

Adjuvant Guidance of T cell Responses

Cellular Technology Limited

Presented by

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Introduction to Cellular Technology Limited (CTL): Shaker Heights, Ohio, USA

- **CTL-Laboratories (CRO)**
 - R&D-Laboratory (Research and Development)
 - CLIA certified, GLP-Laboratory (ELISPOT, ELISA, multiplex cytokine bead arrays, FACS,...) assay development, qualification and validation, and testing
 - Whole blood shipping kits and logistical support
 - Serve as Central Laboratory for whole blood processing and cryo-preservation of PBMC
- **CTL-Consumables**
 - Cryopreserved, uncharacterized, HLA-typed and immune characterized PBMC
 - Standardized solutions (Serum free Media, etc.)
 - Antigens: Peptides
- **CTL- Analyzers**
 - ISO certified: Data Acquisition Systems: ImmunoSpot Analyzer, UV-instrumentations, BioSpot Analyzer etc.
 - Supporting Software Applications, etc..

Current challenges facing broad implementation of Cell Mediated Immune (CMI) monitoring

- Functional, correlative biomarker assays require living sample/cell material
- Sufficient quantity and quality of cell material needs to be collected by diverse clinical sites (and usually can not be tested directly on site)
- Need to establish Effective Monitoring Test Systems, Validation Procedures and Standardization Reagents for primary cell-based assays (not cell-line based systems)

Required characteristics of assays to monitor CMI in regulated environments

- Measures a physiological and clinically relevant response
- Has a adequate, relevant sensitivity that is able to detect low frequency responses
- Lends itself to validation (ability to determining Accuracy, Precision, Specificity, Linearity and Limits of Detection) and is a reproducible assay for testing serial samples
- Objectivity through data analysis equipment that can be integrated in 21 CFR Part 11 compliant laboratory settings (i.e., system validation, computer generated audit trails)

Desirable features of assays for monitoring CMI in regulated environments

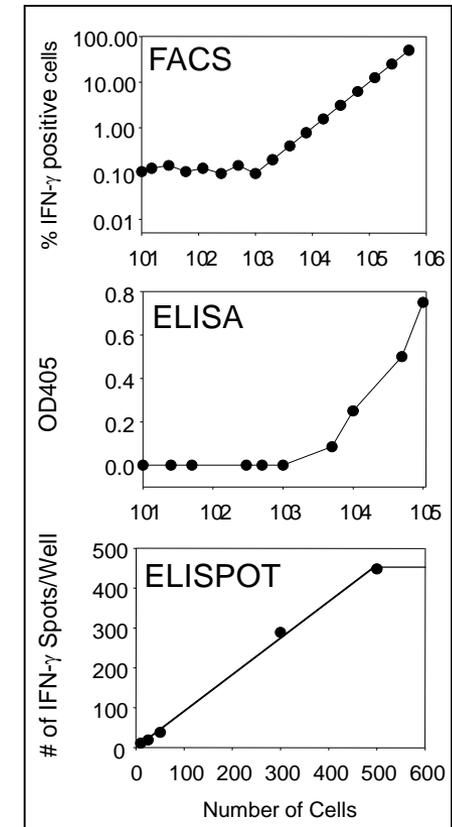
- Performs identical with fresh and previously frozen samples
- Works with out bred, genetically diverse populations
- Uses the least amount of cells and clinical sample material
- Scalable to accommodate large-volume testing with hundreds of samples in later stage clinical trials
- Can be standardized; to enable, for example, inter-study comparisons to make data more robust for multicenter or large international clinical trials

Commonly available assay technologies to evaluate cytokine production CMI

- Enzyme-Linked Immunosorbent Assay (**ELISA**)
 - Luminex & Cytometric Bead Array (**CBA**)
-
- Enzyme-Linked Immunospot Assay (**ELISPOT**)
 - Fluorescence-Activated Cell Sorter (**FACS**)
 - Intracytoplasmatic Cytokine Staining (**ICS**)
 - Tetramer staining
 - Pentamer staining
 - Surface marker staining
- Supernatant based
- Cell based
- Establishes binding or phenotype of cells (Not True Functional Assays)

ELISPOT assay's unique qualification for immune monitoring

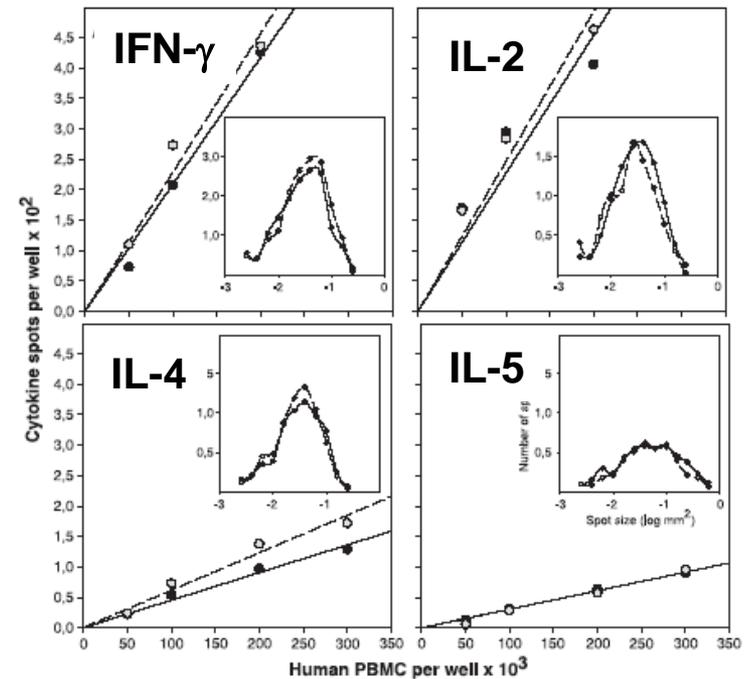
- **Sensitivity:** Routine detection limits of 1 in 500,000, or better. That is several ten to hundred folds more sensitive than ELISA, CBA, ICS, etc.
- **Direct *ex vivo* cell frequencies:** Measures the physiologic magnitude of T-cell immunity, with no need for *in vitro* expansion (not provided by ELISA, CBA, * ICS_{low} , etc.)
- **No need for HLA typing of subjects:** Matching autologous APC are presented within the assay
- **Samples not pharmacologically treated:** No use of secretion inhibitors or cell permeability reagents as with ICS and FACS



**(only applies to low frequency T cell responses)*

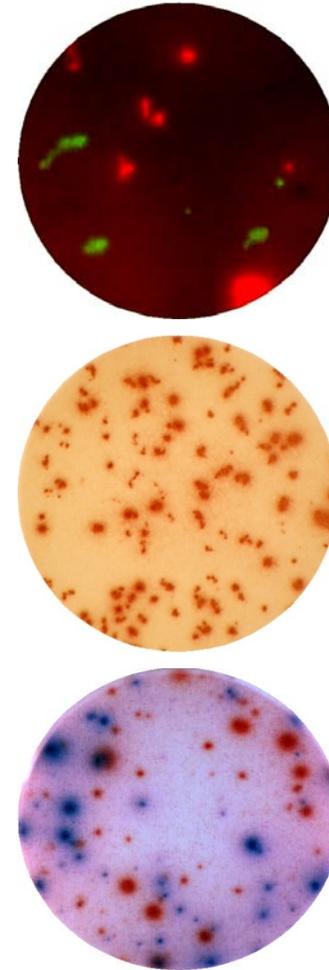
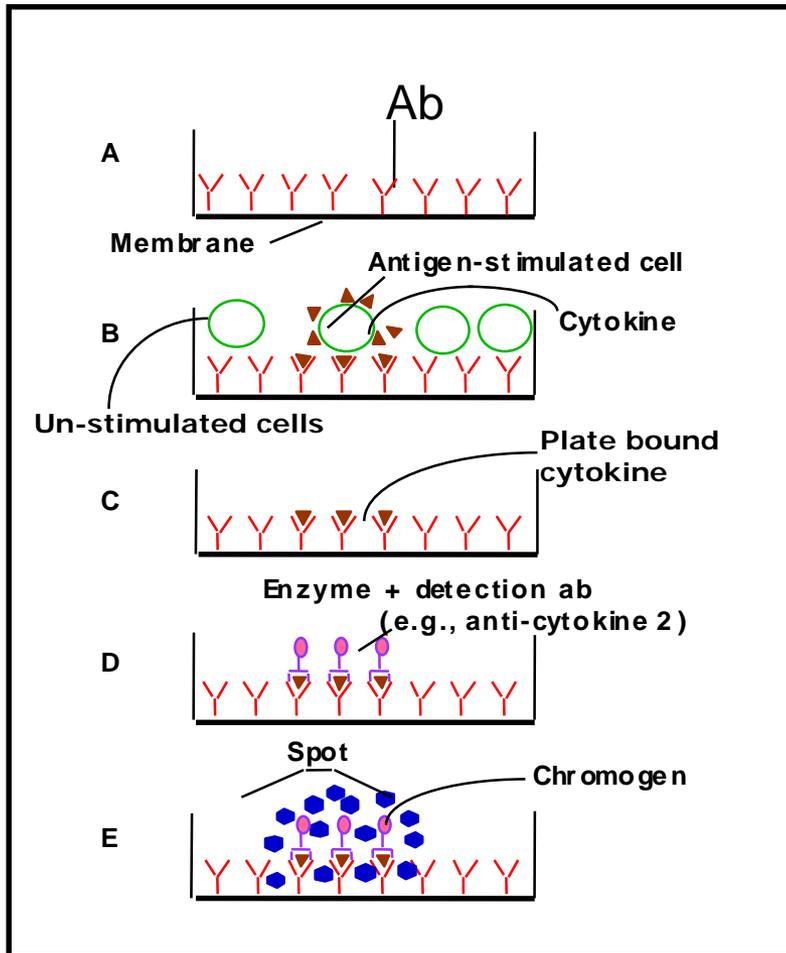
ELISPOT assay's unique qualification for immune monitoring as fresh vs. cryopreserved cells perform equally

- **Robustness:** Cryopreservation allows collection of samples for trials, and testing in high throughput mode at a later time point.
- **Validation Capabilities:** Assays can be validated under GLP with multiple cytokines and test systems (e.g., CTL Laboratories has validated for its clients human, mouse, NHP - non human primates, and pig test systems among others)
- **Linear Performance.**
- **Single cell resolution.**

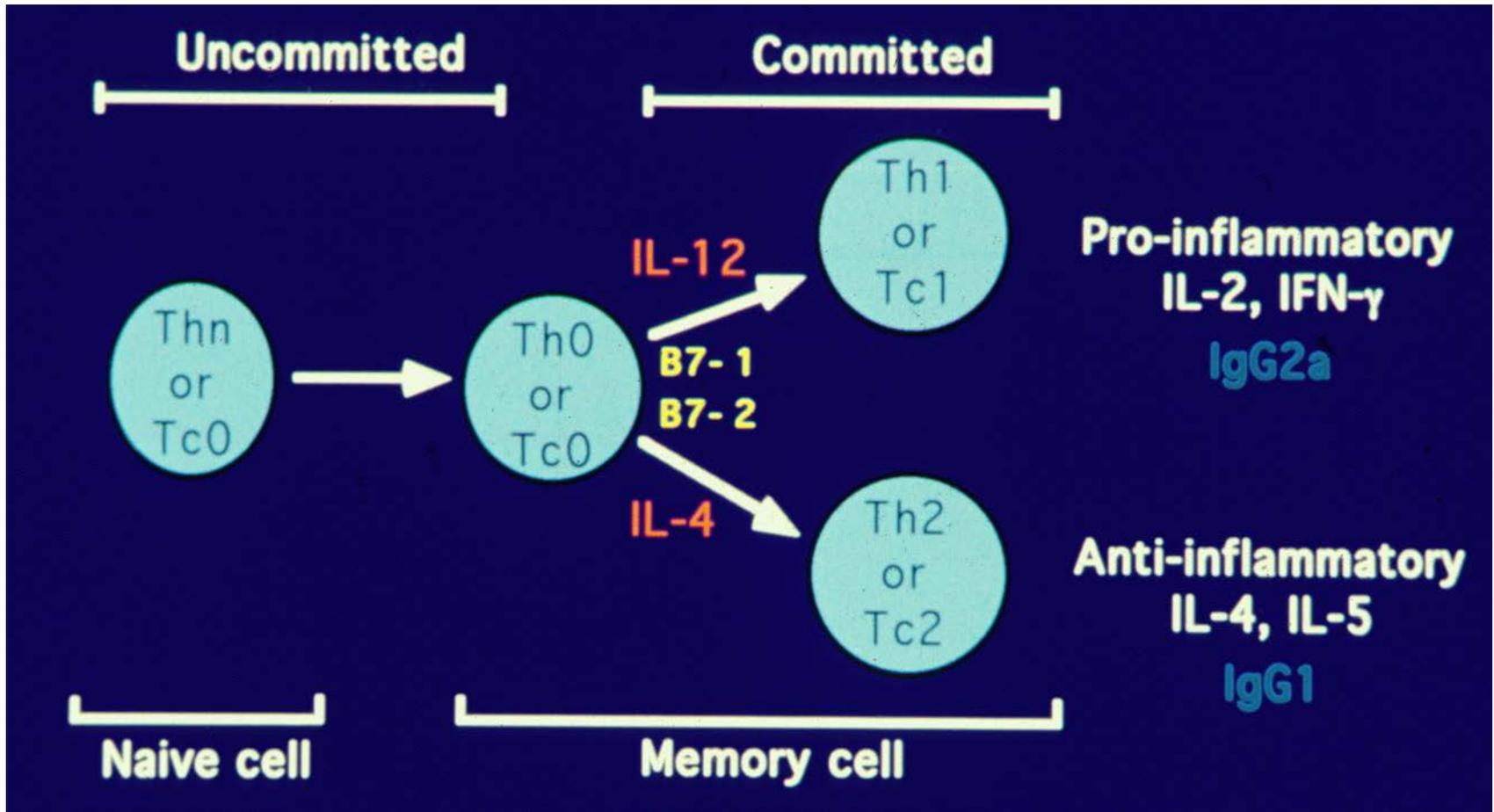


Kreher et al. J. Immunol. Methods, 2003, 278 :79– 93

Principles of ELISPOT



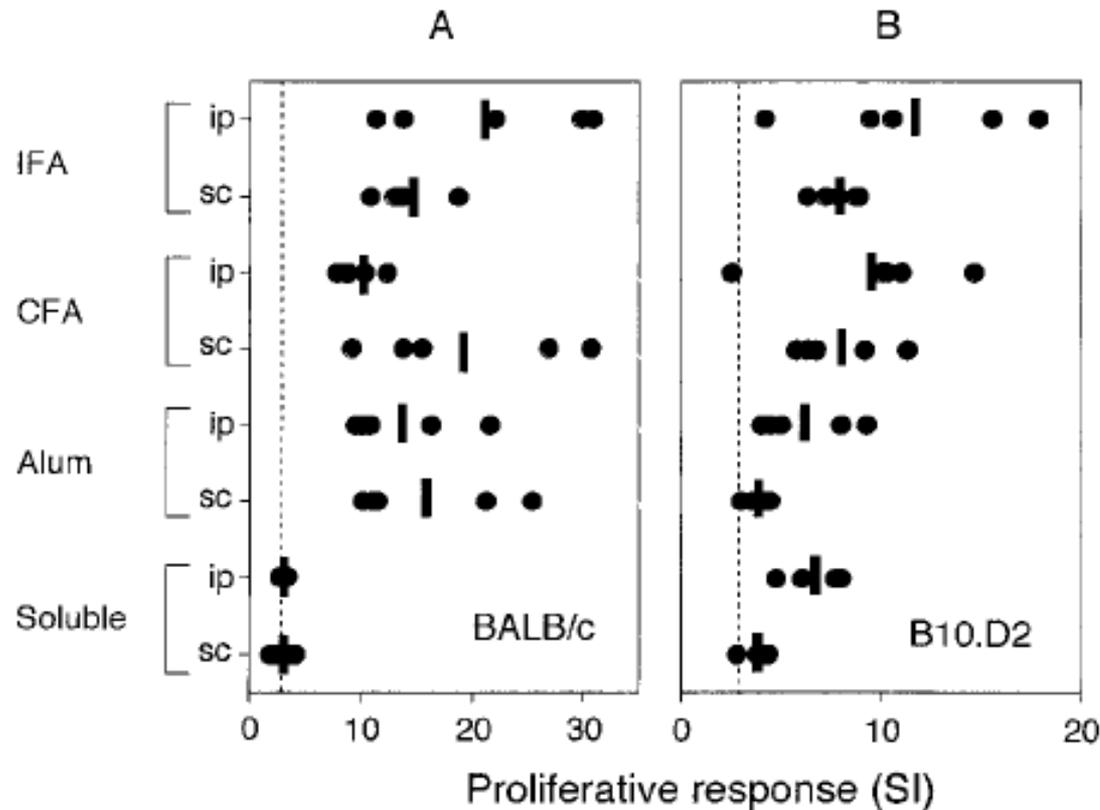
Post-thymic T-cell differentiation



How Adjuvants guide T cell Responses

- Adjuvants are substances which enhance immune responses towards a co-administered antigen
 - e.g. induce enhancement of antibody production, CD4⁺ T cell cytokine secretion, CD8⁺ T cell cytolytic activity
- Some mechanisms of action of adjuvants include:
 - creating an antigen “depot” effect
 - enhancing antigen delivery and uptake
 - creating the appropriate cytokine milieu (immunomodulation)
- Is the influence the of the adjuvants on CD4 or CD8 T cells responses the same?

Proliferation responses induced by all adjuvants used



Yip et. Al., J. Immunology, 162: 3942-3949

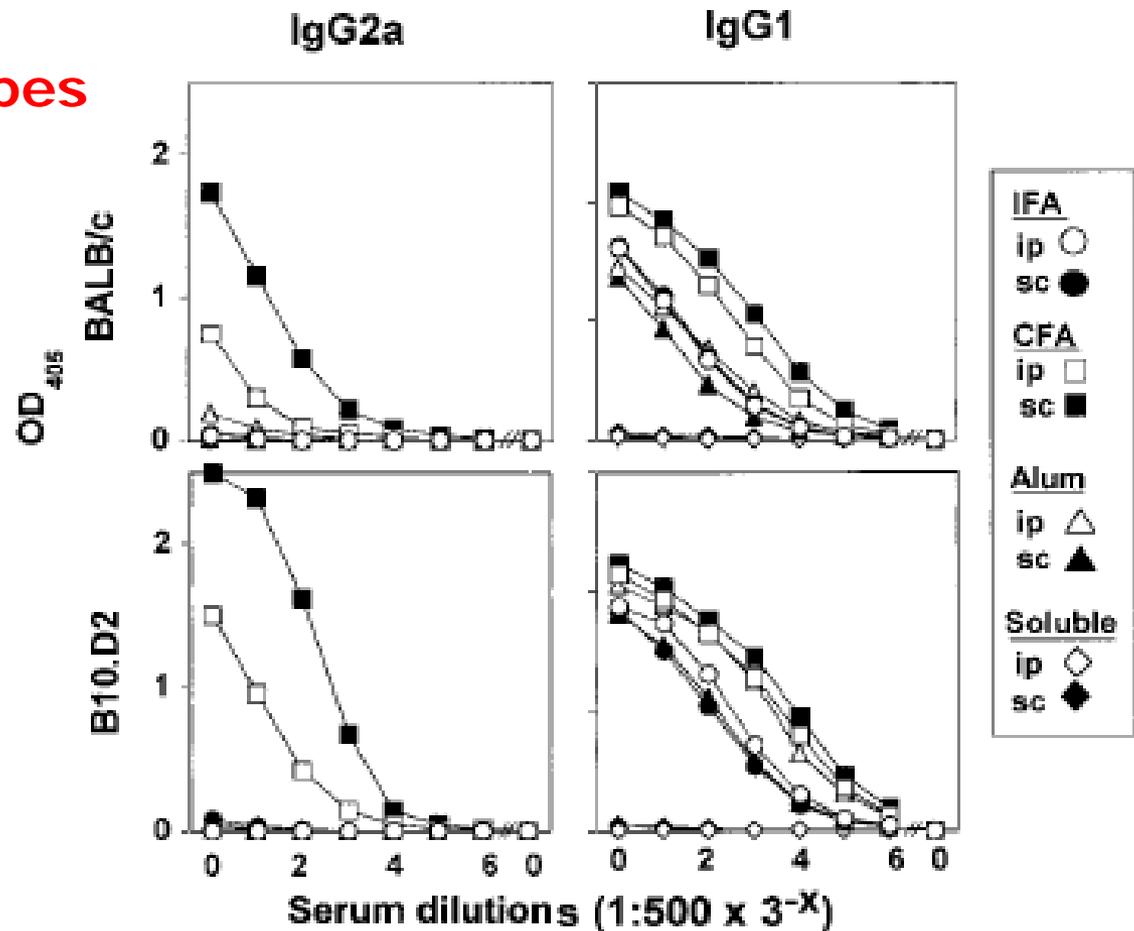
Influence of adjuvants on B-cell responses

- Different Ab isotypes with comparable magnitude were induced

CFA (IgG1 and IgG2a)

IFA (IgG1)

Alum (IgG1)



Yip et. Al., J. Immunology, 162: 3942-3949

Table I. Cytokine recall response in spleens of mice injected with various Ags

Strain	No. of Mice	Ag ^a	Adjuvant and Route	Cytokine Recall Response ^b (no. of cytokine-producing cells/10 ⁶)	
				IL-5	IFN- γ
C57.BL/6	8	OVA	IFA i.p.	40 \pm 23	<3
	8	OVA	CFA i.p.	<3	82 \pm 12
BALB/c	4	<i>Leishmania</i>	IFA i.p.	19 \pm 3	<3
	4	<i>Leishmania</i>	CFA i.p.	<3	31 \pm 5
	4	BSA	IFA i.p.	9 \pm 3	<3
	4	BSA	CFA i.p.	<3	20 \pm 12
	4	BSA	CFA s.c.	<3	42 \pm 20
	4	<i>Leishmania</i>	IFA i.p.	21 \pm 4	<3
	4	<i>Leishmania</i>	CFA i.p.	<3	19 \pm 3
	4	<i>Schistosoma</i>	IFA i.p.	58 \pm 33	<3
	4	<i>Schistosoma</i>	CFA i.p.	<3	40 \pm 24
	24	OVA	IFA i.p.	43 \pm 10	<3
SJL	24	OVA 323-339	IFA i.p.	40 \pm 1	56 \pm 15
	24	OVA 323-339	CFA s.c.	<3	71 \pm 15
	3	RTA	IFA i.p.	55 \pm 21	<3
	3	RTA	CFA s.c.	<3	66 \pm 20
	5	PLP 140-152	IFA i.p.	14 \pm 8	<3
	6	PLP 140-152	CFA s.c.	7 \pm 6	50 \pm 21
B10.PL	5	MBP	IFA i.p.	18 \pm 3	<3
	17	MBP	IFA i.p.	25 \pm 15	<3
	24	MBP	CFA i.p.	<3	46 \pm 8
B10.D2	5	MBP	CFA s.c.	<3	73 \pm 28
	4	BSA	IFA i.p.	24 \pm 15	<3
	4	BSA	CFA i.p.	<3	30 \pm 16
DBA/2	4	BSA	CFA s.c.	<3	35 \pm 28
	2	HEL 106-116	IFA i.p.	29 \pm 4	7 \pm 2
	2	HEL 106-116	CFA s.c.	<3	39 \pm 2

^a A total of 100 μ g of each Ag was injected as described in *Materials and Methods*, with the exception of *Leishmania*, in which 50 μ g was used.

^b Cytokine ELISPOT assays were performed on spleen cells 2–6 wk after injection. The responses were specific for the Ag injected. Mean \pm SD for all mice in the groups are shown. Legend to Fig. 3 applies.

Table 1. Neonates were injected within 24 hours of birth, then tested at 6 weeks of age. Adults were injected at 6 weeks of age, then tested 21 days later. All mice received a single injection of 100 µg of antigen.

Mouse strain and age	Injection	Route	Recall response* (cytokine ELI spots per 10 ⁶ spleen cells)			
			IFN-γ		IL-5	
			HEL	MBP	HEL	MBP
BALB/c						
Neonate	HEL-IFA	i.p.	<5	<5	31.8 ± 7.9	<5
Neonate	HEL-CFA	i.p.	37.8 ± 15.1	<5	<5	<5
Adult	HEL-IFA	i.p.	<5	<5	38.3 ± 12.1	<5
Adult	HEL-CFA	i.p.	51.4 ± 19.9	<5	<5	<5
Adult	HEL-CFA	s.c.	49.3 ± 20.5	<5	<5	<5
B10.PL						
Neonate	MBP-IFA	i.p.	<5	<5	<5	35.6 ± 15.3
Adult	MBP-CFA	i.p.	<5	73.7 ± 28.5	<5	<5

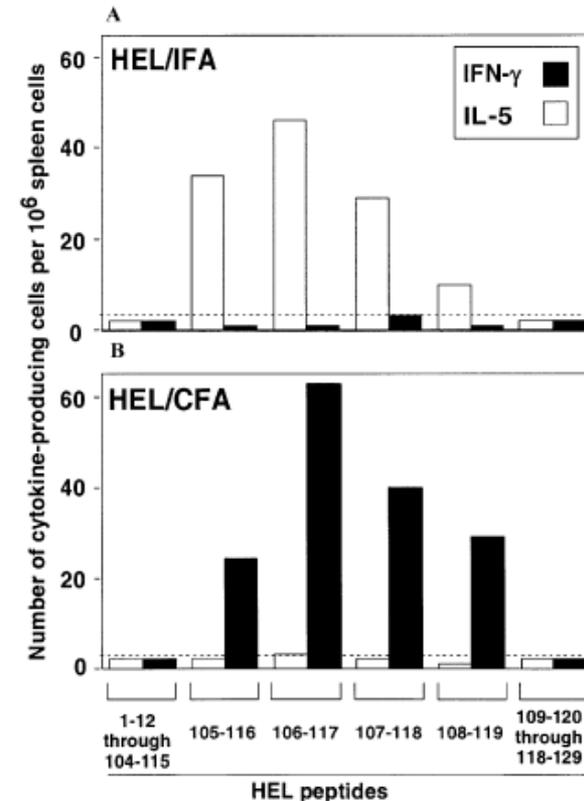
*Data are expressed as an arithmetic mean ± SEM of 9 to 12 spleens independently tested in three experiments.

•Forsthuber T, Yip HC, Lehmann PV. 1996. *Science* 271: 1728-30

Detection of CD4 response modulation requires parallel monitoring of multiple cytokines

- Influence of adjuvants on CD4 T-cells:

The choice of adjuvant during vaccination (i.e. **IFA versus CFA**) can switch peptide specific CD4 recall responses from Th1 (**IFN- γ**) to Th2 (**IL-5**)

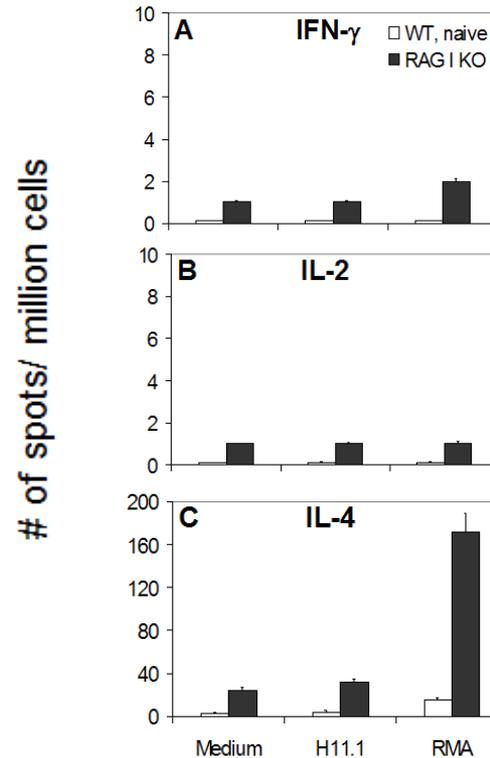
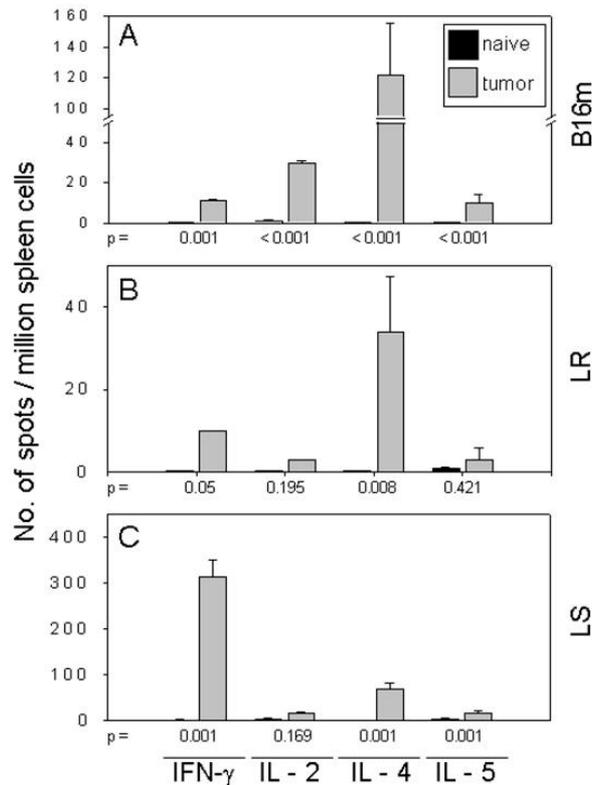


Yip et. Al., J. Immunology, 162: 3942-3949

Induction of protective immunity against tumors continues to be a major challenge

- Tumor cells are immunologically similar to cells with normal growth characteristics.
- Tolerogenic mechanisms that normally prevent induction of autoimmunity may function to prevent protective immune response against the tumor.
- Mechanisms whereby tumors can escape immune recognition.
 - Decreased expression of MHC molecules.
 - Lack of costimulatory molecules.
 - can lead to deletion of specific T cells.
 - make the T cells unresponsive.
 - Tumors can produce immunosuppressive molecules such as TGF- β

Tumor monitoring: Tumors are immunogenic in syngeneic mice

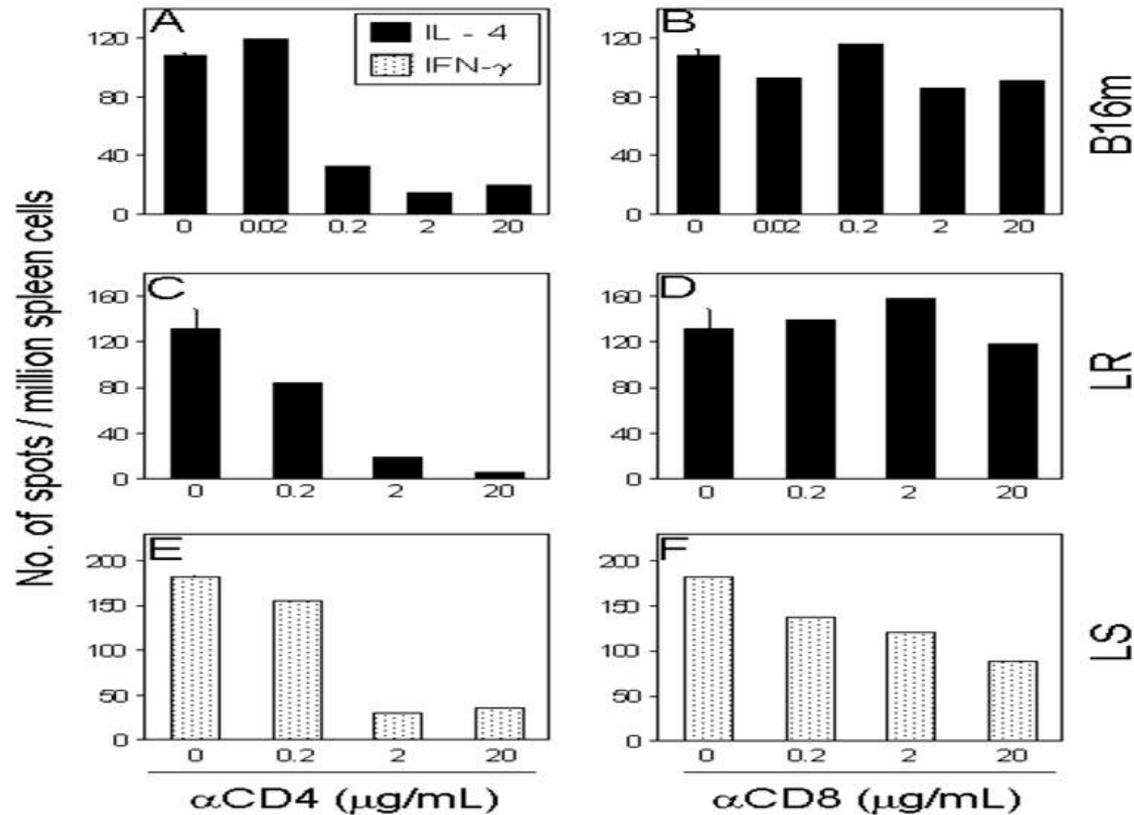


RMA cell-induced
IL-4, IL-6, IL-12 and
TNF- α/β
response in spleen cells
of RAG-1 KO
and naive C57BL/6
mice

Bartholomae W.C. et.al **J. Immunol.** 173: 1012-1022

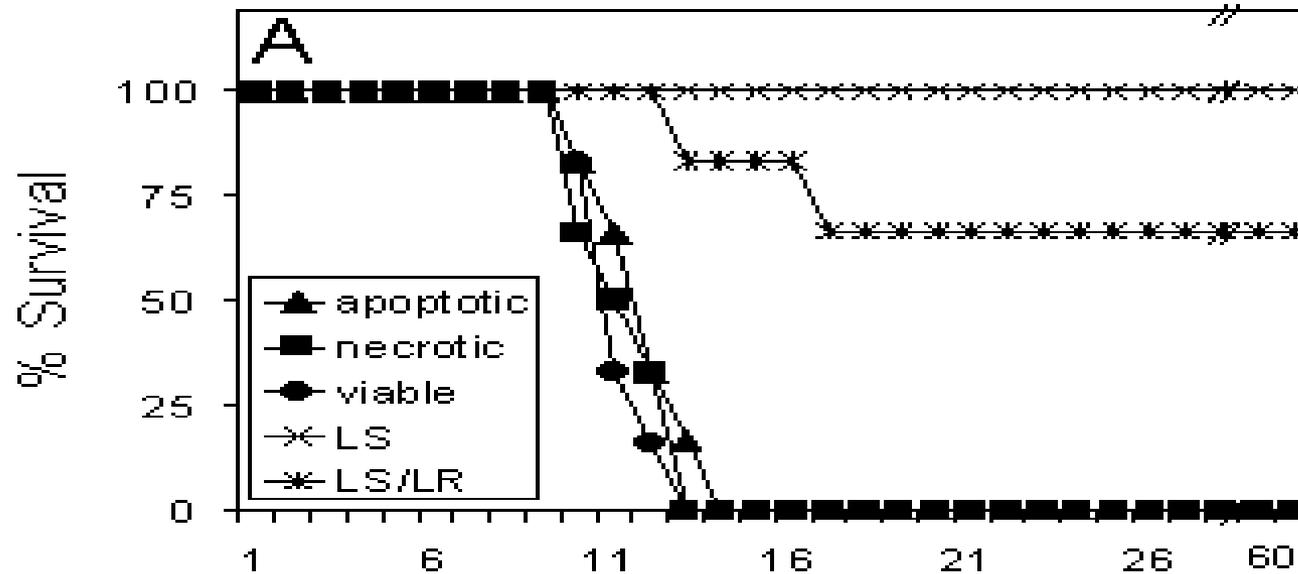
J. Immunol. 168: 6099-6105

Tumors induced T cell immunity

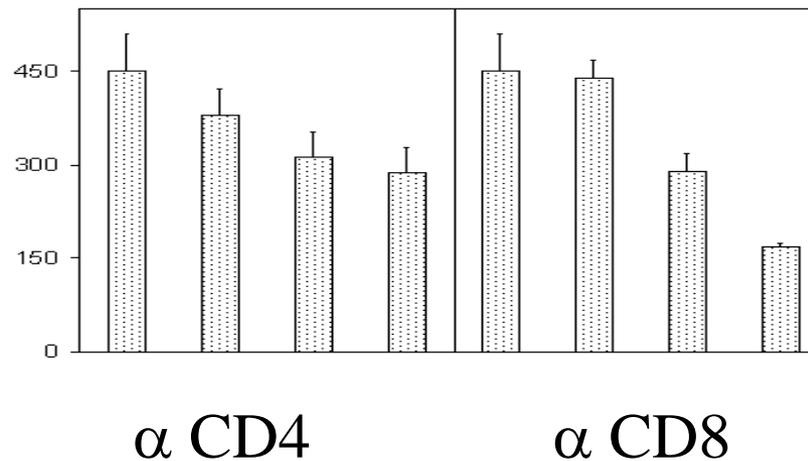
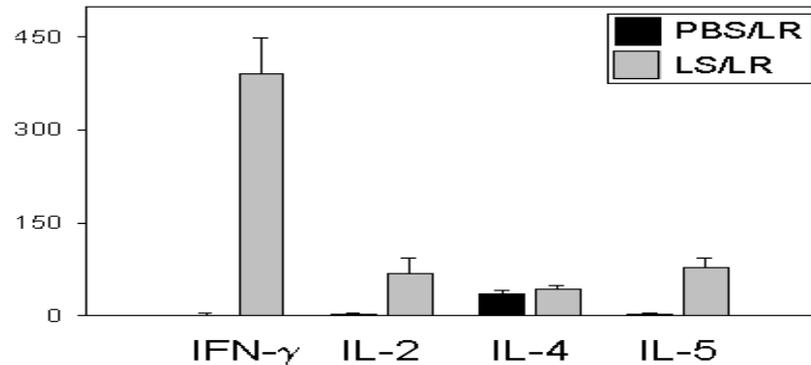


Bartholomae W.C. et.al **J. Immunol.**, 173: 1012-1022

Cellular treatment with non tumorigenic tumor cells leads to protection to tumor challenge



Immunity induced by vaccination with LS tumor is protective against LR tumor and induces a high frequency tumor specific type1 CD8 cell response

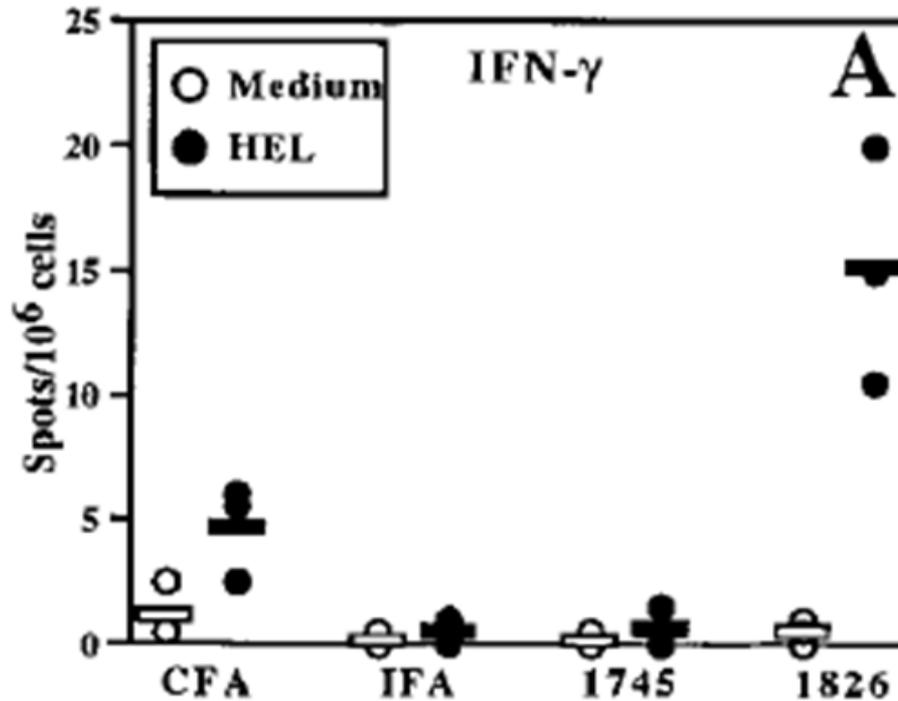


Summary I :

- Tumors are immunogenic.
- Tumors induce a low frequency CD4 T cell response.
- Apoptotic and necrotic tumor cells do not change the cytokine signature, frequency, or CD4 / CD8 class of the T cell response and do not induce protective immunity.
- Immunity induced by vaccination with (non-tumorigenic) LS tumor is protective against (the syngeneic, but tumorigenic) LR tumor and induces a high frequency tumor specific type 1 CD4/CD8 cell response.

**Can “infectious non self “ signals
help to trigger immune responses
against tumor?**

CpG – a type 1 adjuvant



•Chu RS. 1997. *J. Exp. Med.* 186:1623-31

Can vaccination with purified tumor antigens or tumor peptides induce effective anti-tumor immunity?

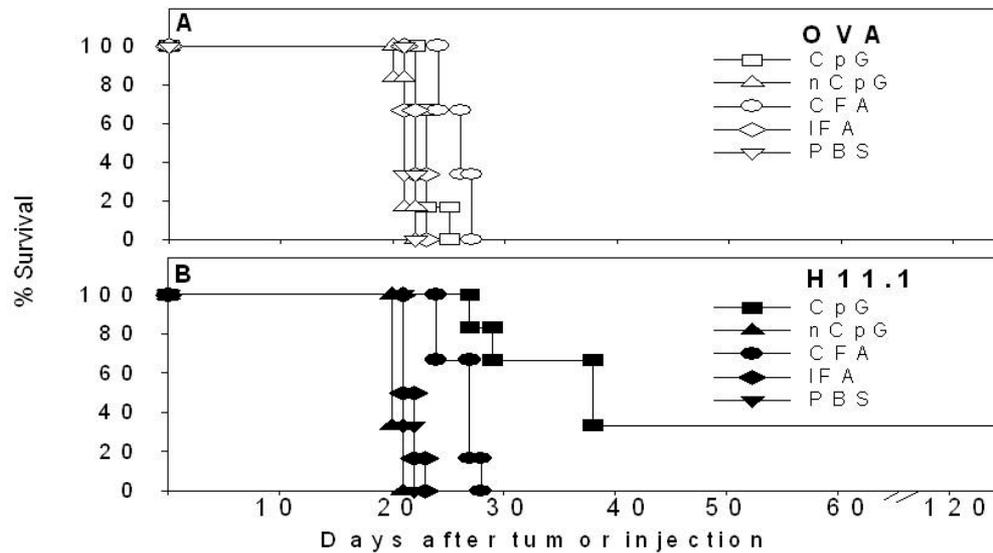
- peptide antigens are not immunogenic unless injected with adjuvant.
- different adjuvants can guide T cell differentiation.
 - Alum: type 2 response
 - IFA : type 2 response
 - CFA : type 1 response
 - CpG : type 1 of T cell response

Adjuvant-guided CD4 cell response to H11.1

	Assay	Recall								Immunization protocol							
		H11.1				H11.1				OVA							
		PBS	CpG	nCpG	CFA	IFA	CpG	nCpG	CFA	IFA	CpG	nCpG	CFA	IFA			
IFN- γ	Medium	0*		1	1	2	3	0	0	10							
	H11.1	1	<u>125</u> †	<u>24</u>	<u>65</u>	3	1	0	0	0							
	RMA	0	<u>60</u>	<u>15</u>	<u>26</u>	5	1	1	1	1							
IL-2	Medium	1	0	1	1	1	0	1	0	0							
	H11.1	0	<u>36</u>	<u>16</u>	<u>35</u>	<u>10</u>	0	0	1	1							
	RMA	0	<u>18</u>	<u>7</u>	<u>11</u>	<u>6</u>	2	0	2	1							
IL-4	Medium	2	1	2	1	3	0	0	0	1							
	H11.1	3	<u>12</u>	9	<u>16</u>	<u>29</u>	1	0	1	1							
	RMA	<u>11</u>	<u>24</u>	<u>19</u>	<u>12</u>	<u>34</u>	1	0	0	2							
IL-5	Medium	0	1	3	2	1	0	1	0	0							
	H11.1	0	<u>23</u>	7	<u>10</u>	<u>15</u>	0	0	1	0							
	RMA	0	<u>20</u>	10	8	<u>10</u>	0	1	1	2							
IL-6	Medium	85	223	44	294	70	221	39	218	75							
	H11.1	103	455	68	464	255	208	42	205	80							
	RMA	181	429	92	443	155	199	44	221	98							
TNF	Medium	60	33	209	83	366	45	180	61	203							
	H11.1	83	86	322	94	408	52	168	65	198							
	RMA	156	77	229	104	380	58	192	70	213							

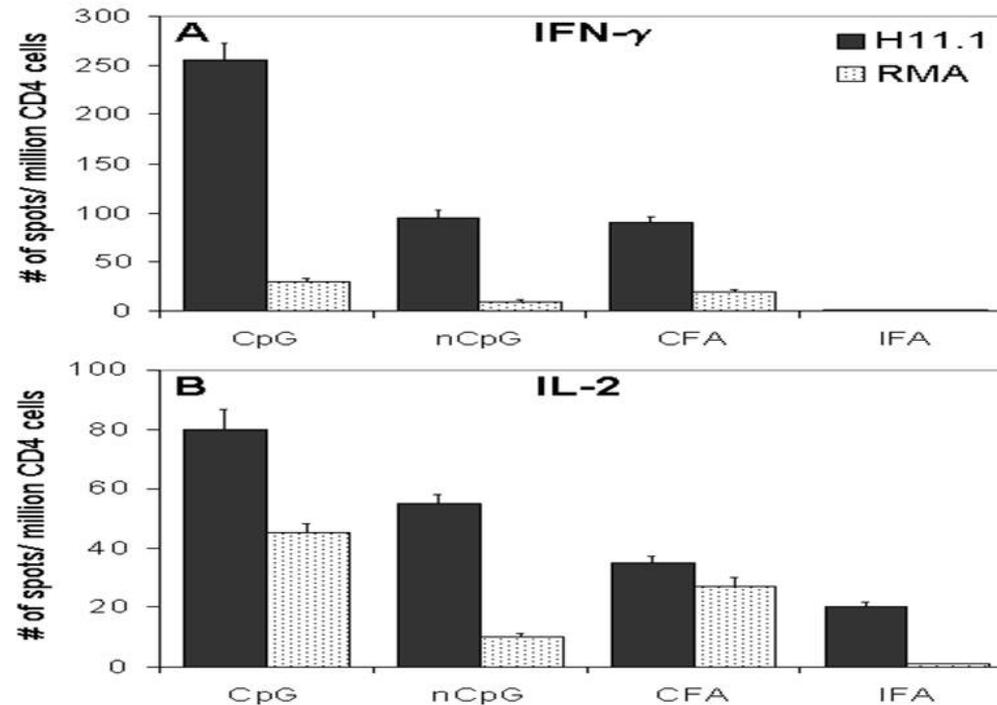
Stern B.V., Boehm B.B. and Tary-Lehmann M. **J. Immunol.** 168: 6099-6105

CpG: H11.1 injection induces specific protection against RMA tumor challenge



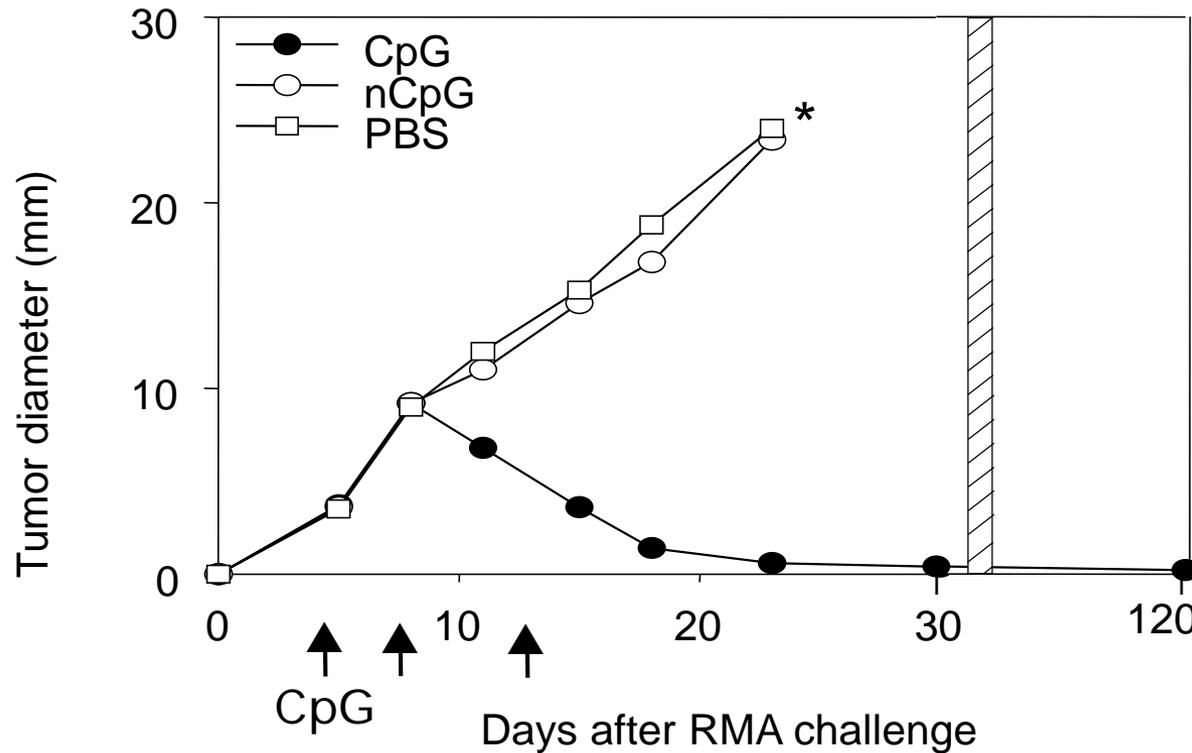
Stern B.V., Boehm B.B. and Tary-Lehmann M. *J. Immunol.*, 168: 6099-6105

CpG induces the highest frequency of H11.1-specific type 1 **CD4** cells



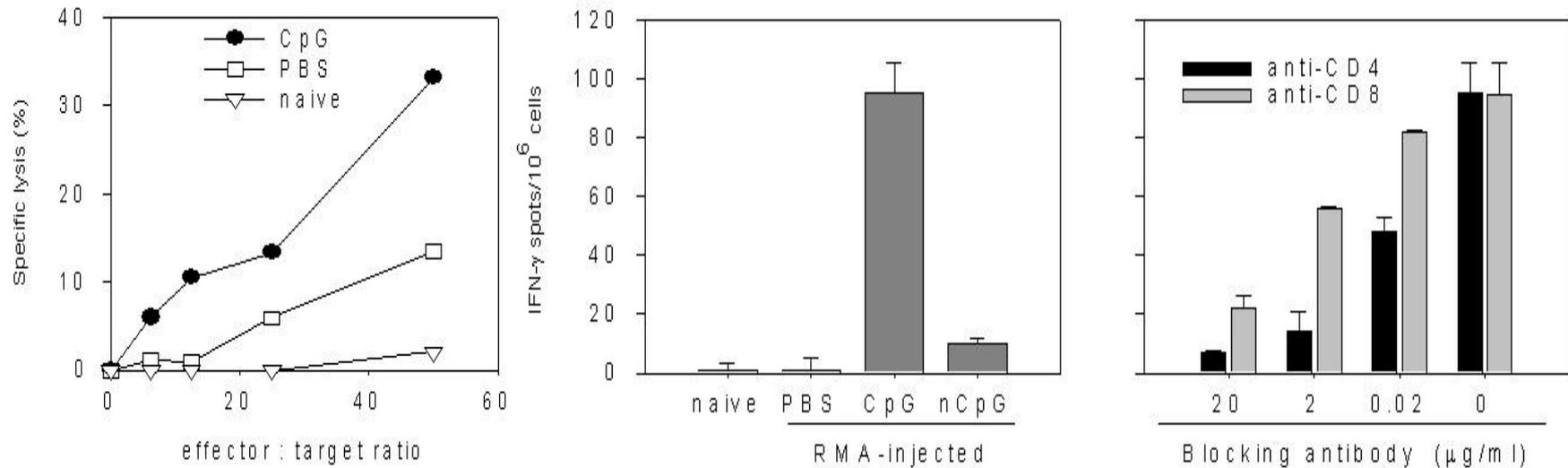
Stern B.V., Boehm B.B. and Tary-Lehmann M. **J. Immunol.**,168: 6099-6105

Intra-tumor injection of CpG ODN leads to tumor remission in immunocompetent B6 mice



Lonsdorf A.S., et.al. **J. Immunol.** 171: 3941-3946

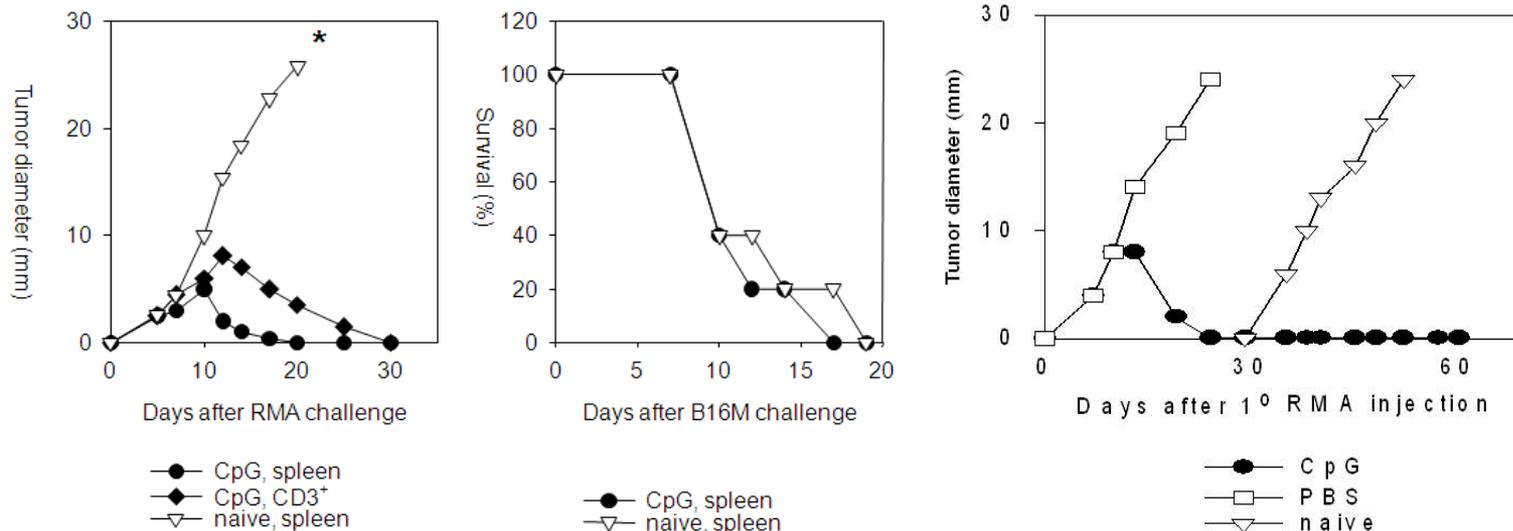
Intra-tumor CpG-injection induces tumor-specific CD4 and CD8 T cells in B6 mice



Lonsdorf A.S., et.al. **J. Immunol.** 171: 3941-3946

CPG as treatment

T cells adoptively transferred provide anti-tumor protection



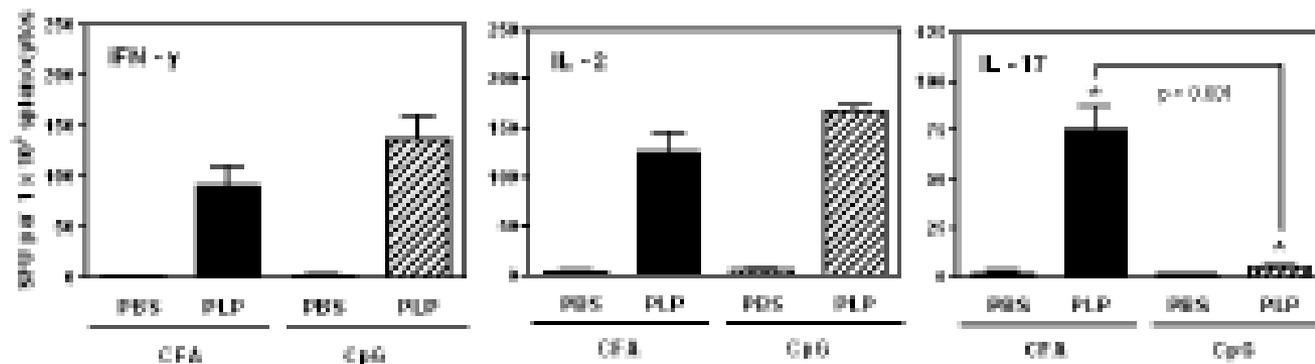
Lonsdorf A.S., et.al. **J. Immunol.** 171: 3941-3946

Summary II: (RMA-model)

- H11.1 peptide immunization in CFA and CpG both induced type 1 CD4 cell responses albeit of different magnitudes.
- Unlike the other adjuvants, H11.1: CpG induced protective immunity against RMA tumor.
- CpG-induced protection was IFN- γ -dependent
- Protection induced by the CD4 cells seems to be an indirect mechanism because the RMA tumor is class II negative.
- Intra-tumor CpG causes tumor rejection in immune competent, but not in T cell-deficient mice.
- Intra-tumor CpG induces anti-tumor T cell response (CD4 and CD8 cells that produce IFN- γ and are cytotoxic).
- These T cells adoptively transfer tumor specific protection.

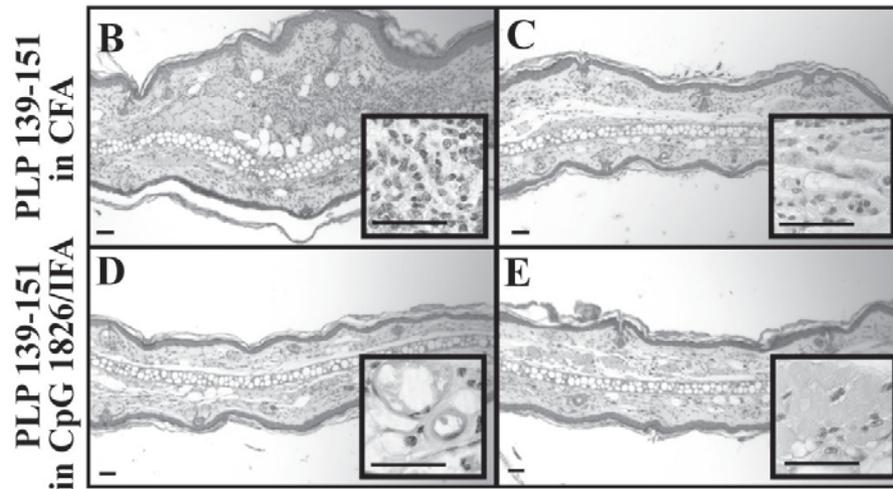
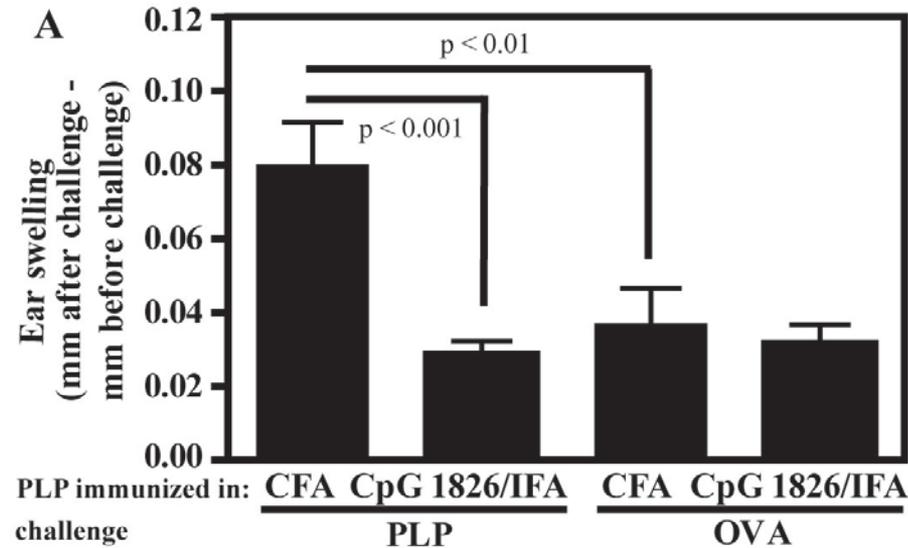
Detection of CD4 response modulation requires parallel monitoring of multiple cytokines (cont.)

- **Influence of adjuvants on CD4 T-cells (cont.):** adjuvant induces similar numbers of IFN- γ and IL-2 producing CD4 cells, but **IL-17** producing CD4 cells are induced by **CFA** only

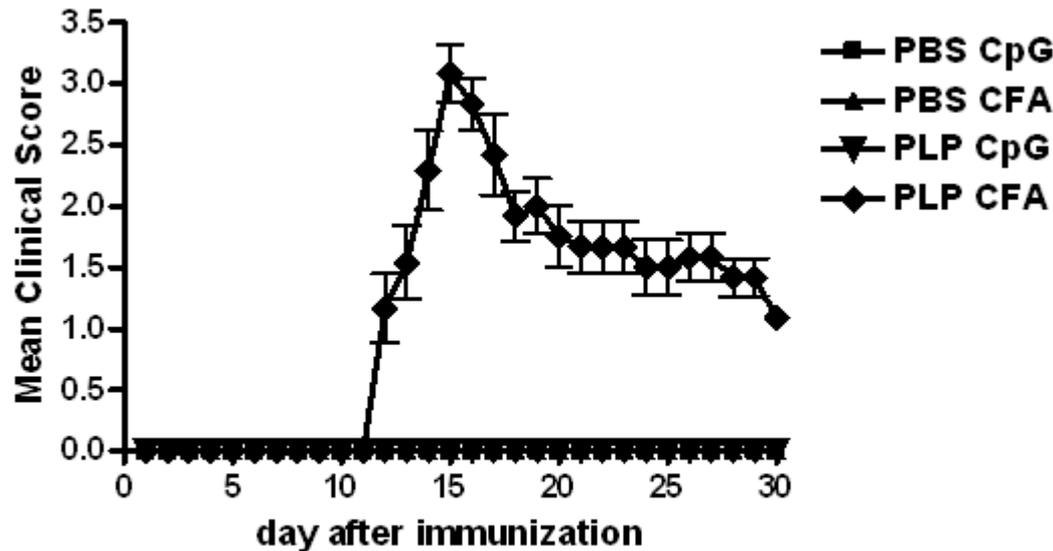


Tigno et al., J. Immunother, 32:389-398

Immunizations using CFA but not CpG 1826/IFA result in DTH reactions

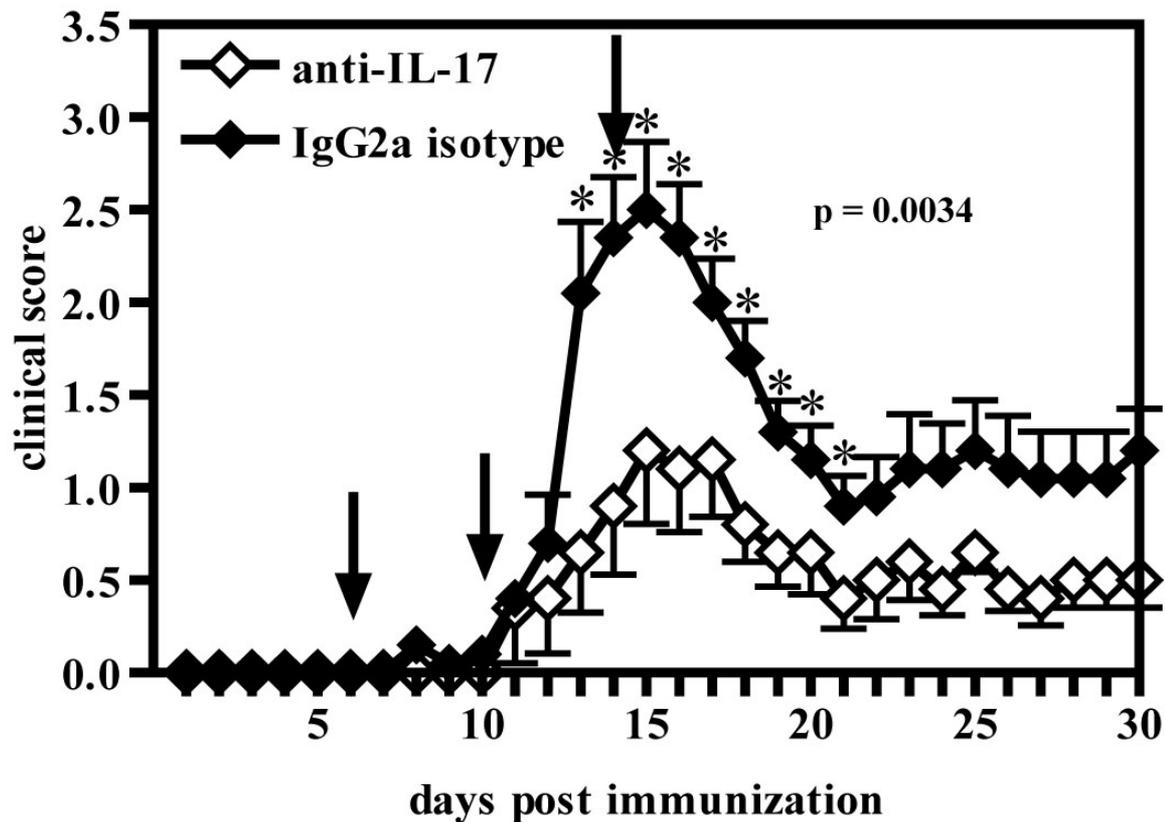


Immunizations with PLP:139-151 peptide in CFA induces EAE while immunization with CpG does not induce EAE



Tigno et al., J. Immunother, 32:389-398, 2010

IL-17 neutralization reduces severity of clinical disease induced by CFA immunizations



Tigno et al., J. Immunother, 32:389-398

Differential induction of IFN- γ vs. IL-17 producing by CD4 cells, and of EAE by CFA and CpG immunizations

- In mice, injection of class II-restricted PLP peptide in different type 1 adjuvants induces:

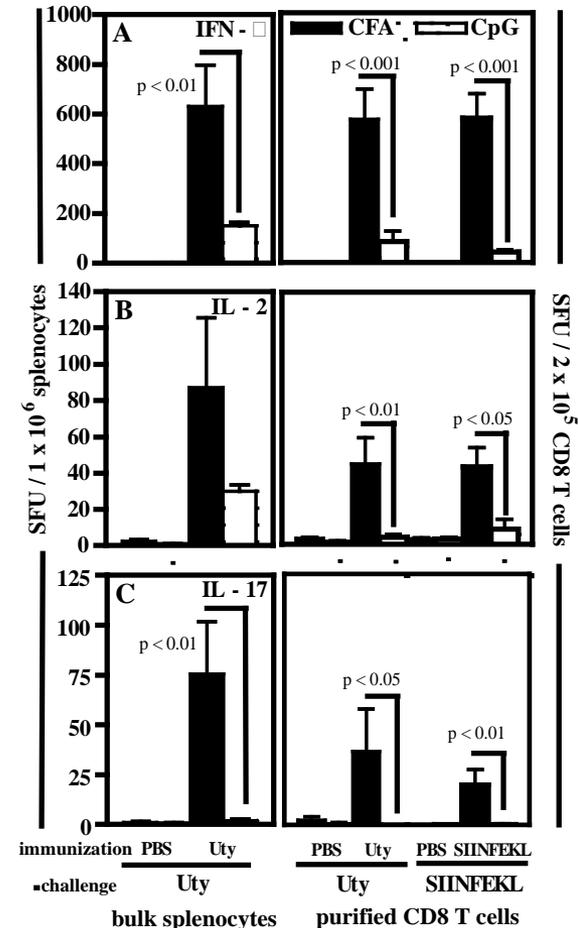
	<u>IFN-γ</u>	<u>IL-2</u>	<u>IL-4</u>	<u>IL-5</u>	<u>IL-17</u>	<u>EAE</u>	<u>DTH</u>
CFA	+++	+++	-	-	+++	+++	++
CpG	+++	+++	-	-	-	-	-

Tigno et al., J. Immunother, 32: 389-398

Detection of **CD8** response modulation requires parallel monitoring of multiple cytokines

- Use of Adjuvants with CD8 T-cells:

Immunization with "Uty" or SIINFEKL peptides plus **CFA** as **adjuvant** induce higher numbers of **IFN- γ** , **IL-2** and **IL-17** producing CD8 cells than immunizations with CpG

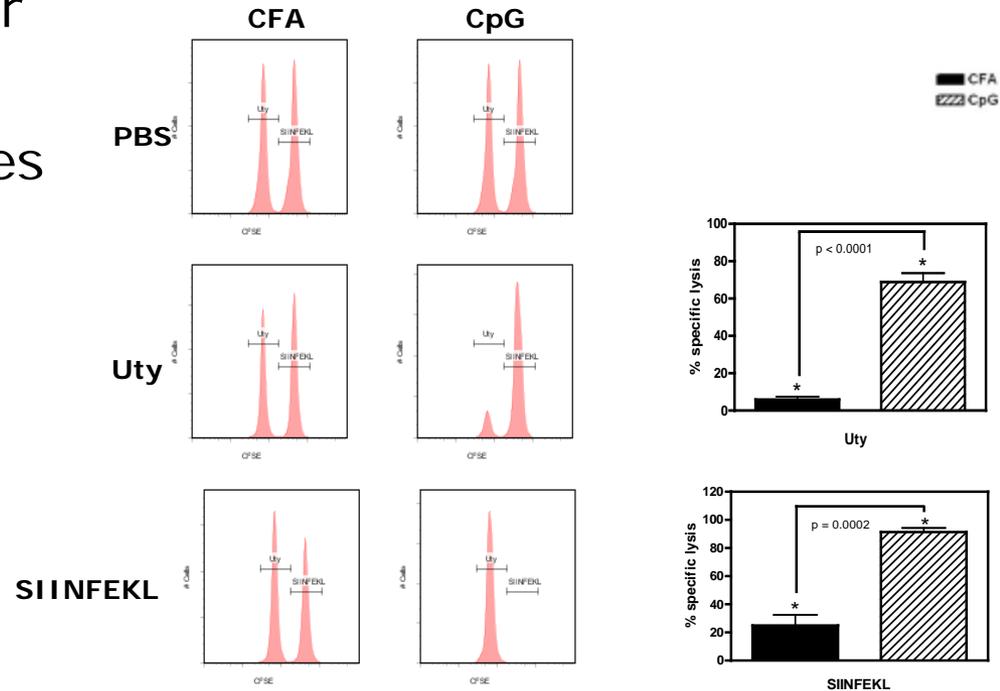


Tigno et al., J. Immunother, 32: 389-398

Use of adjuvants with CD8 T-cells (cont.):

In vivo cytotoxicity

Immunization with Uty or SIINFEKL peptides and **CpG as adjuvant** induces stronger *in vivo* killing than immunization with CFA



Tigno et al., J. Immunother, 32:389-398

Detection of CD8 response modulation requires parallel monitoring of multiple Cytokines (Summary)

- **Use of Adjuvants with CD8 cells (cont.):**

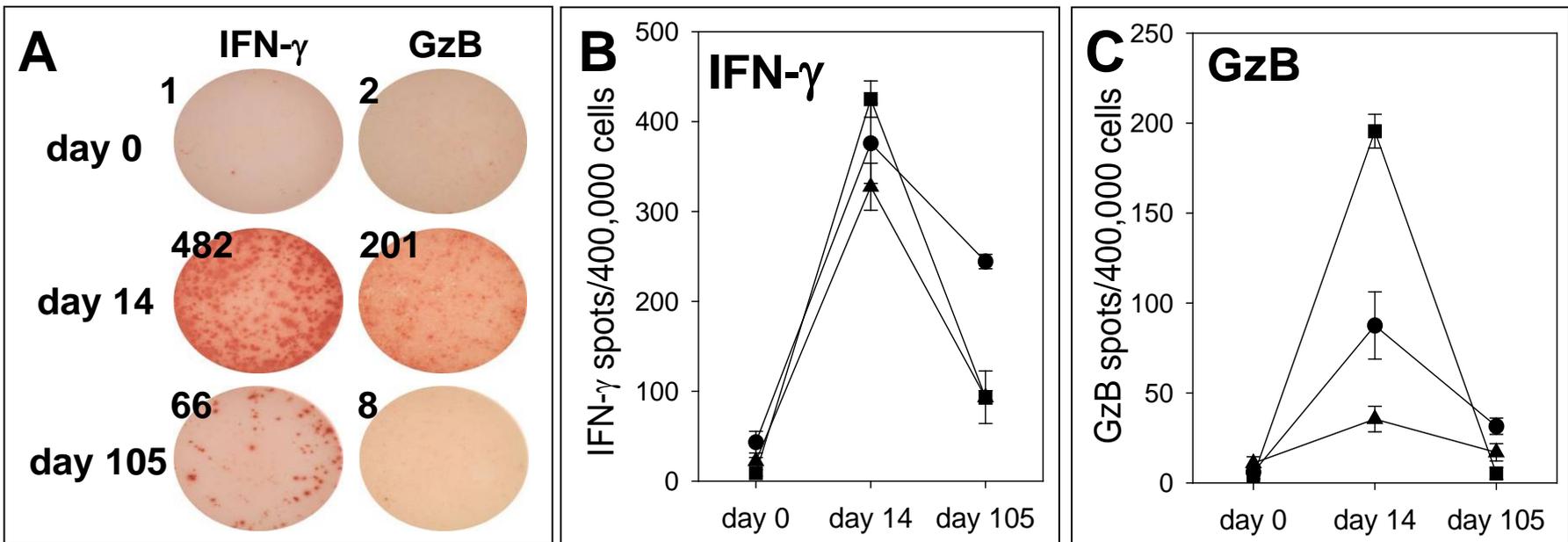
The choice of adjuvant during vaccination (i.e. **CFA versus CPG**) can switch peptide specific CD8 recall responses between mainly DTH mediating or mainly cytotoxic responses

	IFN-γ	IL-2	IL-17	Cytotoxicity	DTH
CFA	+++	+++	+++	+	+++
CPG	+	+	+	+++	+

Tigno et al., J. Immunother, 32: 389-398

ELISPOT applications:

Detection of vaccinia-specific CD8⁺ cells producing IFN- γ and GzB (cytolytic response) post vaccinia immunization



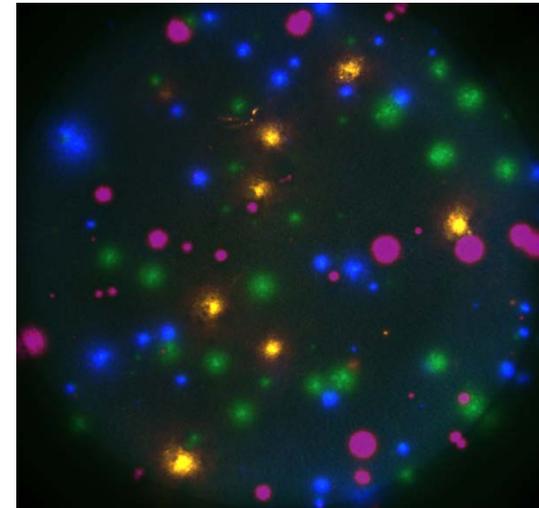
Nowacki T.M. et al.
Cell. Immunol. 2007 May; 247 (1):36-48.

Other ELISPOT applications:

B-cell monitoring: antigen-specific antibody production from PBMC

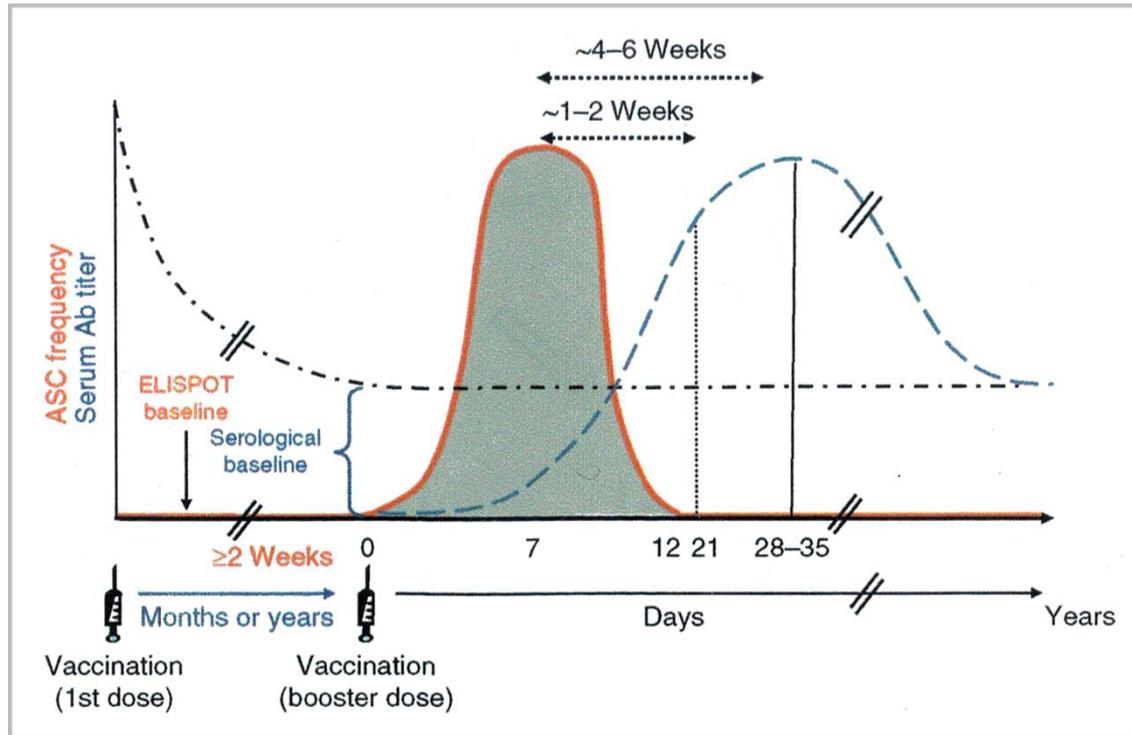


Example of antigen-specific secretion of IgG (red) and IgA (blue) from PBMC



Example of antigen-specific secretion of IgA, IgG2b, IgG3, IgG2a

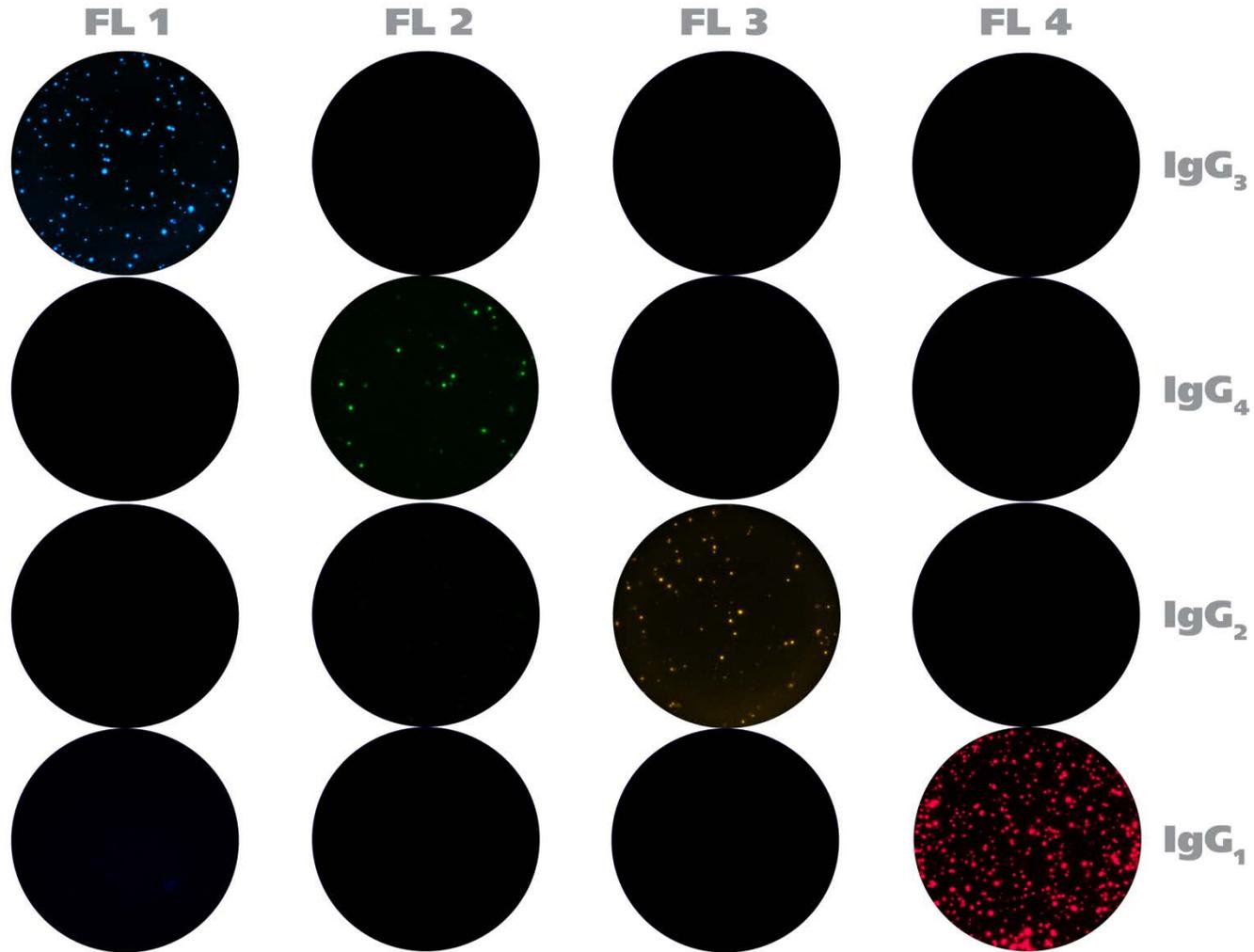
B Cell ELISPOT vs. Serum Antibodies #1



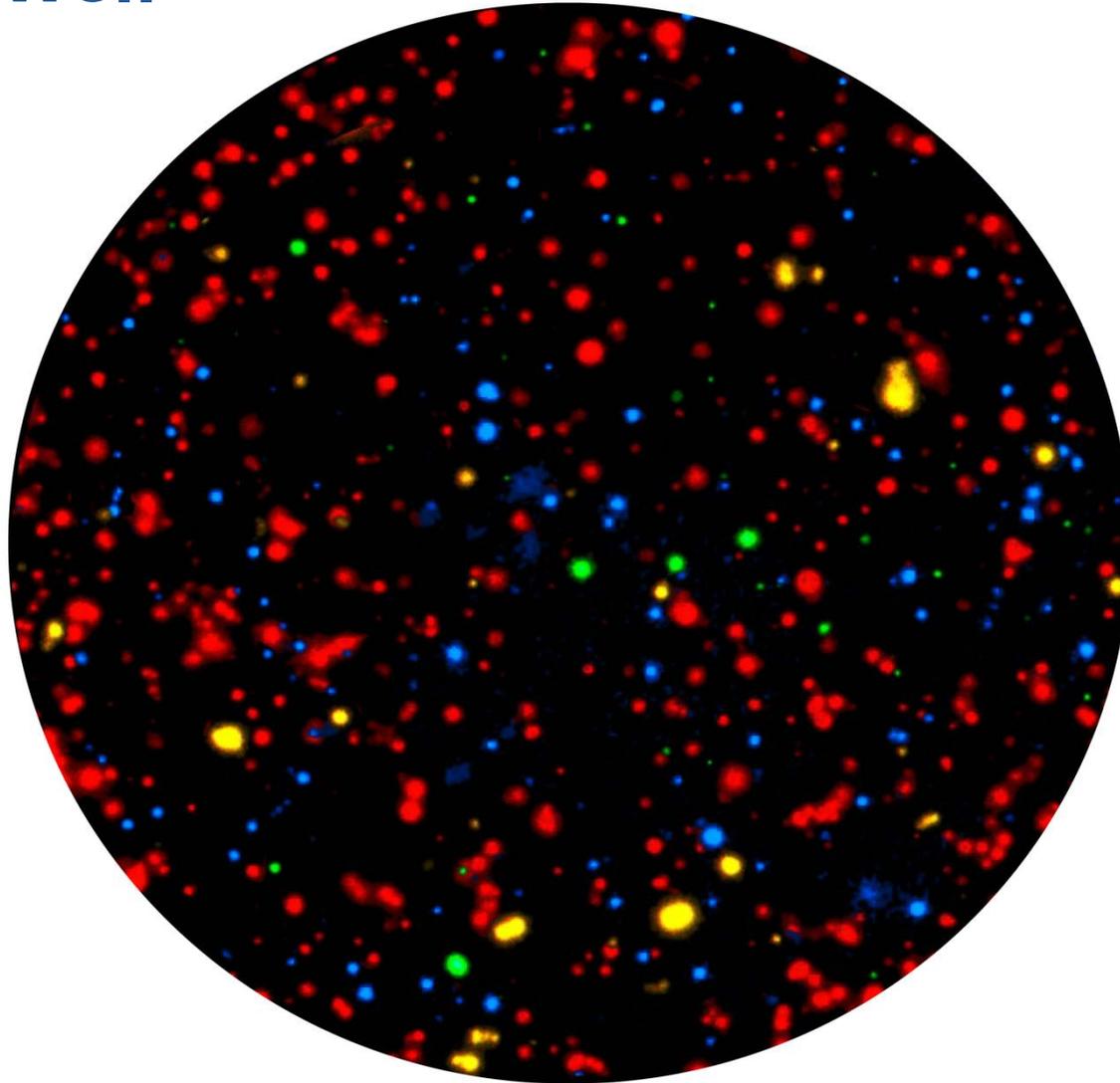
**Spontaneous
antibody
secreting cells
(ASC) identify
active B cell
response**

— Saletti, et al.: *Nature Protocols*; Vol. 8, No. 6; 2013; 1073-86

4-color B Cell ELISPOT



4-color Well



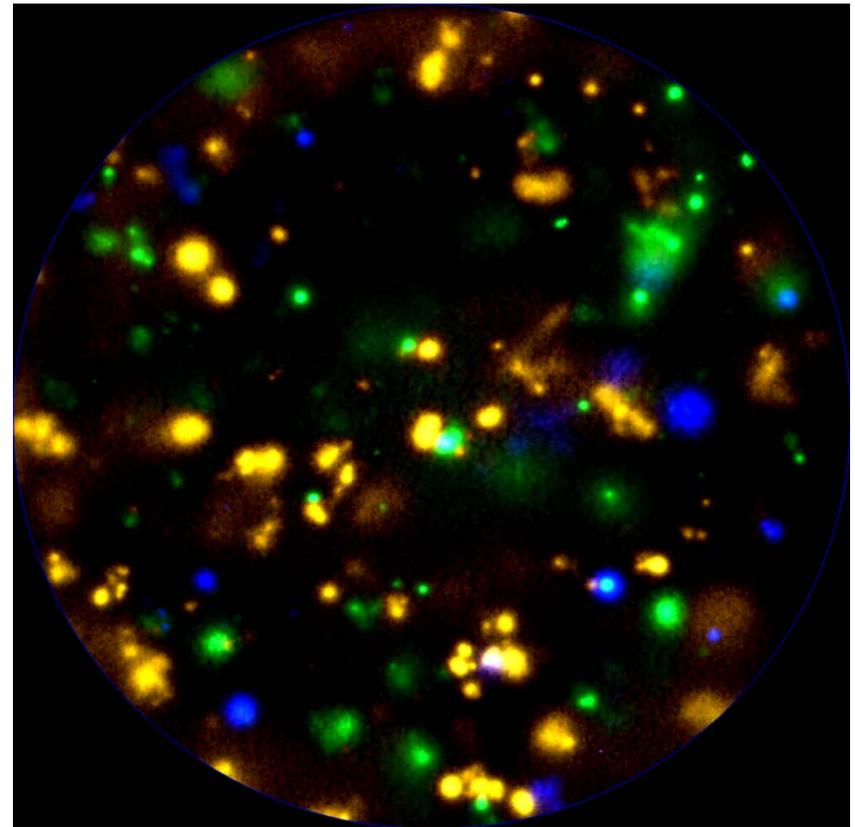
Dissecting the Ig classes: three-color detection of the HCMV-specific antibody classes produced by polyclonally stimulated B cells: all three classes were produced

yellow: IgG

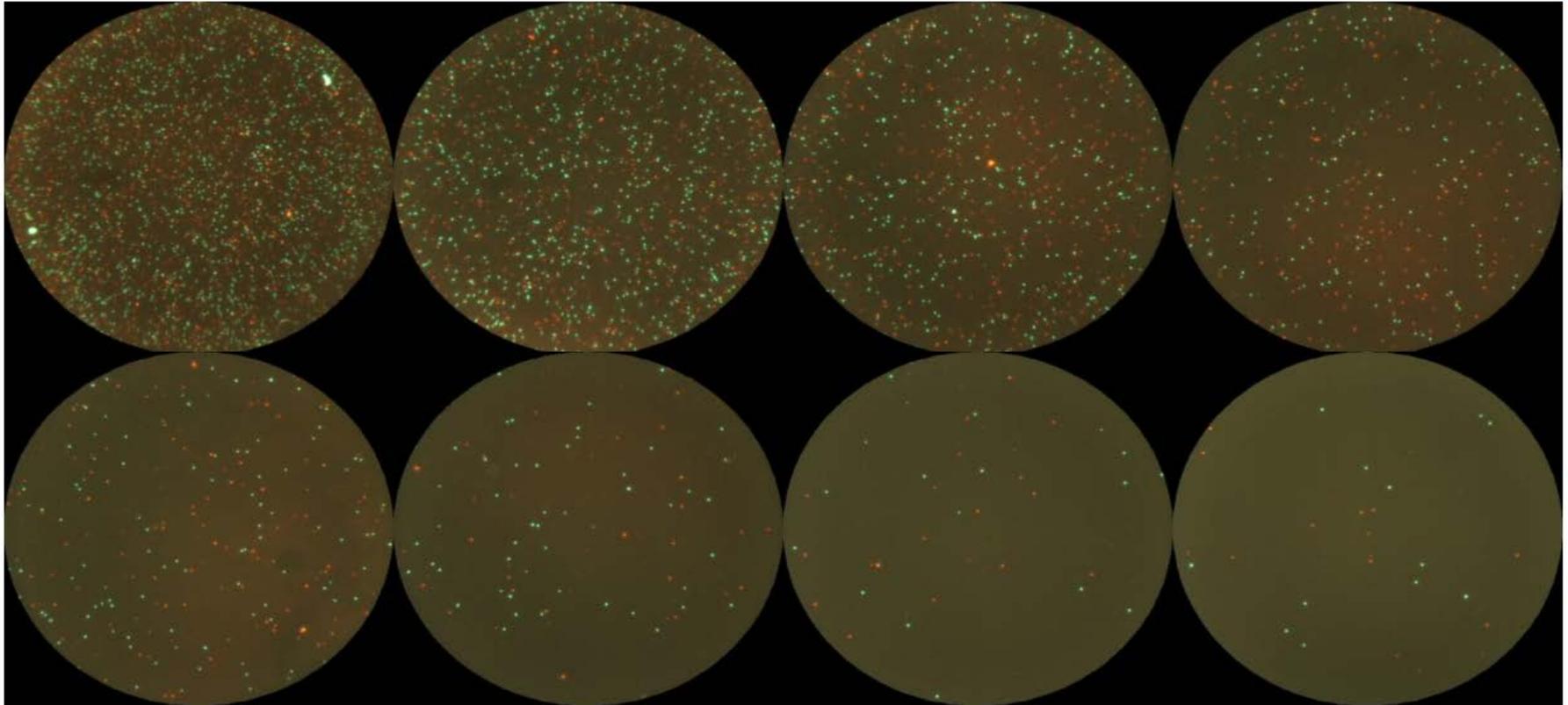
green: IgM

blue: IgA

PBMC were polyclonally stimulated for 4 days, then plated at 1 million cells per well in HCMV coated plate, followed by three color detection of Ig classes bound by HCMV.

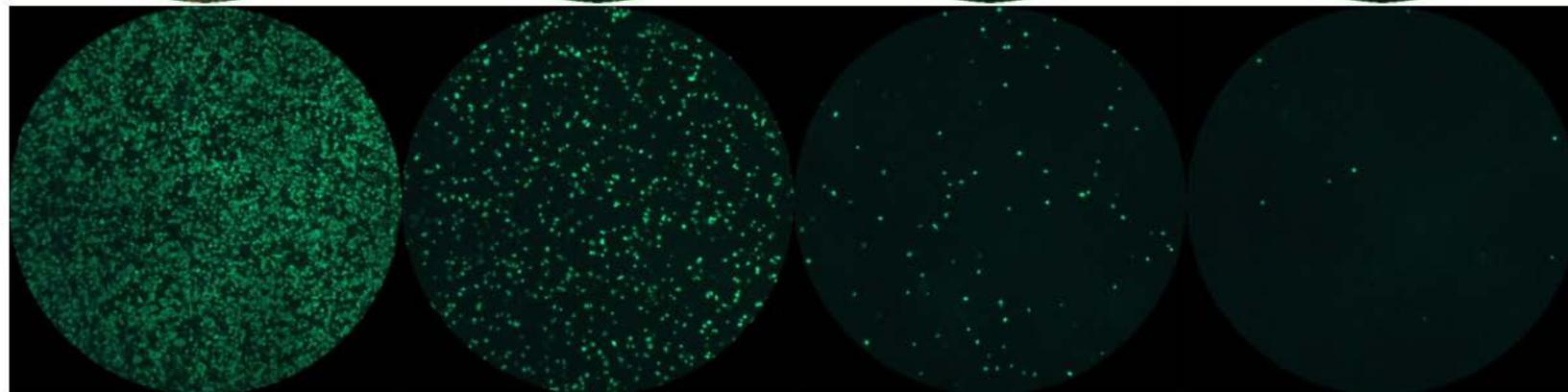
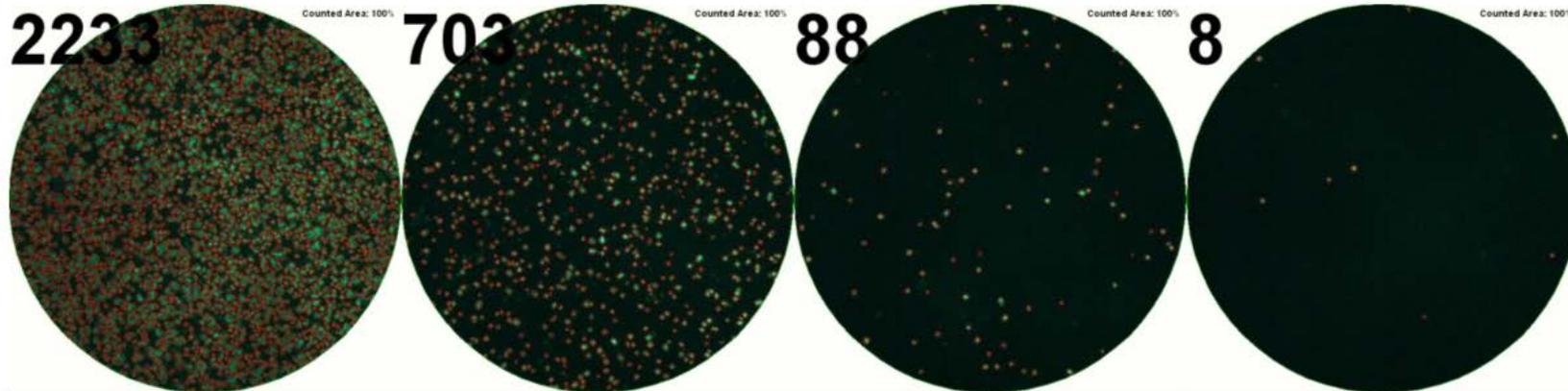


Other ELISPOT platform applications: Example of *in vitro* Killing Assay



Target CFSC-labeled cells (human PBMC) were mixed at different ratio with the effector cells (activated with peptide for 5 days) plus peptide.

Other ELISPOT platform applications: FluoroSpot™ virus Neutralization Assay



1:1500

1:500

1:100

1:20

Neutralizing serum dilution

Standardization strategies for CMI monitoring and ELISPOT

- Harmonize sample collection at clinical sites – Training and qualification of all clinical sites in proper sample handling, PBMC processing, cryopreservation and shipping standard operating procedures (SOPs)
- Harmonize methods across all participating laboratories
- Implementation of detailed SOPs for cell thawing, cell counting, assay procedures and the analysis of assay results
- Standardize all assay materials, including plates (CTL-kit), antibodies (CTL-kit), media (CTL) and enzymes
- Utilize cell-based reference sample PBMC (ePBMC) to optimize assays and compare performance between laboratories

Inter-Lab standardization of ELISPOT: NEUCAPS Effort

- **S. McArdle, R. Rees (UK, Nottingham)**
- **N. Haicheur, E. Tartour (France, Paris)**
- **A. Abdelsalam, A. DeLeo (USA, Pittsburgh)**
- **K. Johannesen, E.M. Inderberg-Suso, G. Gaudernack (Norway)**
- **Virginie Vignard, N. Labarriere, F. Jotereau (France, Nantes)**
- **P. Dudzik, K. Garula, Piotr Laidler (Poland)**
- **K. Georgakopoulou, S. Perez, C. Baxevanis (Greece)**
- **A. Mihaylova, E. Naumova (Bulgaria)**
- **K. Silina, A. Line (Latvia)**
- **N. Aptsiauri, F. Garrido (Spain)**
- **V. Adams (ONYVAX, UK)**
- **CTL reference laboratory (USA)**

Zhang W, et.al. J Immunotoxicol. Dec;6(4):227-34.

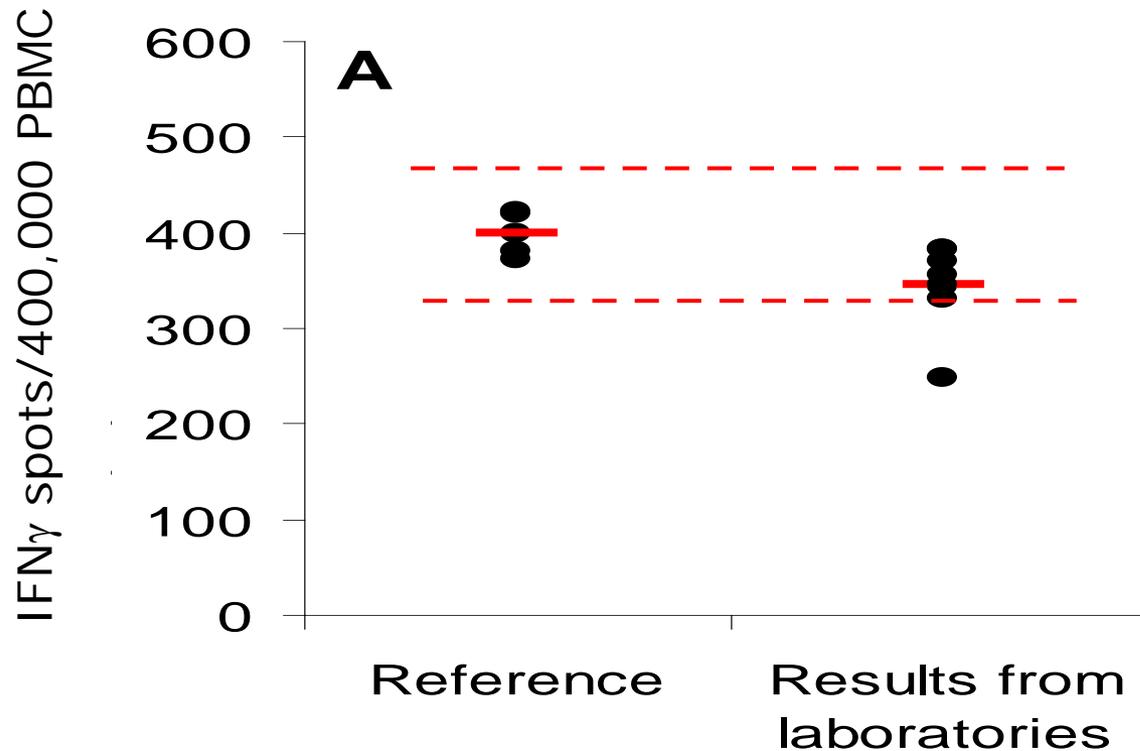
All laboratories were provided with THE SAME:

- Cryopreserved PBMC of three donors (provided by CTL)
- Serum free thawing, washing and testing reagents (by CTL)
- In addition, each lab also used its “favorite tested serum”
- HLA Class I restricted peptide (CMV pp65 NLVPMVATV – frozen and ready to use concentration – provided by CTL)
- Human IFN- γ ELISPOT Kit (provided by BD)
- Protocol (provided by CTL)

All individuals performing the assay were ELISPOT inexperienced (except CTL reference laboratory)

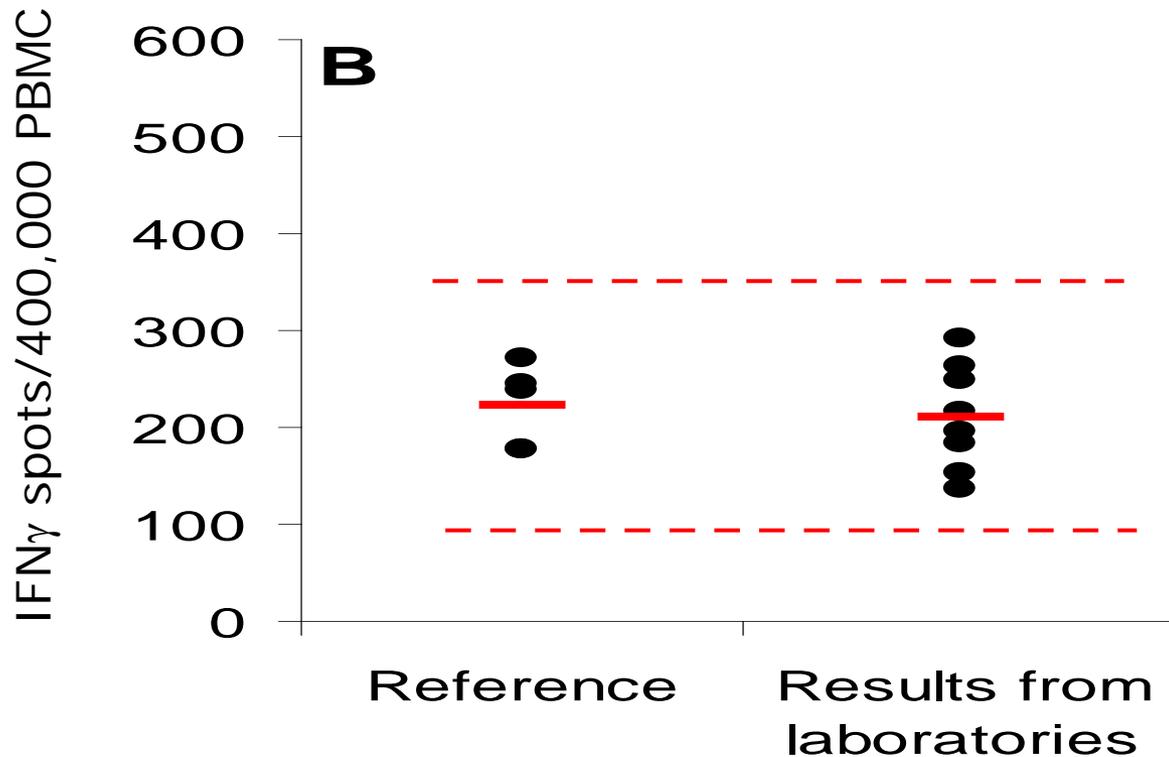
Zhang W, et.al. J Immunotoxicol. 2009 Dec;6(4):227-34.

Inter-laboratory reproducibility of data for Reference Sample 1.



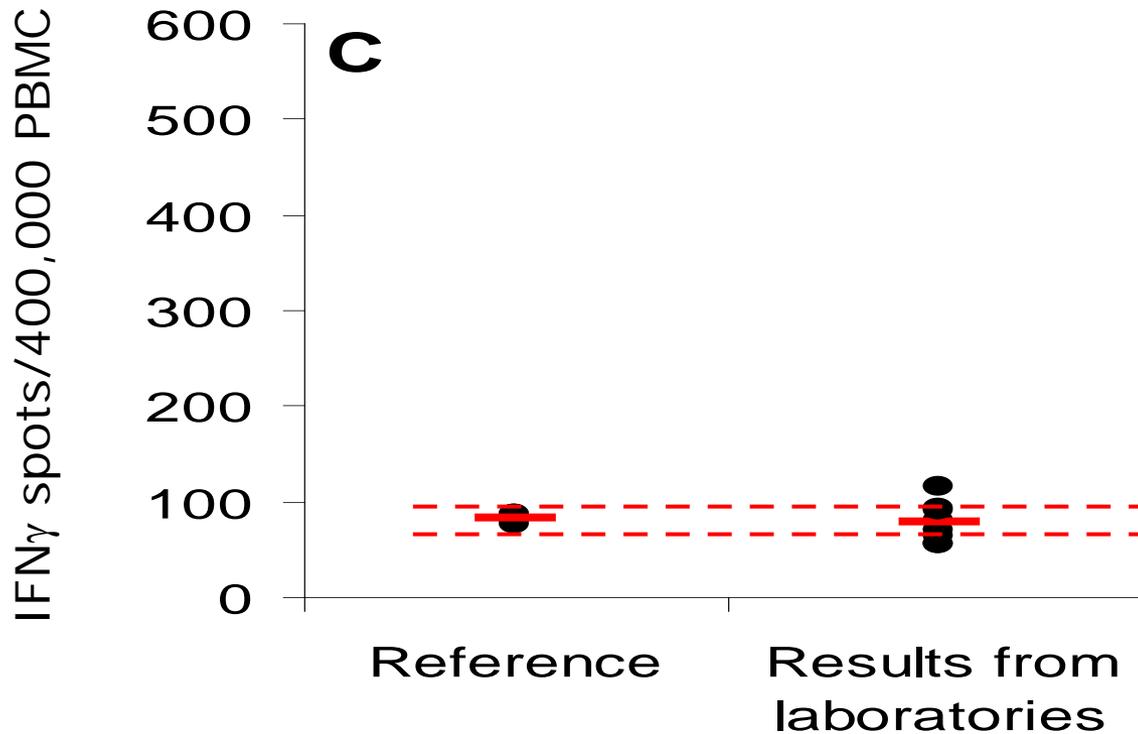
- 100% of labs detected the response
- Mean of the 9 labs results is within 3 standard deviation of the reference value

Inter-laboratory reproducibility of data for Reference Sample 2.



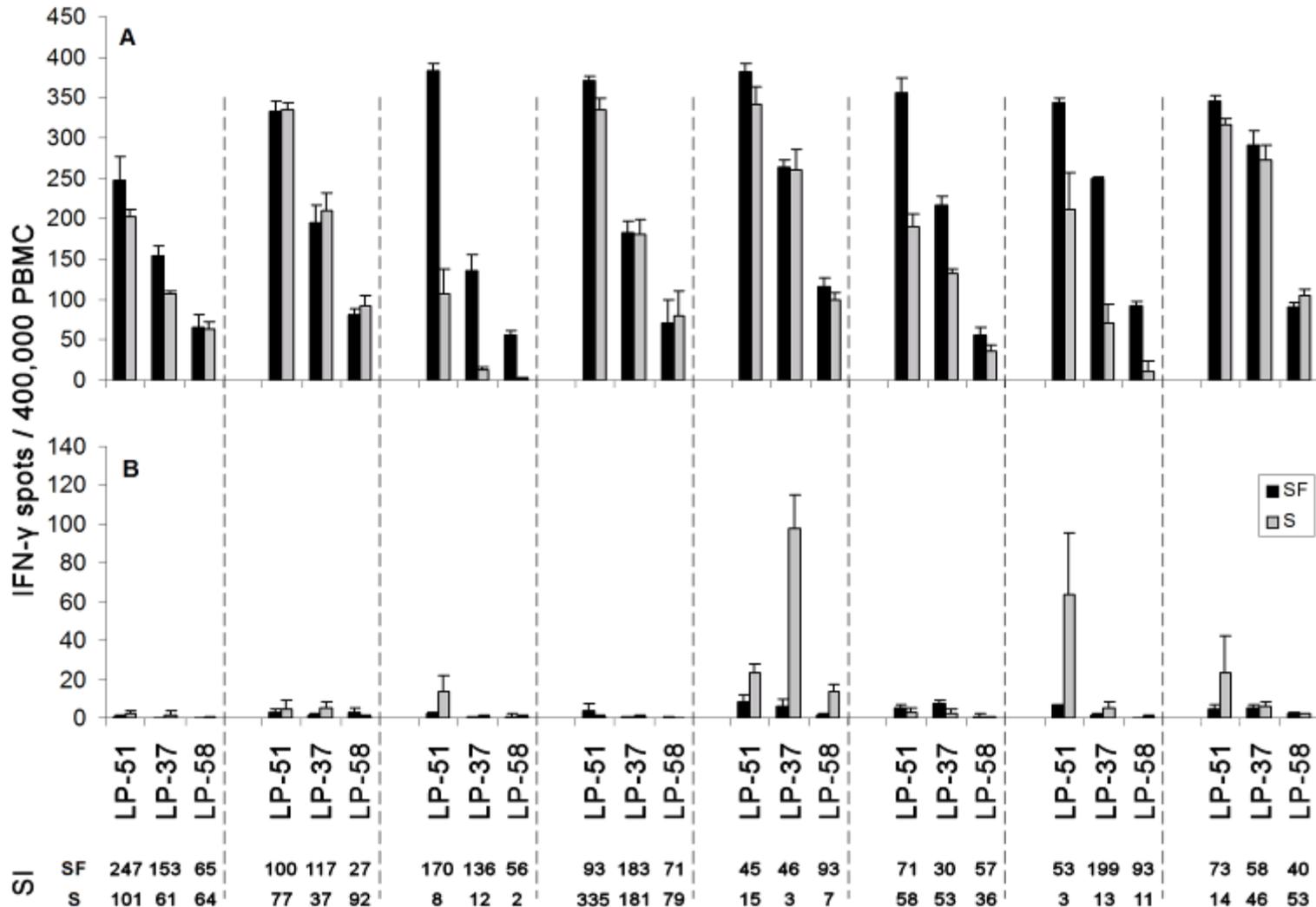
- 100% of labs detected the response
- Mean of the 9 labs results are within 3 standard deviation of the reference value

Inter-laboratory reproducibility of data for Reference Sample 3



- 100% of labs detected the response
- Mean of the 9 labs results is within 3 standard deviation of the reference value

Assay performance in CTL media (serum free) versus serum containing media.



Conclusion

- Immunological assays are pivotal for the clinical development and monitoring phase.
- Immunology assays are scientifically sound and precise.
- ELISPOT is an ideal assay system for immune monitoring
 - Works with frozen cells identical as with fresh samples
 - Uses physiological stimuli (e.g. antigens, peptides, proteins)
 - Very sensitive
 - Robust
 - High throughput capable (CTL can test up to 450 samples per week)
 - Can be standardized
 - Reproducible
- Appropriate readout system are essential to evaluate your drug/biologic or vaccine.
- Using inappropriate readout systems to assess the potential advocacy and effectiveness of drugs, biologics, vaccines etc. may render them undetected and for this reason, may have been rejected.

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