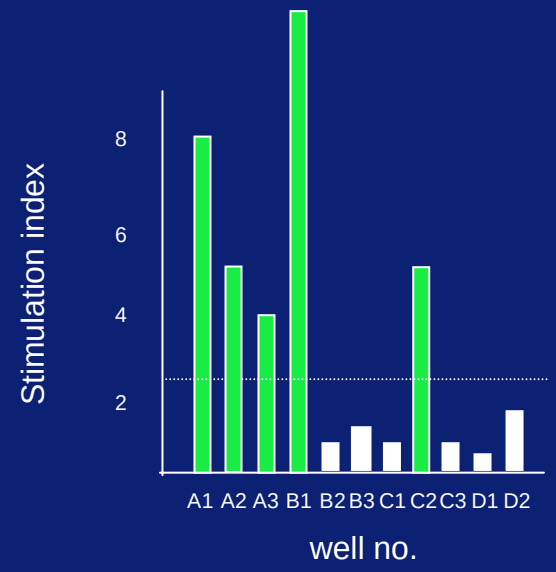
1. Proliferation of virus specific T lymphocytes
   - 3H-thymidine incorporation
   - CFSE dilution assay
2. Functional properties of virus specific T cells
   - Lytic activity
   - Cytokine production
   - Activation markers, e.g. CD69, CD154
3. Epitope specificity
   - Use of peptides
   - Multimers of MHC class I/Peptide complexes
4. A combination of 1. and 2. or 3.

In vitro proliferation of antigen-specific T cells

- ^{3}H-incorporation assay -

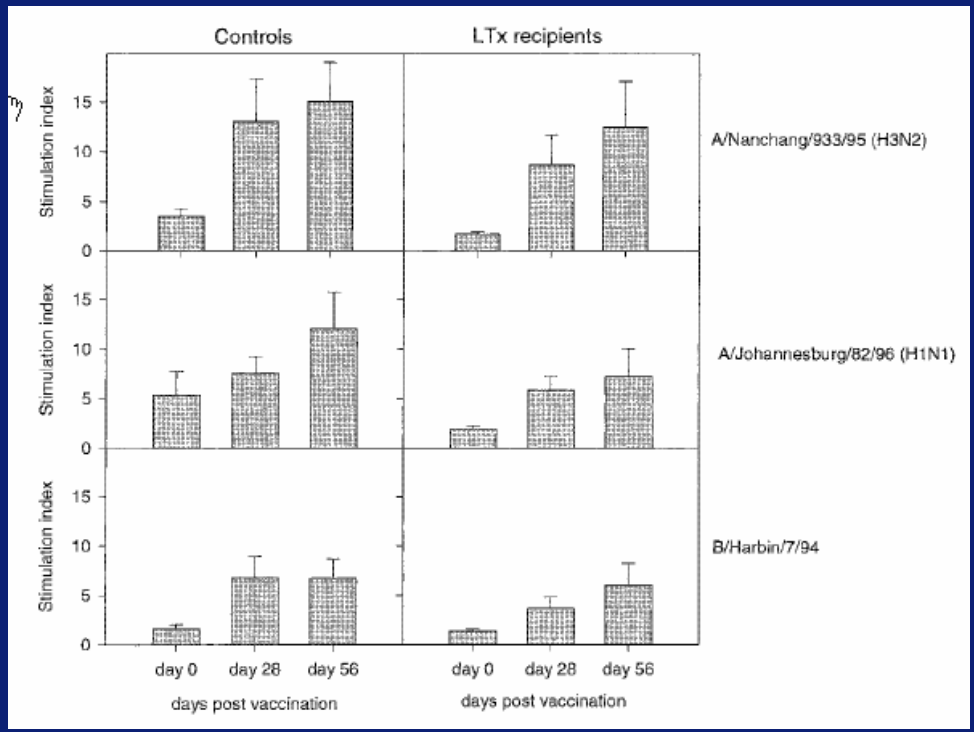
- Based on the incorporation of radioactive ^{3}H-TTP into the DNA of dividing cells
- ^{3}H-TTP is added for 14-18h to *in vitro* stimulated cell-culture
- Cells are harvested and lysed, DNA is captured onto glass-fiber filter
- Radioactivity (cpm) as measure for proliferation
- Often expressed as Stimulation index (antigen specific cpm/control cpm)



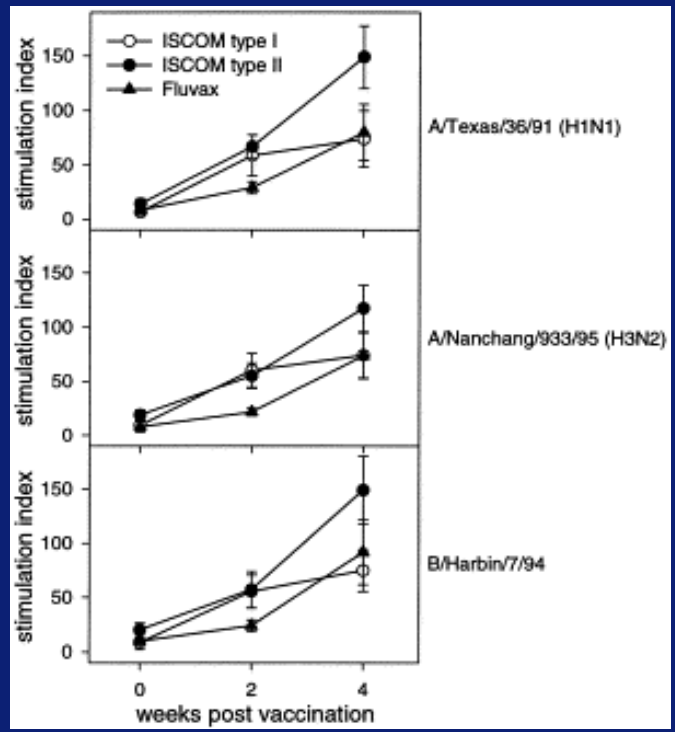


In vitro proliferation of antigen-specific T cells

-3H-thymidine incorporation: examples-



Soesman et al.
J. Med. Virol. 61:85-93 (2000)



Rimmelzwaan et al.
Vaccine 19(9-10):1180-1187 (2000)

In vitro proliferation of antigen-specific T cells
-3H-thymidine incorporation-

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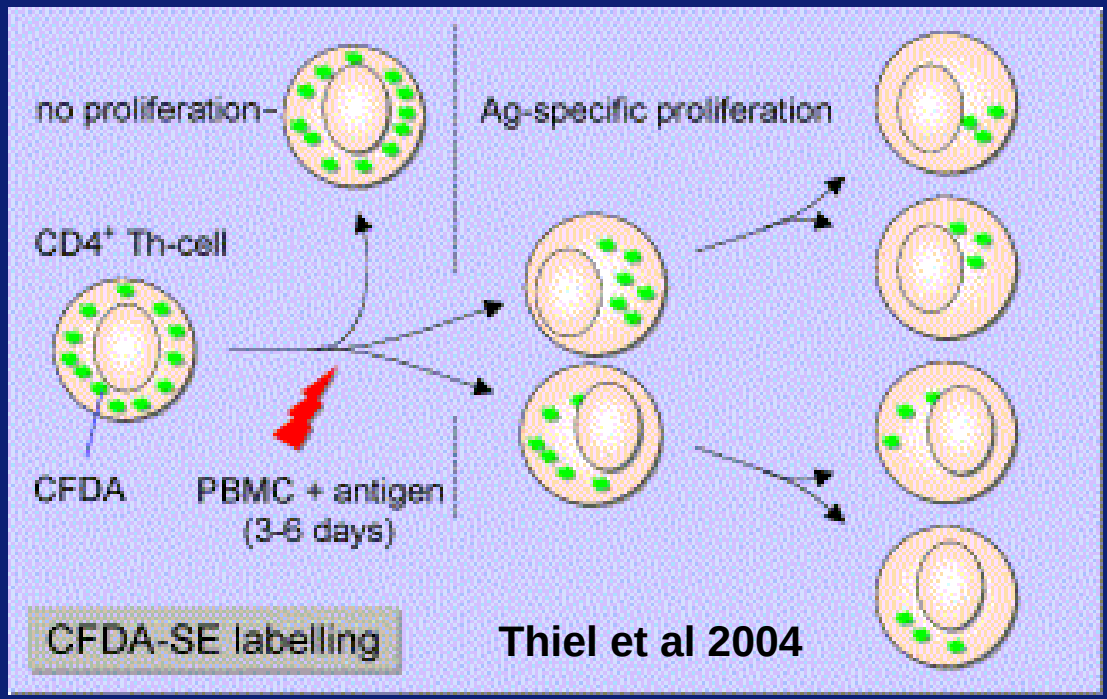
Advantages	Disadvantages
• Relatively easy to perform	• Use of isotopes • Identity proliferating cells unknown • Functional properties unknown • Bystander activation?

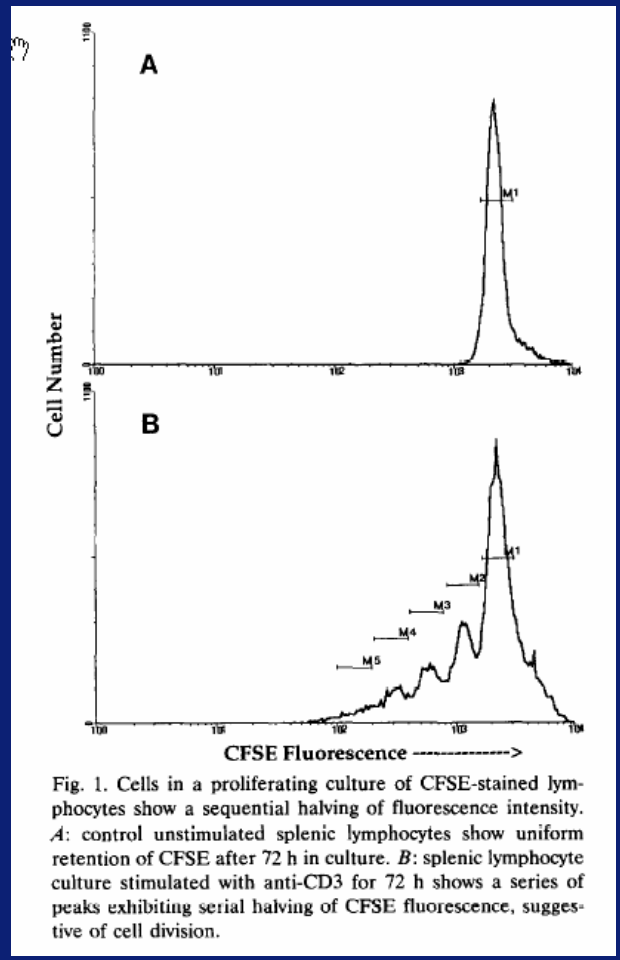
In use since early 80’s…

In vitro proliferation of antigen-specific T cells

-5,6-carboxyfluorecein diacetate succinimidyl ester (CFSE) labelling-



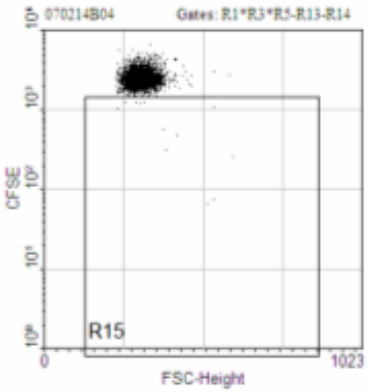
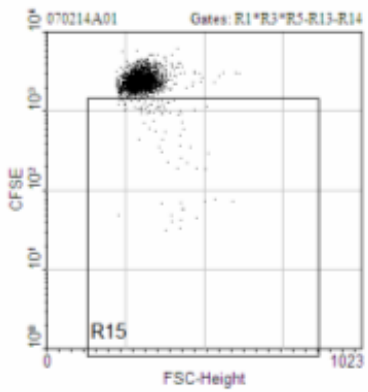
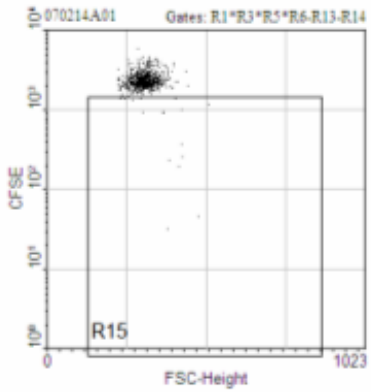
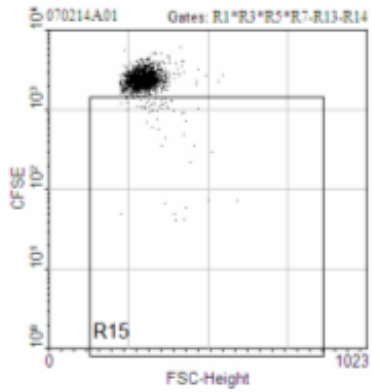
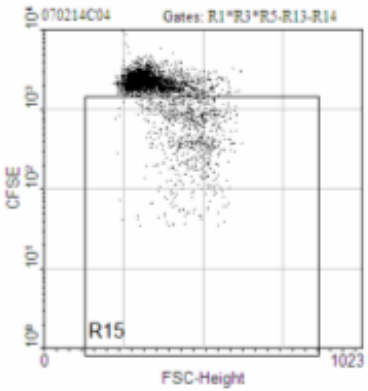
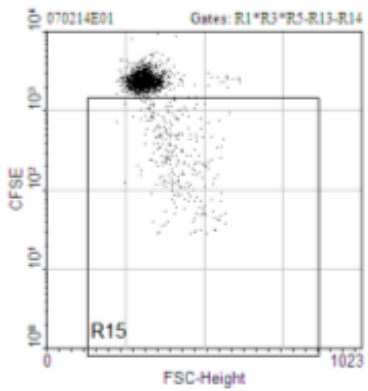
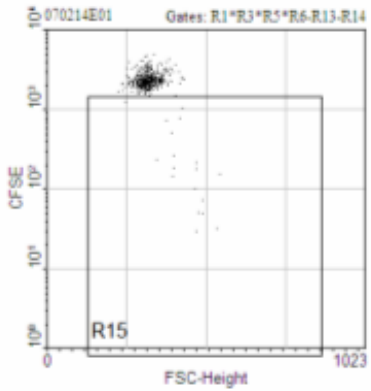
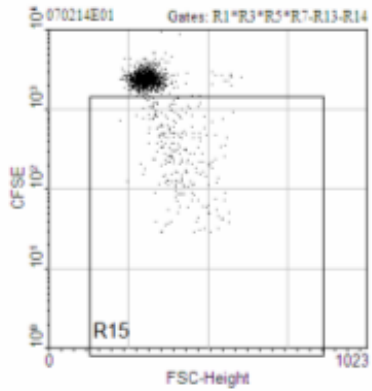
Thiel et al 2004



Lyons et al 1994 JIM 171:131-137

In vitro proliferation of antigen-specific T cells

-CFSE labeling, an example-

CD3+	CD3+	CD3+CD4+	CD3+CD8+
Medium	DC-mock		
 0.3%	 1.8%	 1.4%	 1.7%
PHA	DC-Gag		
 22.1%	 12.7%	 3.6%	 15.4%

Van Baalen personal communication

In vitro proliferation of antigen-specific T cells
-CFSE labeling-

Advantages
- Relatively easy to perform
    - Flow cytometry
- Allows identification of cells
    - Functional profile and differentiation
- No use of isotopes

Disadvantages
- Identifies proliferating cells only
- Bystander activation?

Lytic activity of virus specific CTL
-^{51}Chromium release assay-

- Traditional method to assess cell-mediated cytotoxicity
  - Label target cells with $Na_2[\ ^{51}Cr]O_4$
  - Usually autologous or MHC class I matched EBV-transformed BLCL
  - pulsed with peptides or infected with (recombinant) virus

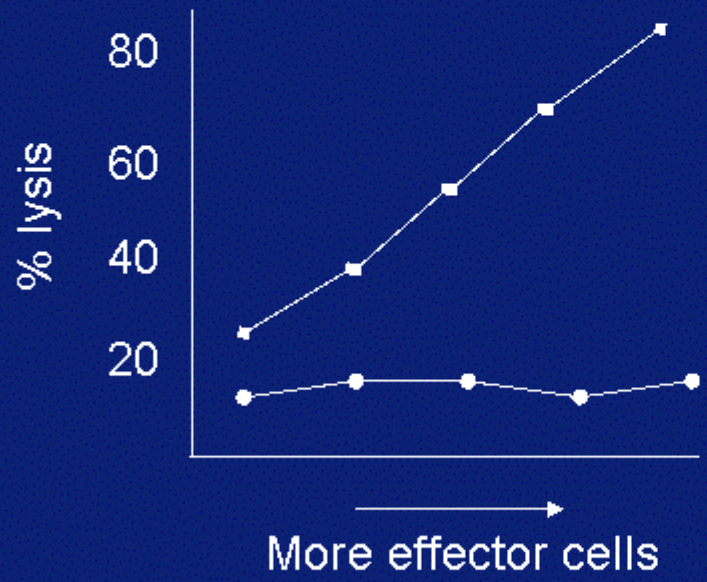
  - Add effector cells and measure ^{51}Cr-release
    - Virus specific expanded PBMC in vitro
    - Limiting dilution (after 7-21 days of in vitro culture)
    - CTL clones

  - After 4 hours radioactivity is measured in culture supernatants

Lytic activity of virus specific CTL

-^{51}Chromium release assay, example-

Detection of the release of $Na_2[\ ^{51}Cr]O_4$ from lysed target cells



Specific lysis = ((experimental release – spontaneous release)/(maximum release – spontaneous release))*100%

Lytic activity of virus specific CTL

-51Chromium release assay-

Disadvantages

- Use of isotopes
- PBMC as effector cells identity unknown
- Relatively insensitive
- Expansion/enrichment of specific T cells required
- Need for autologous target cell (EBV-transformed BLCL)

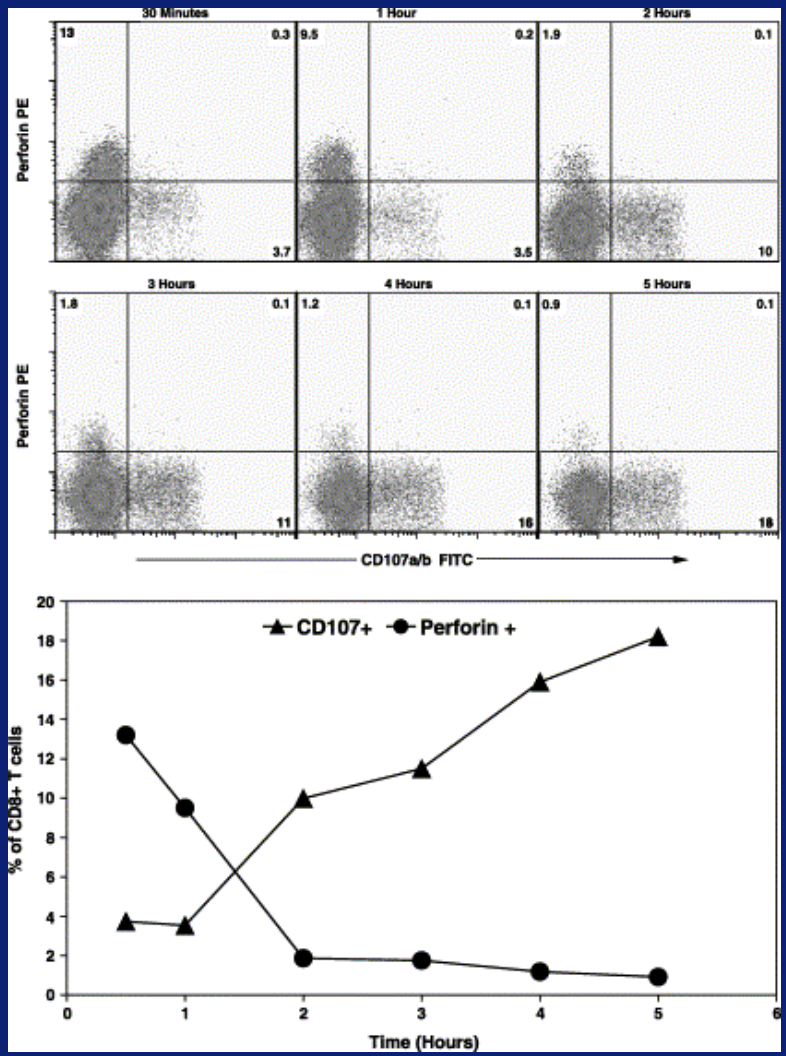
Lytic activity of virus specific CTL

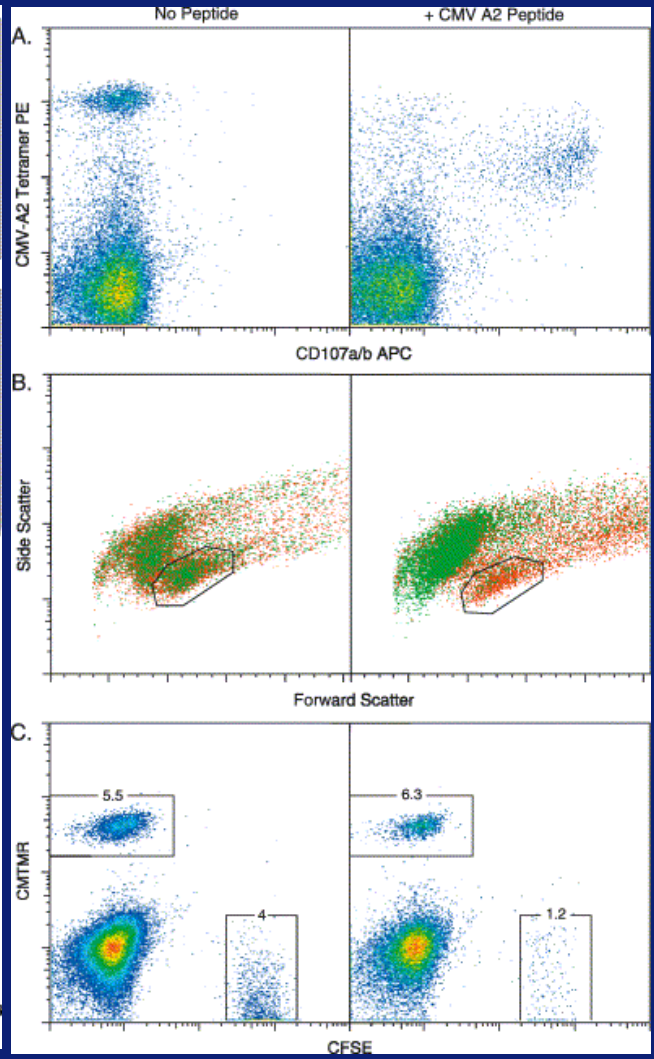
-Alternative assays, example-

- Non-radioactive alternatives
  - Flow cytometric methods
  - Label target cells with fluorescent dyes
  - Add effector cells and measure release of fluorescent dyes or target cell viability
- CTL clones, peptides

Lytic activity of virus specific CTL

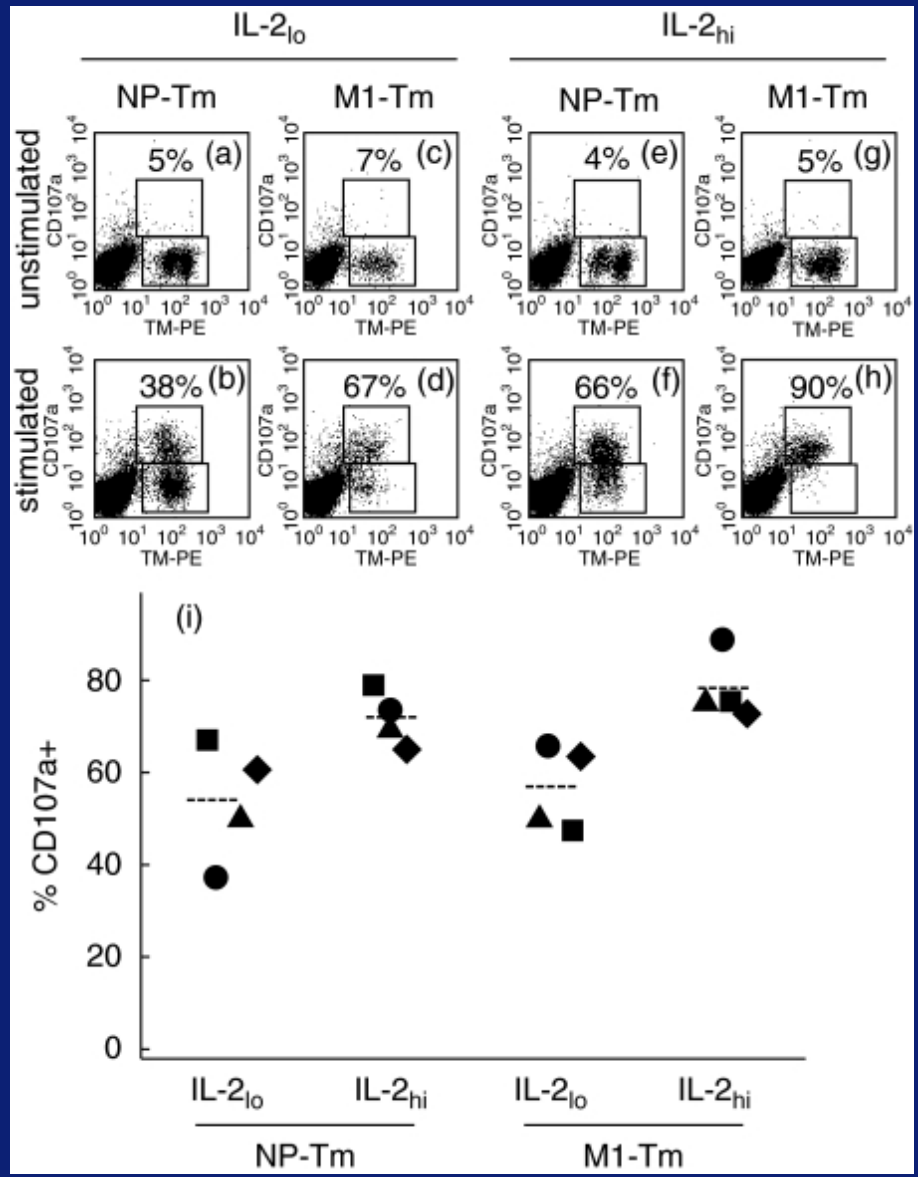
-CD107a expression, marker for degranulation and cytotoxicity-





Betts et al. 2003 JIM 281:65-78

CD107a expression
-marker for degranulation and cytotoxicity-

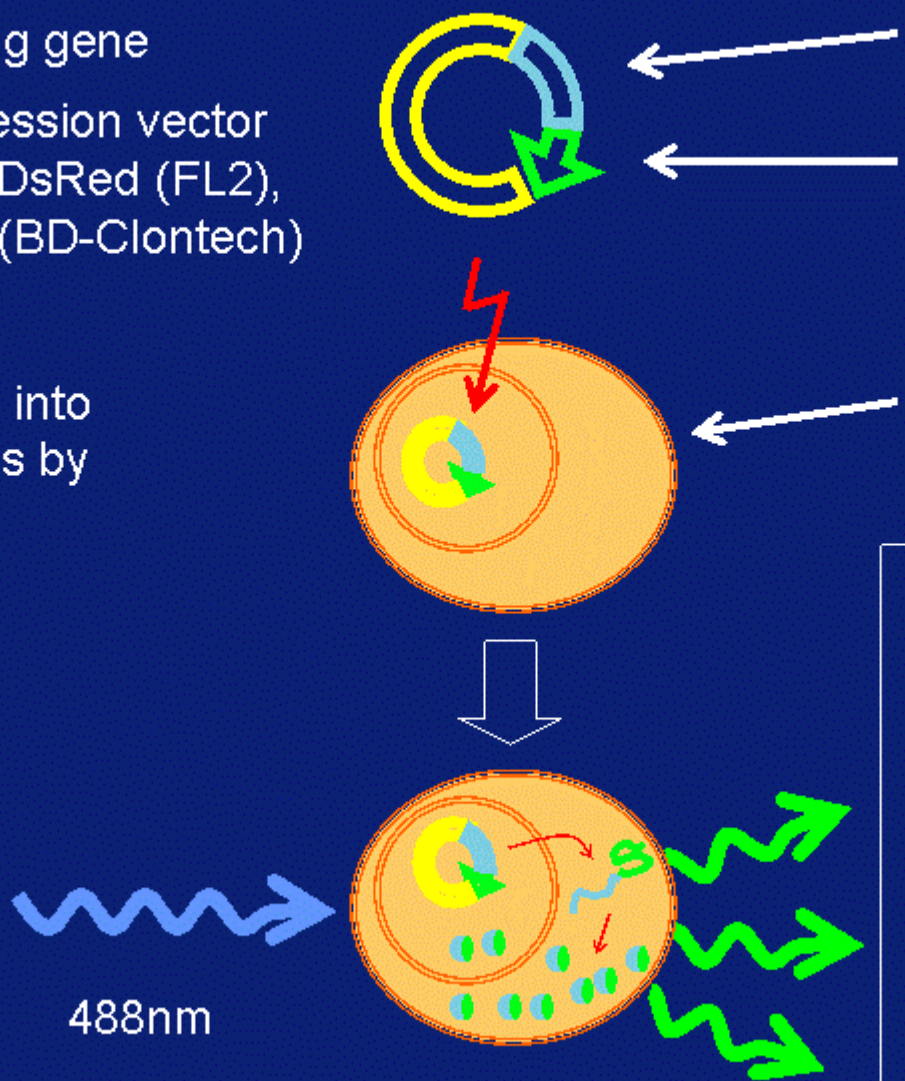


Lytic activity of virus specific CTL

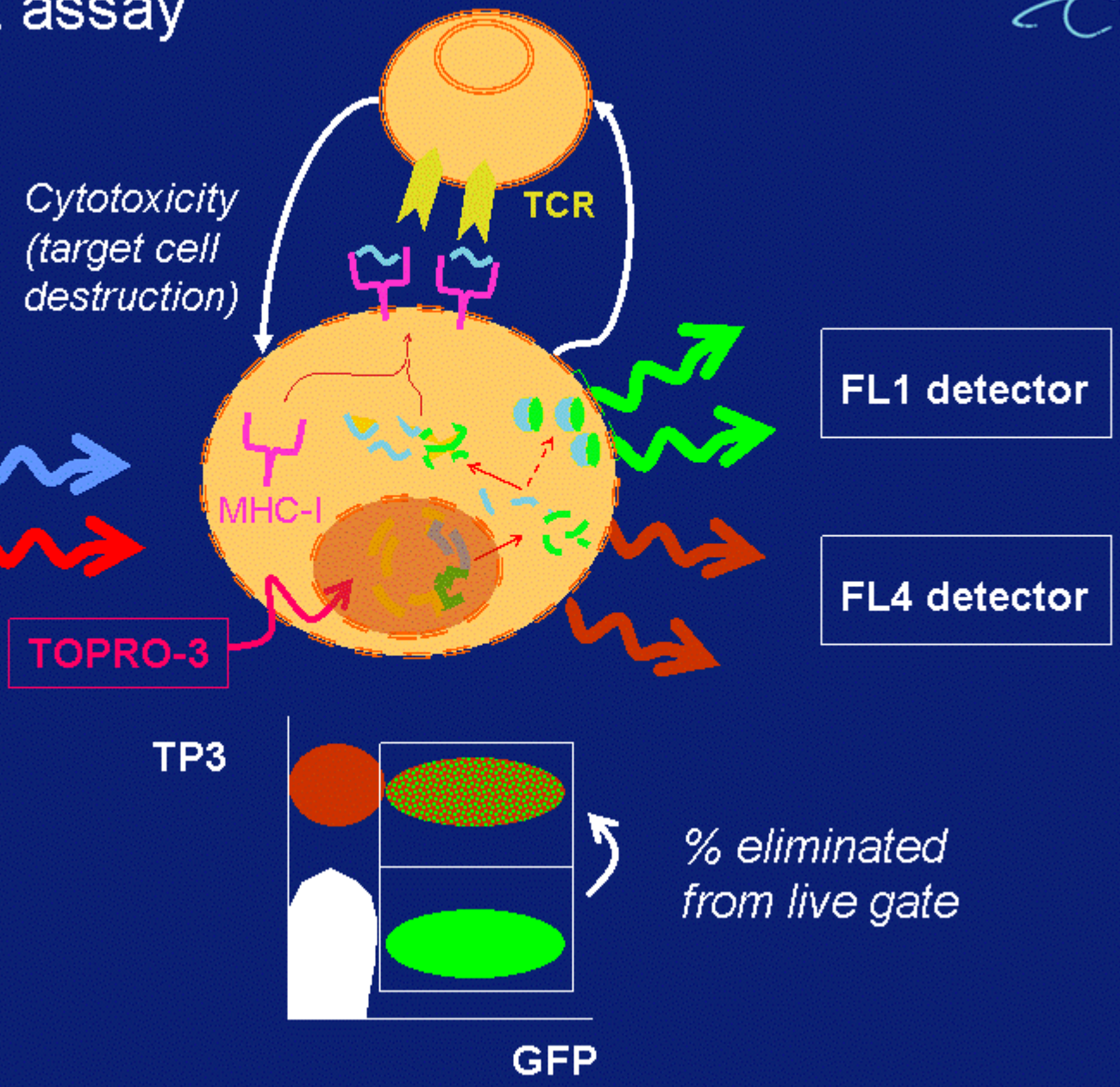
- Fluorescent-antigen-transfected target (FATT) cells CTL assay-

Erasmus MC

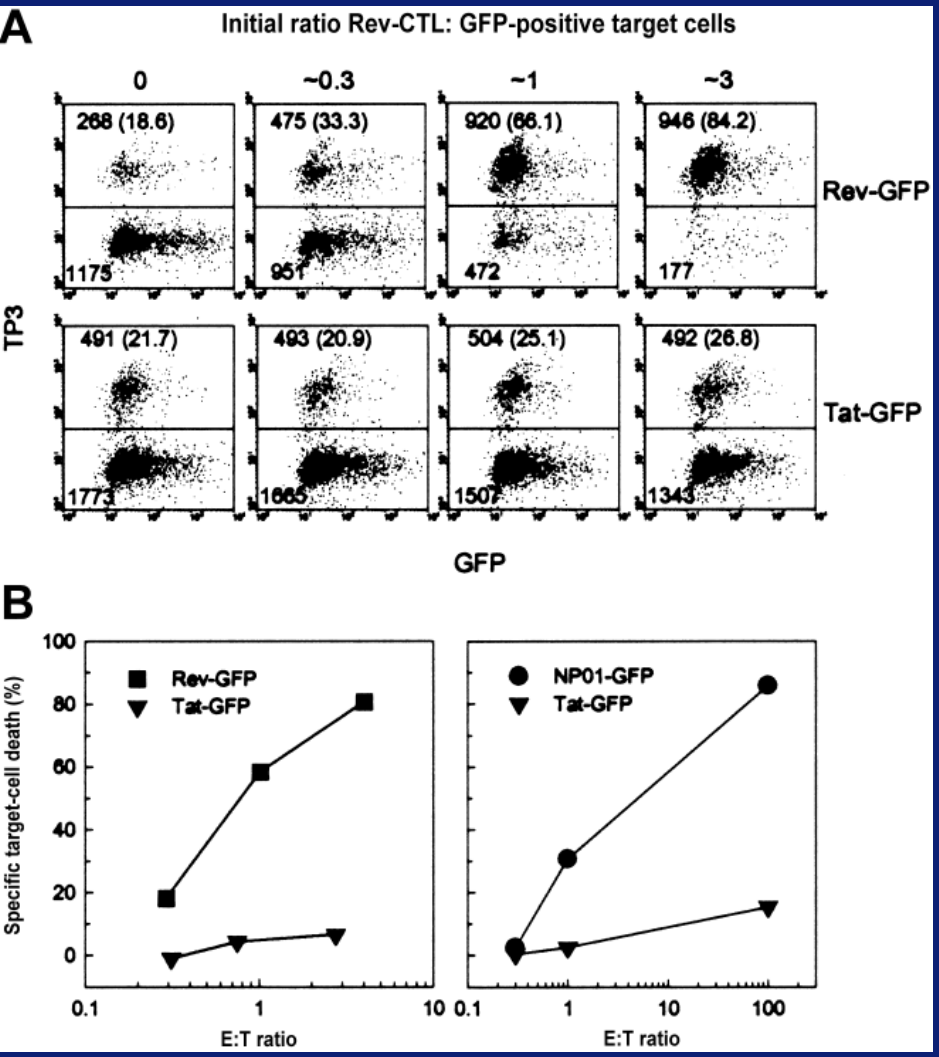
- Antigen-encoding gene
- Eukaryotic expression vector pEGFP (FL1), pDsRed (FL2), pHcRed1 (FL3) (BD-Clontech)
- Nucleofection: Transfer of DNA into the nuclei of cells by electroporation
- Incubation: 4h – 7d 37°C
- Flow cytometry



FATT-CTL assay



Lytic activity of virus specific CTL
-FATT CTL assay-

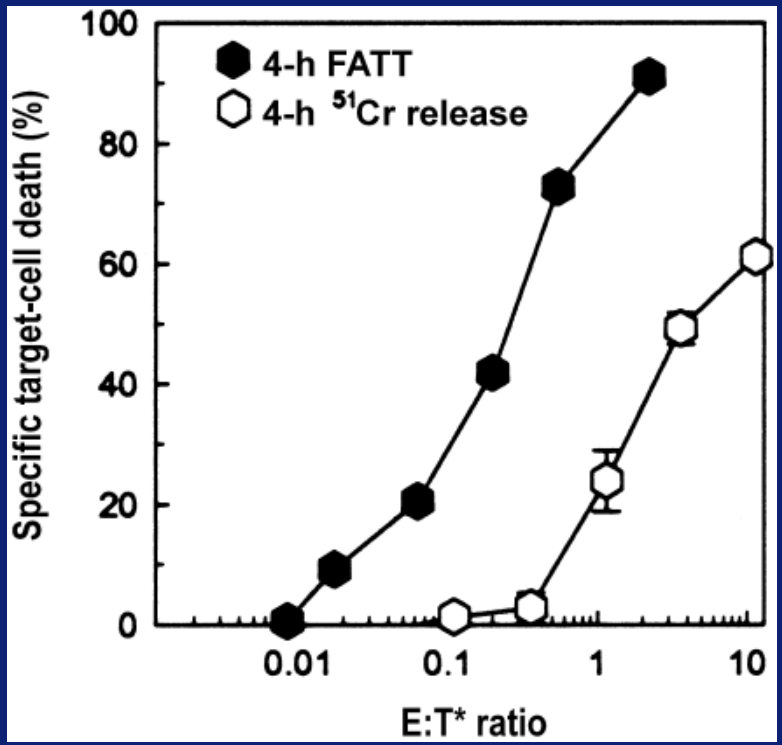


Effector cells:	HIV-1 Rev-specific CTL clone
Target cells:	HLA-matched BLCL cells
Antigen:	pRev-GFP; pTat-GFP

Effector cells:	Flu NP-CTL clone
Target cells:	HLA-matched PBMC
Antigen:	pNP01-GFP; pTat-GFP

Van Baalen et al. J. Infect. Dis. 192:1183-1190 (2005)

Lytic activity of virus specific CTL
-FATT CTL assay-

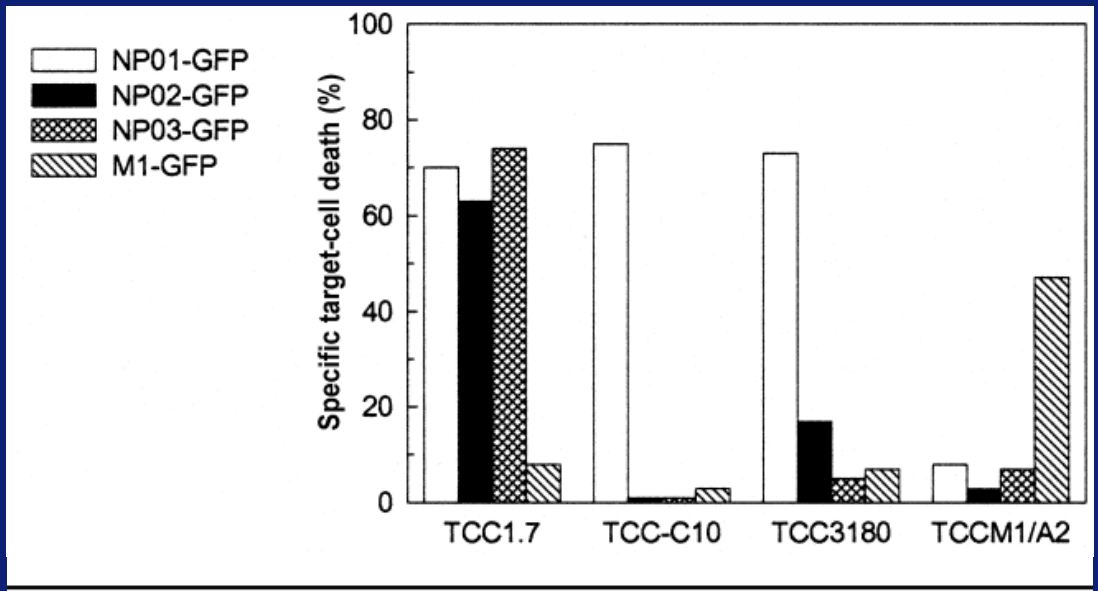


Effector cells: HIV-1 Rev-specific CTL clone
Target cells: HLA-matched BLCL cells
Antigen: pRev-GFP

Van Baalen et al. J. Infect. Dis. 192:1183-1190 (2005)

FATT CTL assay

-lysis correlated to functional avidity-



		CD8+ T cell clone			
Gene	Epitope	TCC1.7	TCC-C10	TCC3180	TCCM1/A2
NP01/02/03	CTELKLSDY	50[a]	…	…	…
NP01	LPFEKSTVM	…	0.8	0.5	…
NP02	---D-P-I-	…	>5 × 10^3	26	…
NP03	---DRT-I-	…	>10^4	1100	…
M1	GILGFVFTL	…	…	…	50

NOTE. M1, matrix protein 1.

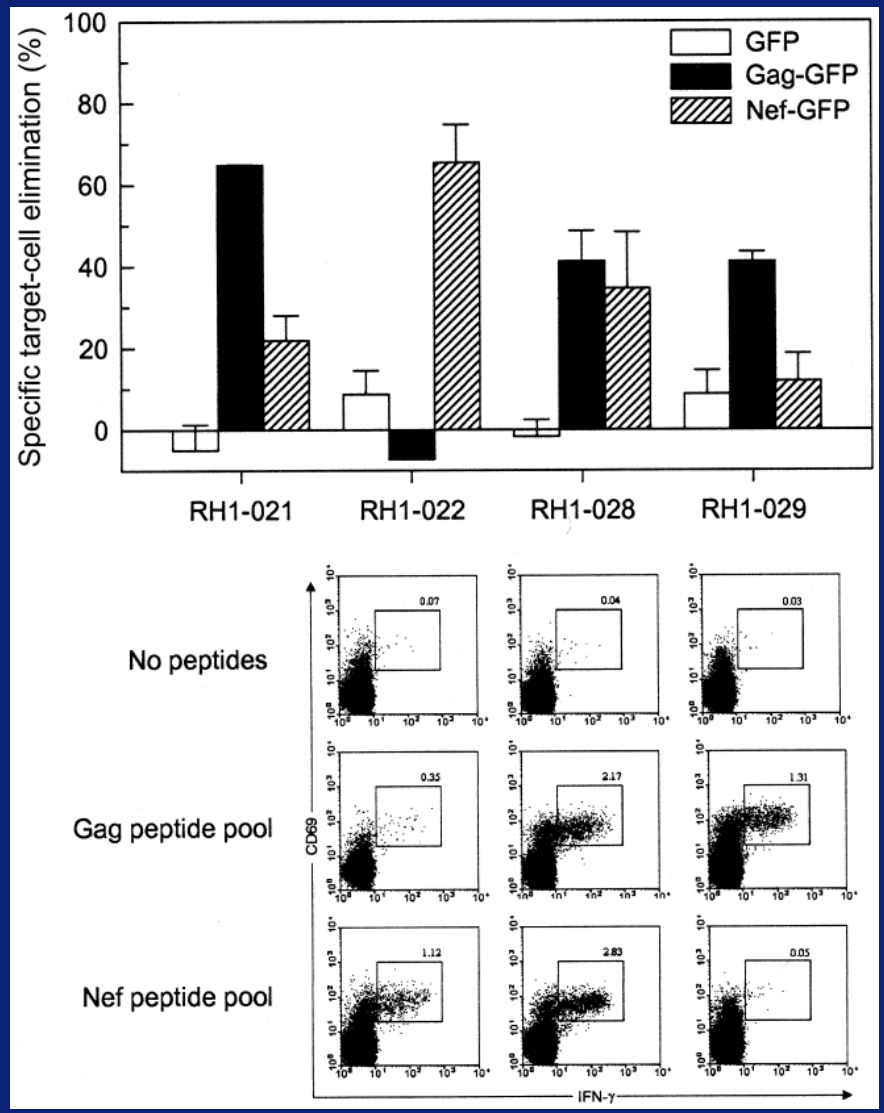
[a] Functional avidity: EC_{50} (nanomolar) of the cytotoxic T lymphocyte clones for the epitope variants, as determined in a ^{51}Cr-release assay [24].

*Functional avidity: EC_{50} value (nM) (^{51}Cr-release) (Boon et al. 2004. J. Immunol **172**:2453)

Ex vivo antigen-specific PBMC-mediated cytotoxicity

-16 hour incubation time-

Mesurement of lytic activity without prior expansion of virus specific T cells



Van Baalen et al. J. Infect. Dis. 192:1183-1190 (2005)

FATT CTL assay

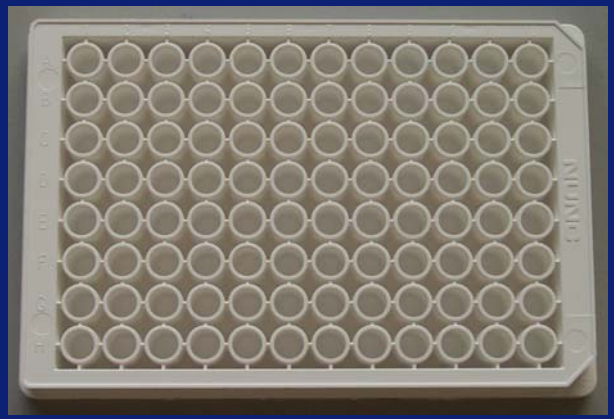
Advantages

- No use of isotopes
- Endogenous antigen processing and presentation
    - No use of peptides
- No autologous cell lines required
    - PBMC as target cells
- No need for HLA typing of study subjects
- Use plasmid DNA vectors, easy to prepare
    - No viral vectors required
- Sensitive

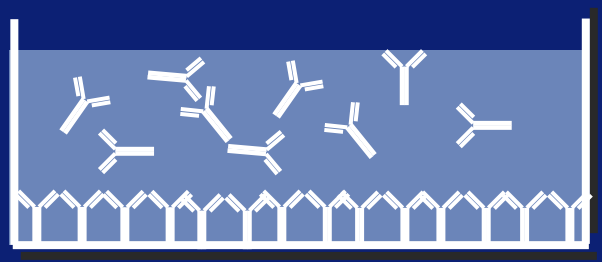
- Prolonged incubation times allow detection of lytic activity without prior expansion of T cells
    - When frequency of specific T cell is high enough
    - e.g. chronic infections, HIV-1

Cytokine production by CTL

-Elispot assay, general principle-



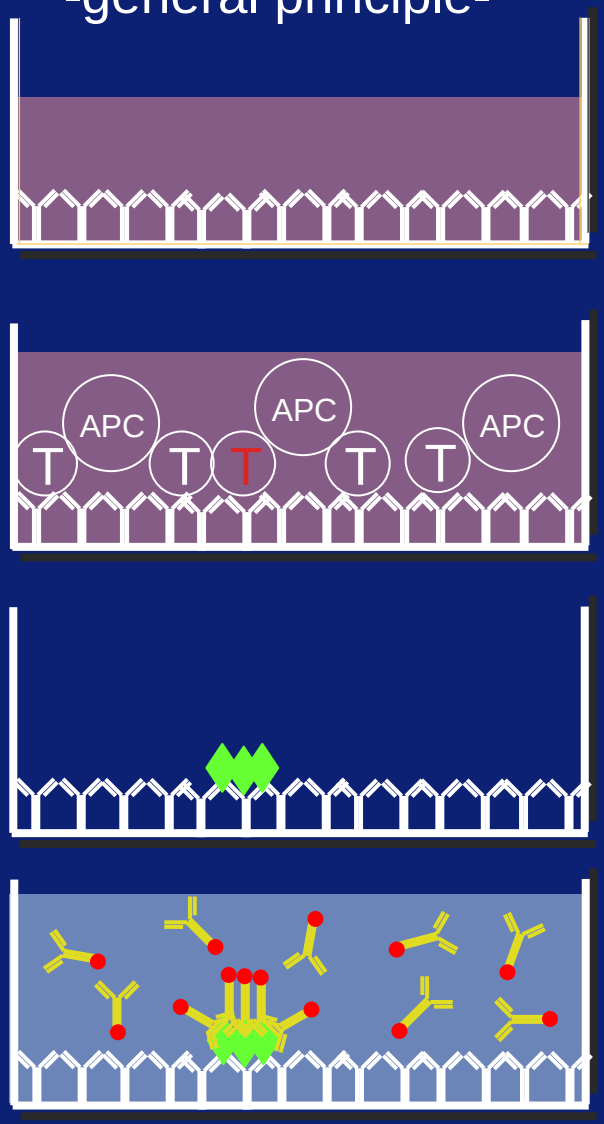
- Standardized reagents
- Commercially available



- Coat with antibody specific for cytokine, e.g IFN-γ

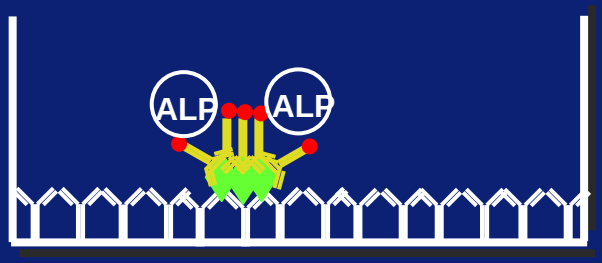
- Wash away excess antibody

ELIspot assay
-general principle-

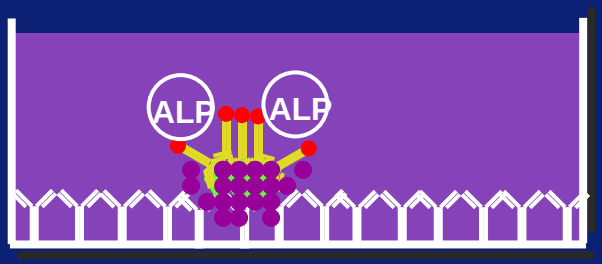


- To prevent non-specific binding block 10% human serum
- Add stimulated cells and incubate 3-6 hours.
- Wash the cells away
- Incubate with secondary biotinylated anti-cytokine antibody. 1 h @37°C or o/n @ 4°C

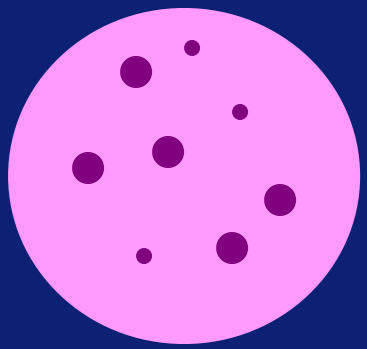
ELIspot assay
-general principle-



• Wash and incubate with alkaline-phosphatase conjugated streptavidin 1 hr@20°C-37°C



• Wash and add substrate (BCIP/NBT substrate) 15-30 minutes at 20°C



• Each spot represents a cytokine producing cell

• Spots are counted with aid of a digital camera!

ELIspot assay
-an example-

IFN-γ production by a $NP_{418-426}$ specific CTL clone

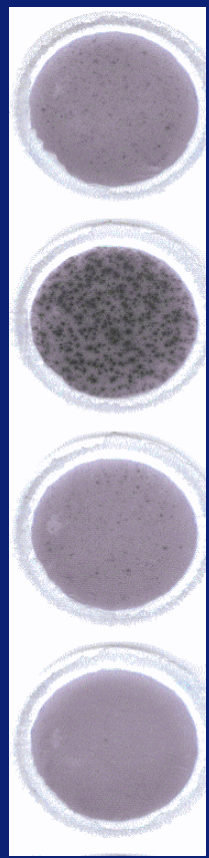
Stimulus

Cells loaded with $M1_{58-66}$

Cells loaded with $NP_{418-426}$

Cells

Negative control



ELIspot assay

Advantages

- Sensitive
- Can be performed without prior enrichment of virus specific cells
- Suitable for high thru-put testing

Disadvantages

- Identity of cytokine producing cells not always known
- Depends on antigen used for stimulation
    - peptides (pools)
    - proteins
    - Live virus
    - Inactivated virus preparations
- Unless cells are sorted prior to testing

Cytokine production by CTL

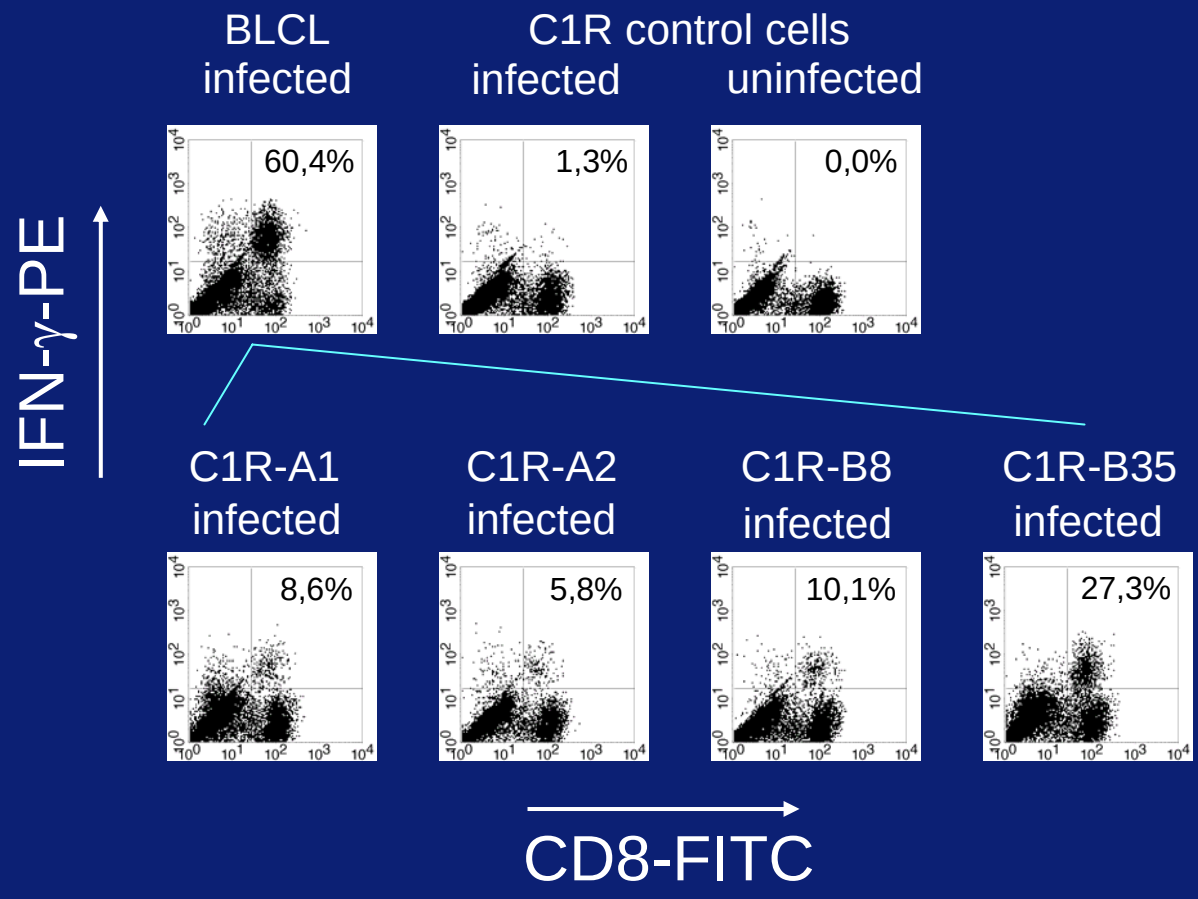
-Intracellular cytokine staining ICS, general principle-

- Effector cells
  - (in vitro expanded) PBMC
  - T cell clones
- Stimulate with MHC class I matched target cells
  - infected with recombinant virus
  - loaded with peptides
- Treat T cells with blockers of the secretory pathway
  - monensin, brefeldin A
- Incubate 3-6 hours
- Fix cells
- Permeabilize
- Stain for cytokine, e.g. IFN-γ and for appropriate CD markers
  - CD3, CD4, CD8
- Analyse by flow cytometry

Intracellular IFN-γ staining

-HLA-usage in influenza virus specific CTL response, an example-

HLA-A*0101, -A*0201, -B*0801, -B*3501 positive donor



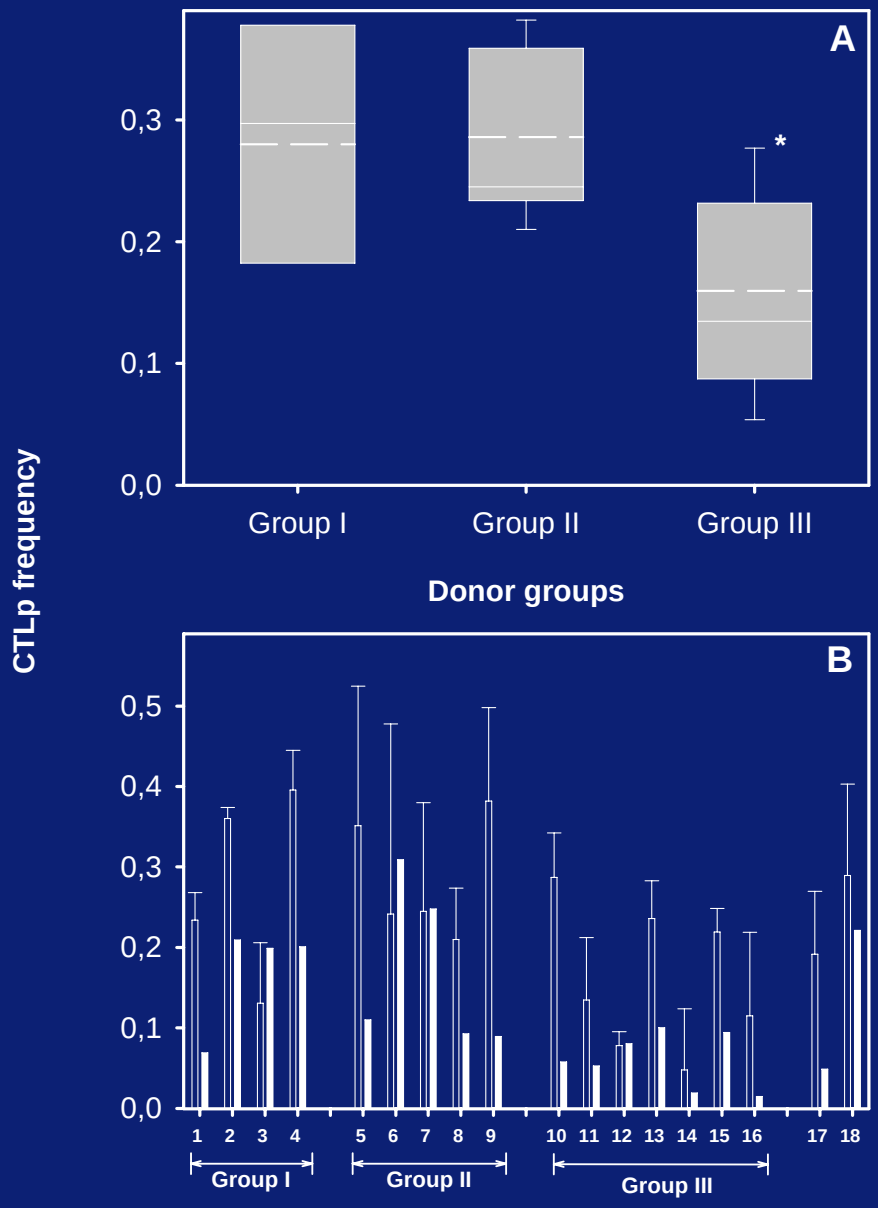
Boon et al, 2004, J. Immunol. 172(7):4435

Comparison of HLA usage in donors with different HLA alleles

- Avg. % IFN-γ+ cells of the CD8+ T cell fraction -

	Group I	Group II	Group III
BLCL	46	41	32
C1R-A1	5,5	2,4	3,5
C1R-A2	10,2	10,5	
C1R-A3			10
C1R-B8	9,2	2,9	7,8
C1R-B27		23,8	
C1R-B35	16,4		15,9

Frequencies of influenza virus specific CTL



- Immunodominant and conserved M1 58-66 epitope is HLA-A*0201 restricted
- Group III is HLA-A*0201 negative
- Overall lower influenza virus specific CTL response in this group

Boon et al, J Virol. 2002 76:582-90

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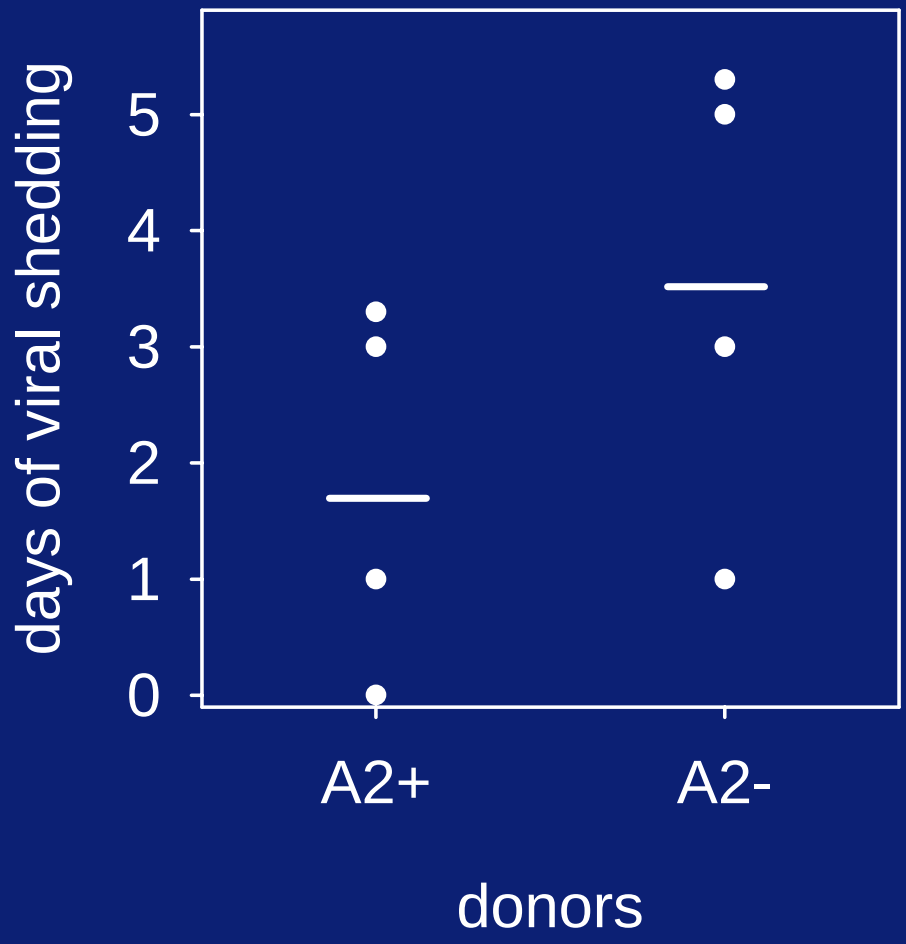
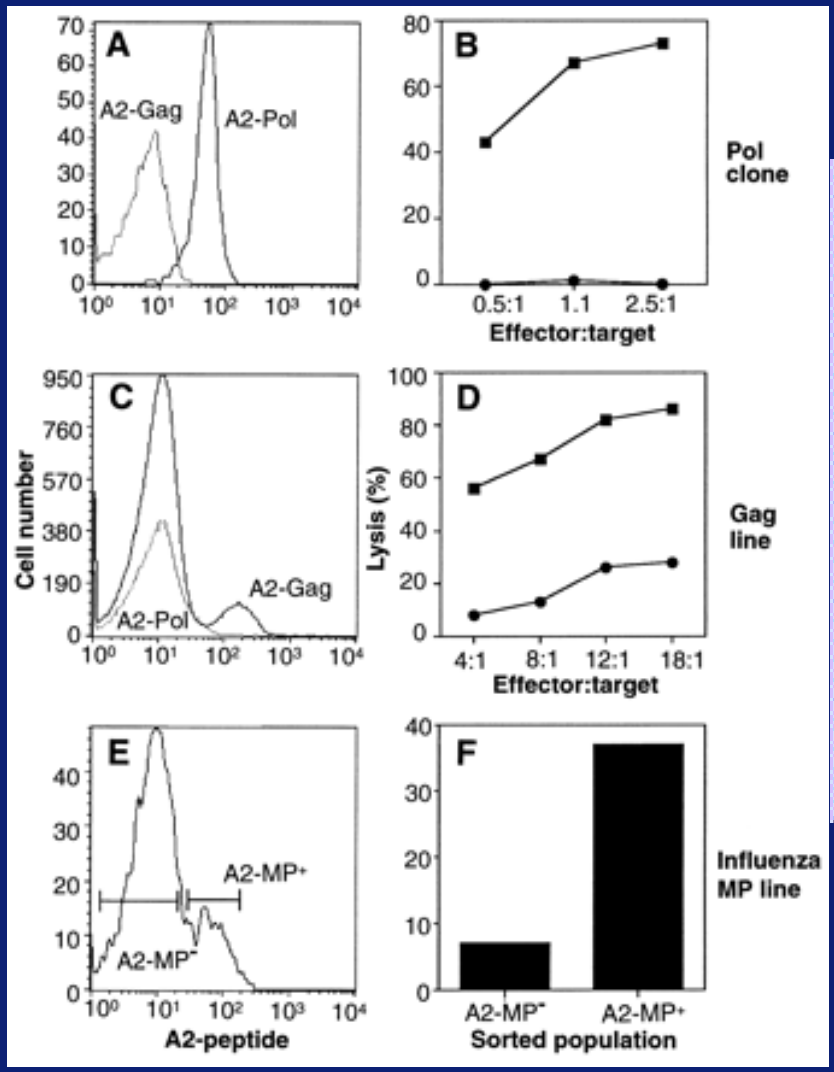
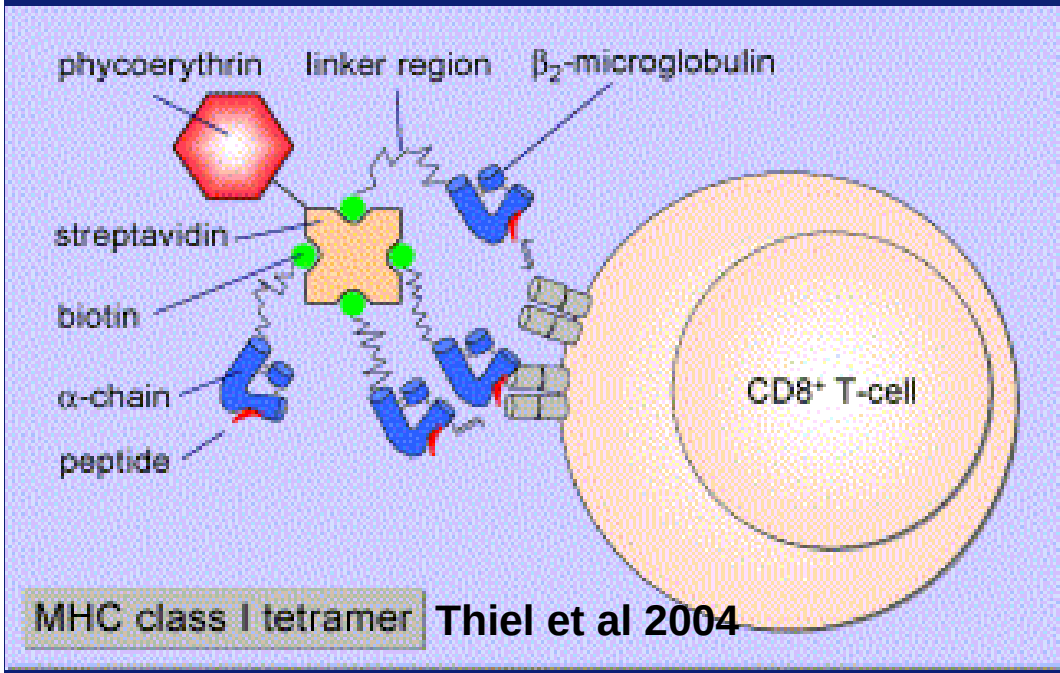
Prolonged viral shedding in HLA-A2 negative donors? (P=0.2)



Epitope specificity

-Multimers of MHC class I/Peptide complexes-





Altman et al, 1996 Science 274:94-96

Epitope specificity

-Multimers of MHC class I/Peptide complexes-

Advantages

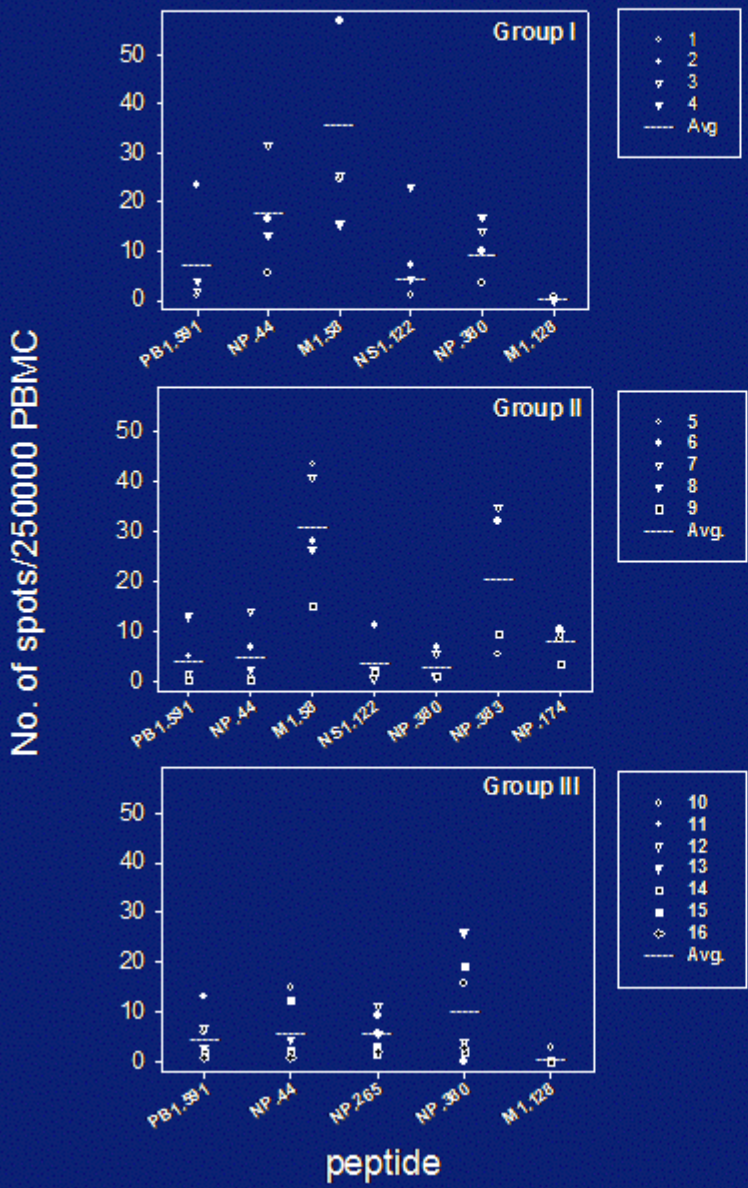
- Ease!
- Identifies ALL epitope specific T cells
- Simple detection by flow cytometry
- Can be performed ex vivo
- Depends on frequencies

Disadvantages

- Number of available MHC class I peptide complexes is limited
- For defined epitopes only
- Must know HLA background of study subjects!
- No information on functionality of T cells, unless co-stain for cytokines, CD107a etc.

Epitope specificity

-Use of peptides in IFN-γ ELIspot



Considerations

- Many different technologies
- Some require prior re-stimulation and expansion of specific cells in vitro.
- Source of antigen used for stimulation of T cells
    - peptide (pools)
    - (recombinant) virus expressing viral proteins
    - protein expression from plasmids
- Immunodominance
    - hierarchy
- HLA system is highly polymorphic!!
- HLA phenotype dictates which epitopes are recognized and which are not e.g. HLA A*0201 restricted M1 58-66 epitope
- Exposure history!
- HLA back-ground of non-corresponding HLA alleles can influence response
- Variation in T cell epitopes
    - anchor residues
    - T cell receptor contact residues

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