Highly immunogenic C-terminal binding domain of *Clostridium difficile* toxin a stimulates dendritic cell maturation

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黃瑞鑫, 吳家瑋, 連淑培, 蕭光男, 冷治湘, 劉士任 & 莊再成

Vaccine R&D Center, NHRI

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Missions of Vaccine Center

1. To establish the infra-structure and facility for conducting vaccine research and development to meet regional needs,

2. To build and implement cGMP facilities in compliance with FDA regulatory and quality guidelines for manufacturing vaccines, vaccine candidates and anti-venom for regional use,

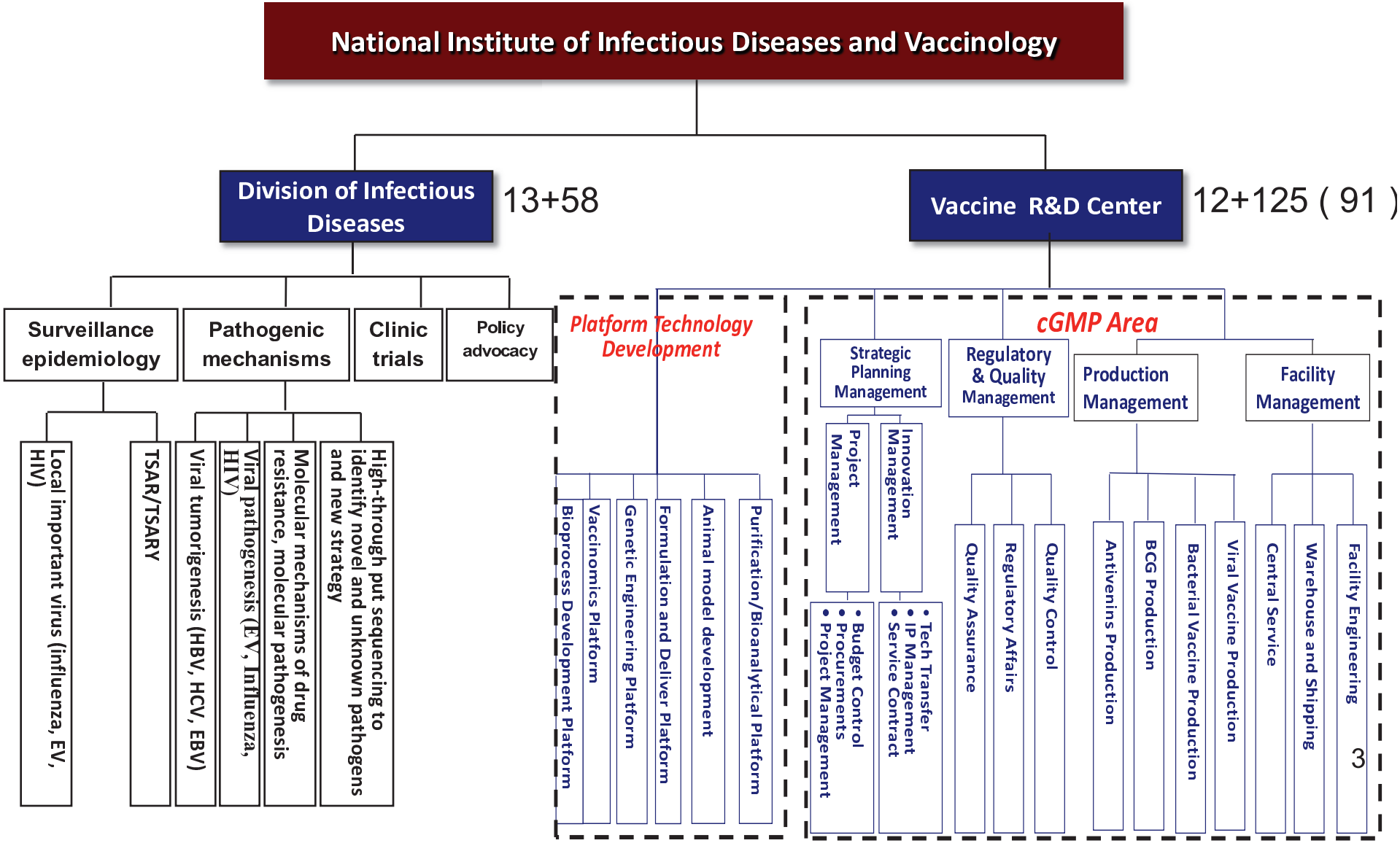
3. To develop the ability to respond to Taiwan government emergency requests for vaccines against pandemic diseases and bioterrorism, and

4. To serve as the forum for training and educating young scientists in vaccine-related biotechnology.

1. Taiwan Vaccine Self-Manufacturing program (2004-2007)
2. Human Vaccine R&D Priority Programs (2007-2011)

NIIDV Organization & Structure

Effective, May, 2011



Roles of Vaccine Center

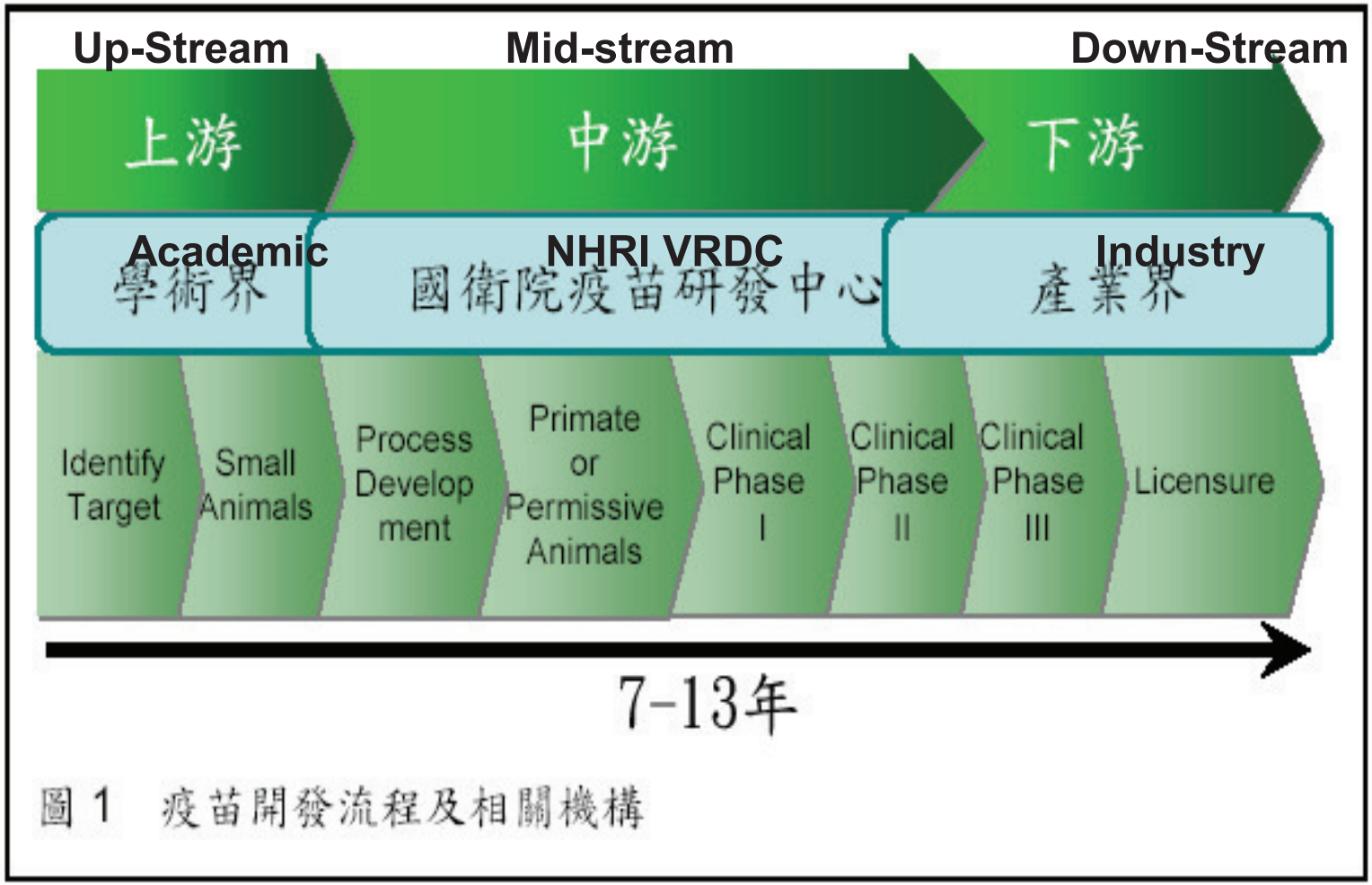
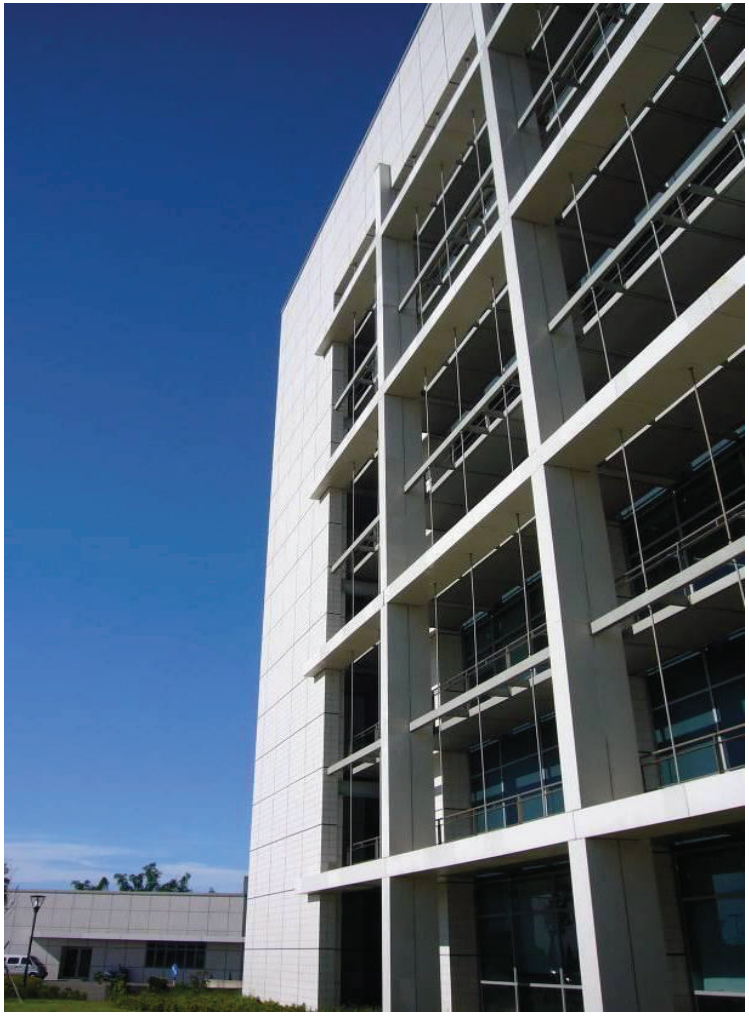


圖 1　疫苗開發流程及相關機構

Vaccine Research and Development Center

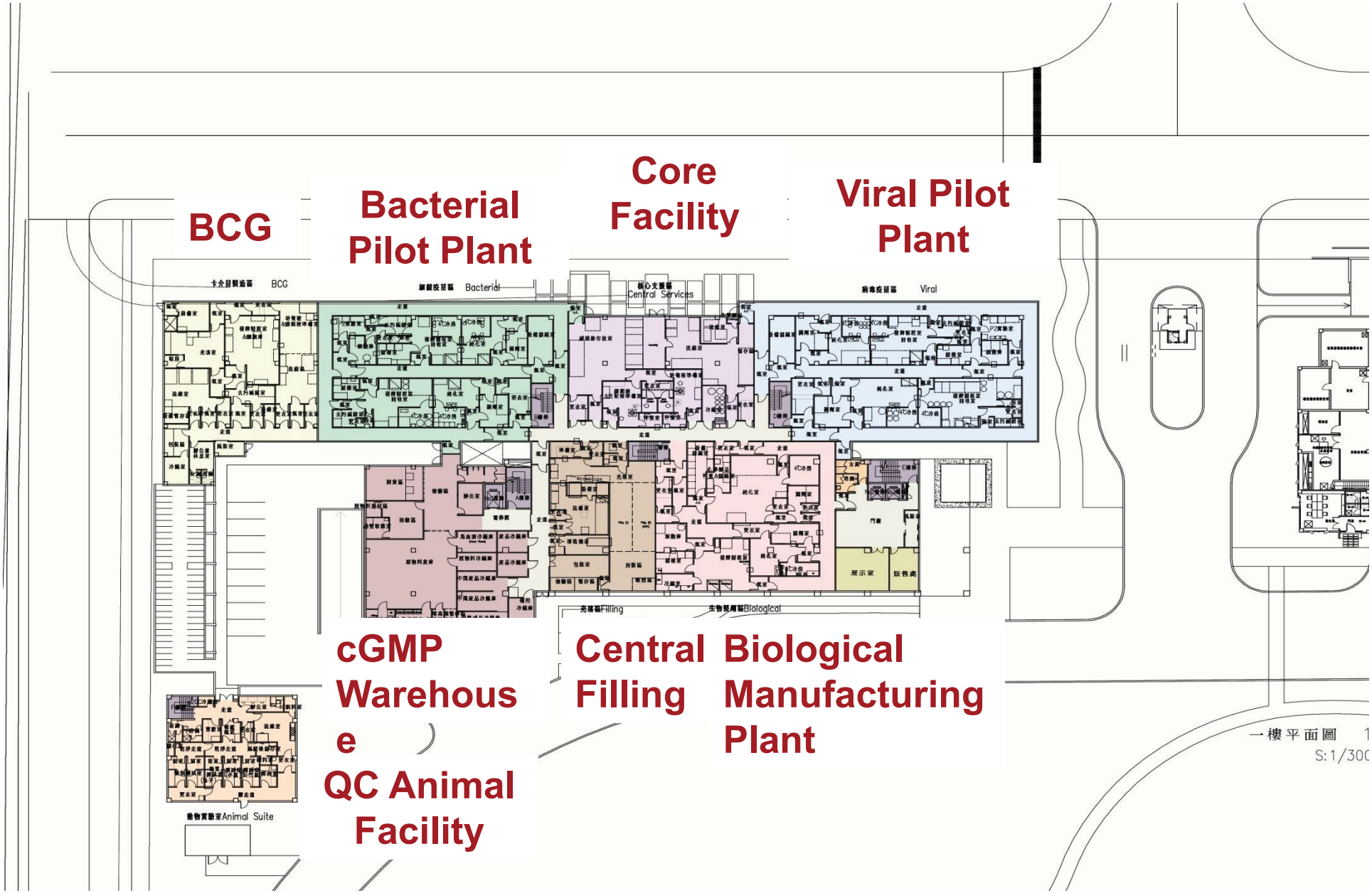


Vaccine Center & cGMP facility



NHRI R1-7F (R&D)

Vaccine Center Floor Plan (1st floor)



Clostridium difficile



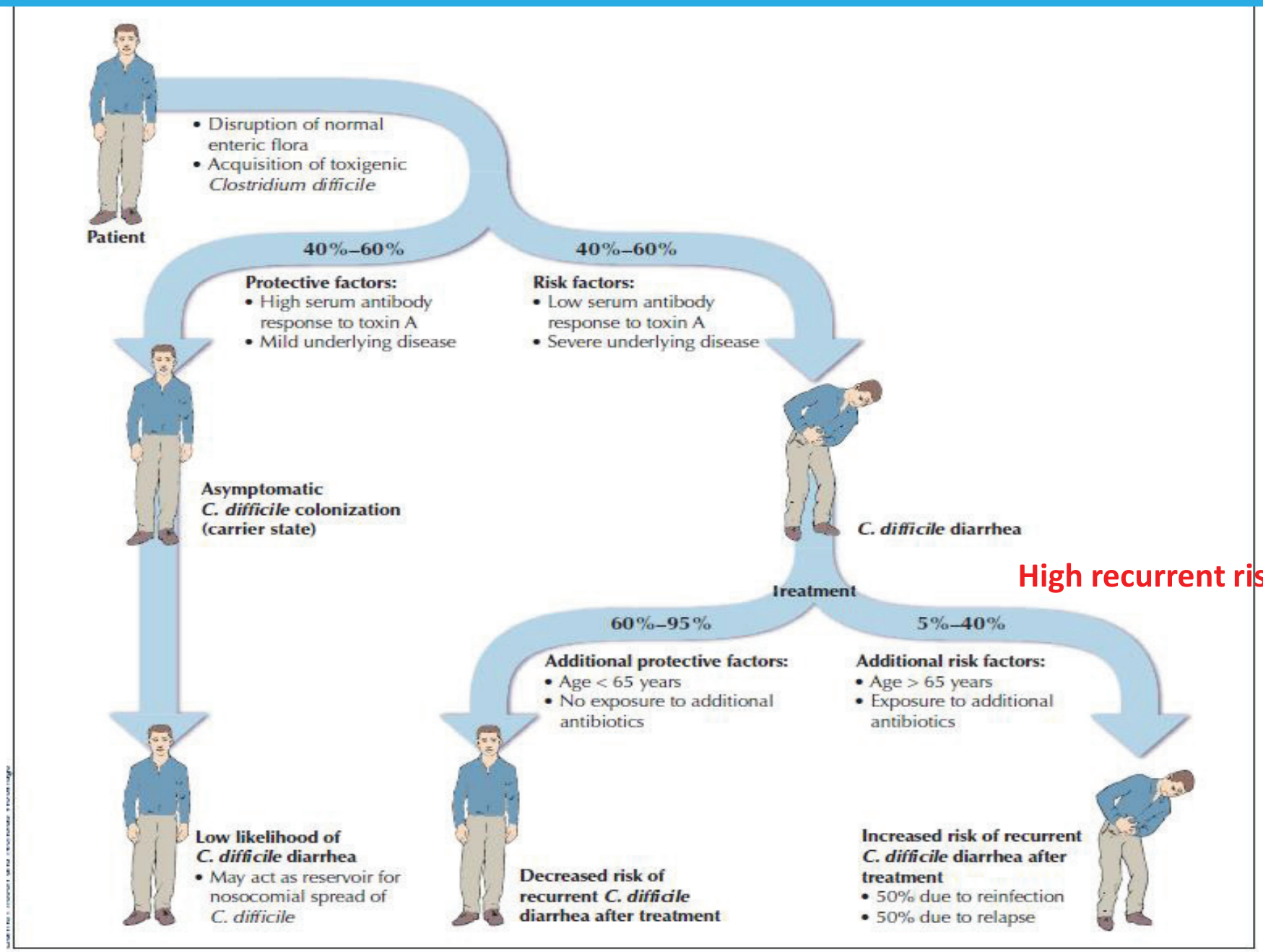
- A gram-positive bacteria
- A anaerobic, spore-forming rods (bacilli)
- Present as one of the 'normal' bacteria in the gut of healthy adults. It is much more common in babies(up to 70%).
- Eradication of the normal gut flora by antibiotics results with *C. difficile* colonization.
- *C. difficile*-associated diarrhea(CDAD)
  Antibiotics-associated diarrhea are caused by Toxin A and/or Toxin B

Scientific classification	
Kingdom:	Bacteria
Phylum:	Firmicutes
Class:	Clostridia
Order:	Clostridiales
Family:	Clostridiaceae
Genus:	*Clostridium*
Species:	***C. difficile***

Binomial name

Clostridium difficile
Hall & O'Toole, 1935

Opportunistic infection in hospital

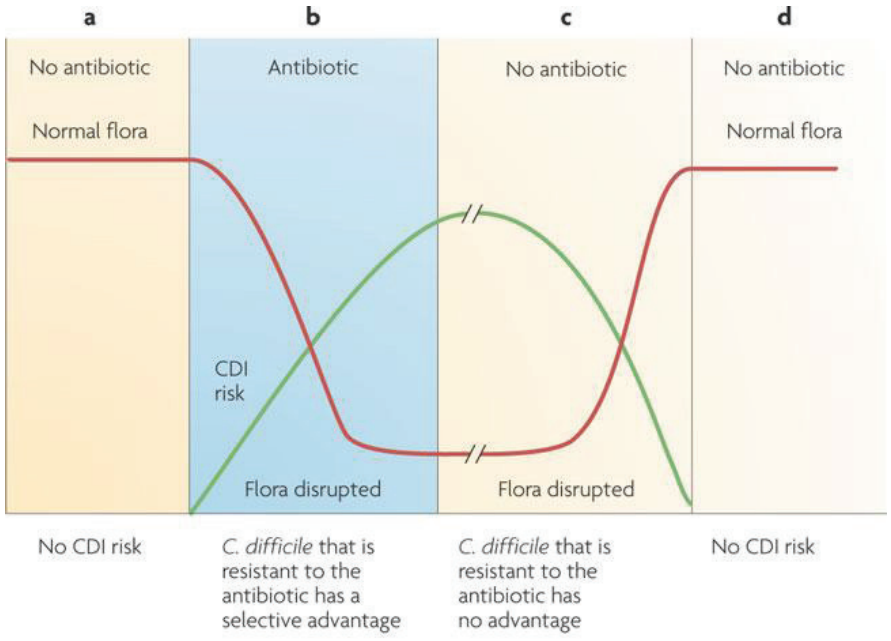


High recurrent risk

Double-edged sword of antibiotics in *C. difficile* treatment

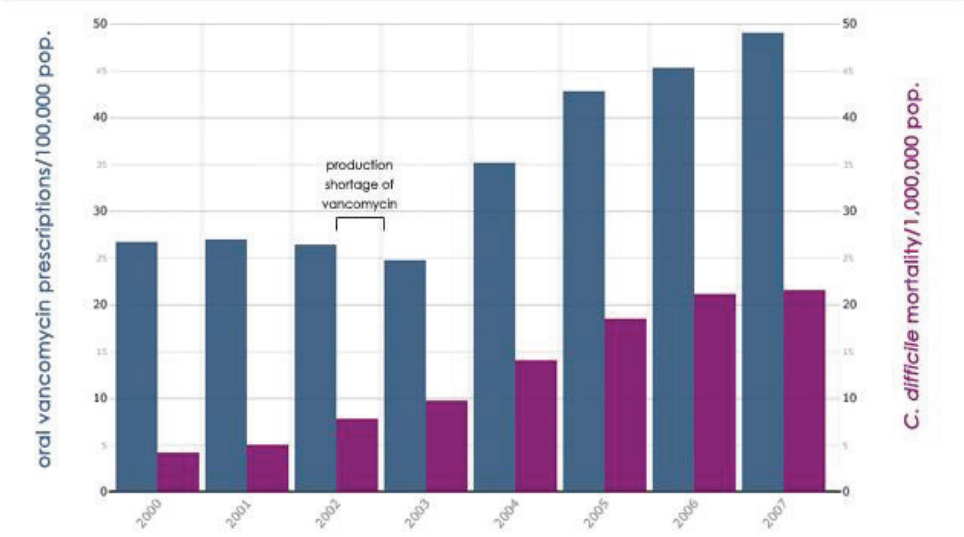
Antibiotic treatment result two major concerns in *C. difficile* infection:

Opportunistic infection after disruption of flora in small intestine



Nature Reviews Microbiology (2009) **7**, 526-536 .

More virulent and antibiotics-resistant strain of *C. difficile* bacteria



Source: Centers for Disease Contol and Prevention, National Center for Health Statistics

Treatment of *Clostridium difficile*-associated diarrhea in adults

Current antibiotics treatment:

metronidazole, vancomycin

Vaccine Development

Passive immunization:

Antibody-mediated antitoxin immunity

Sanofi in Phase 2 trials

Active immunization:

Vaccination to produce long-term protection

Sanofi used chemical inactivated whole toxin A as vaccine in phase 3

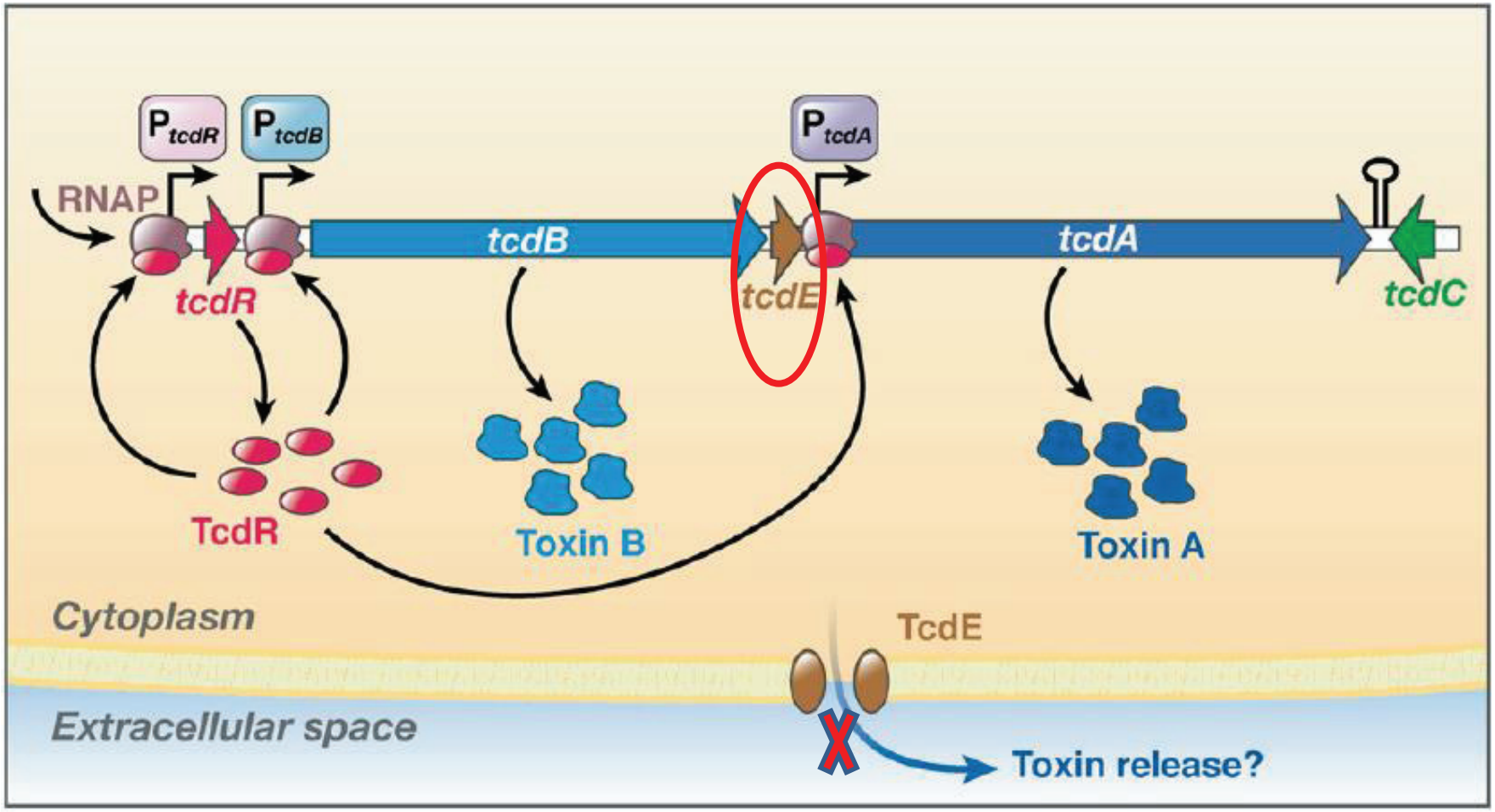
Subunit vaccine based on C-terminal repeat domain + adjuvant

Synthetic peptide based on the Repeating sequences + adjuvant

Recombinant chimeric toxin (A enzymatic domain + B RBD) + adjuvant

NHRI use lipoprotein based on the receptor binding domain of Cd Toxin

Aims of Vaccine Development: Preventing Diseases

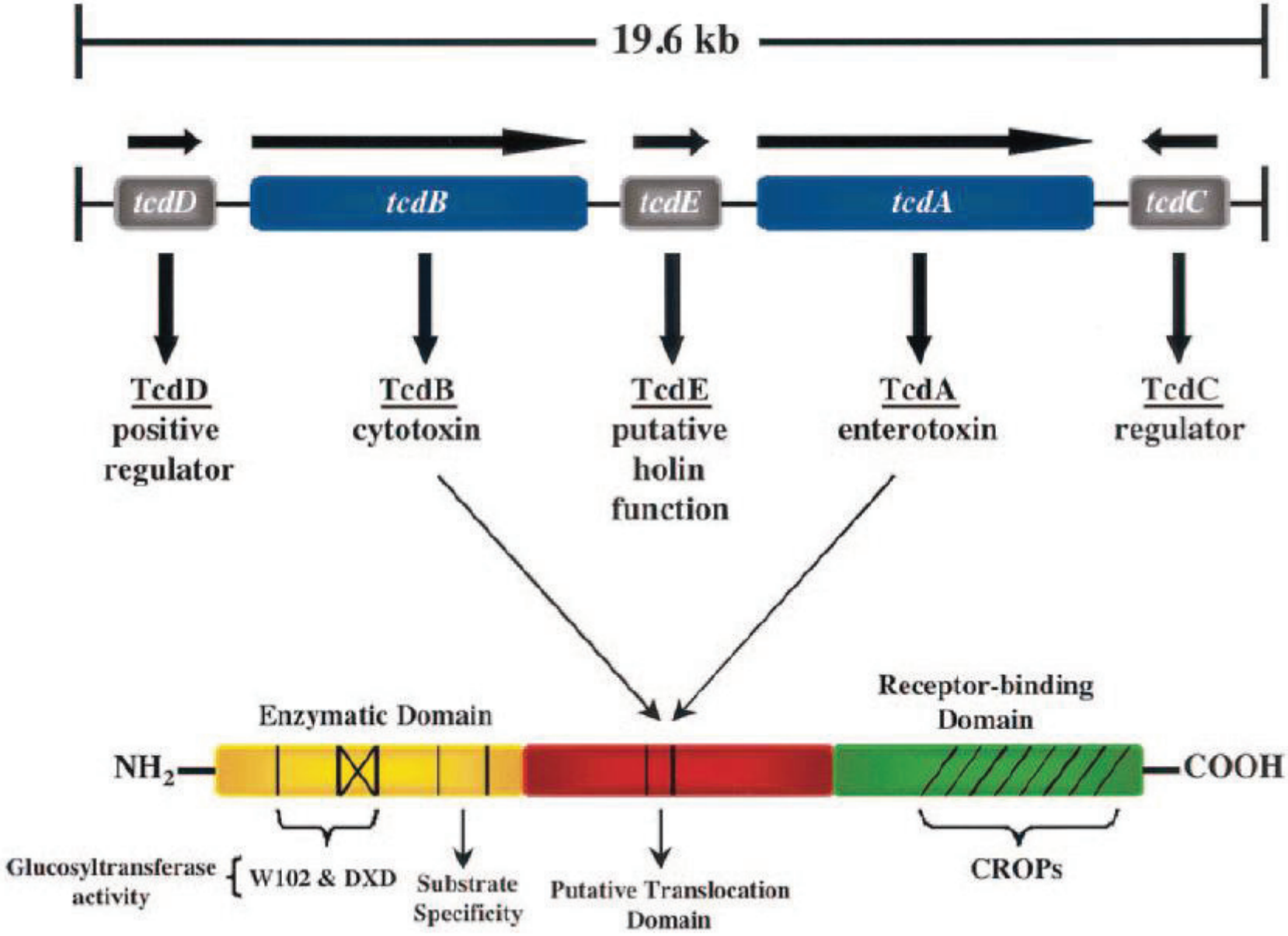


(Modify from O'Connor JR et al., 2009)

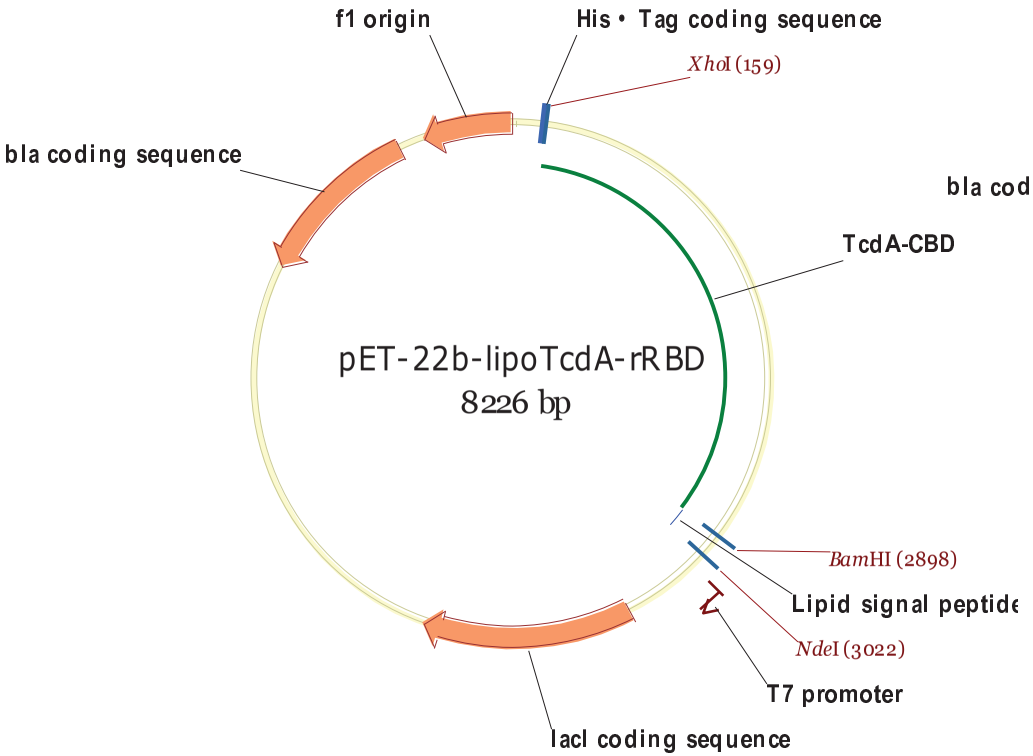
Neutralize the toxins

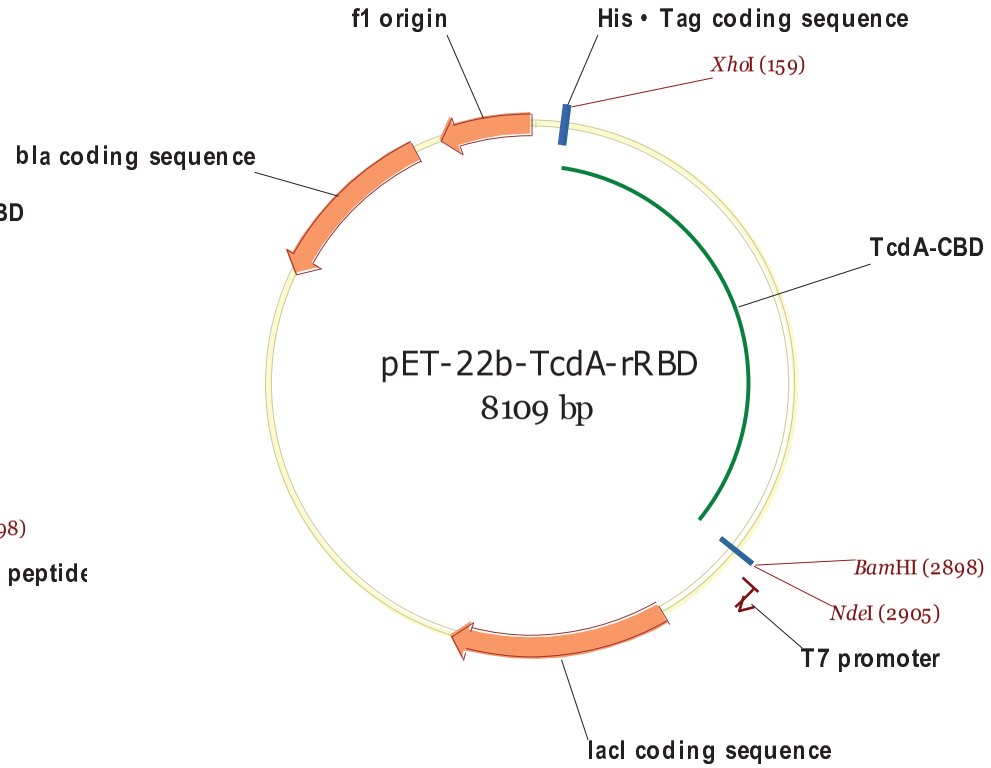
Block the releases of toxins by tcde specific antibodies

Structure of *C. difficile* Toxin A and B



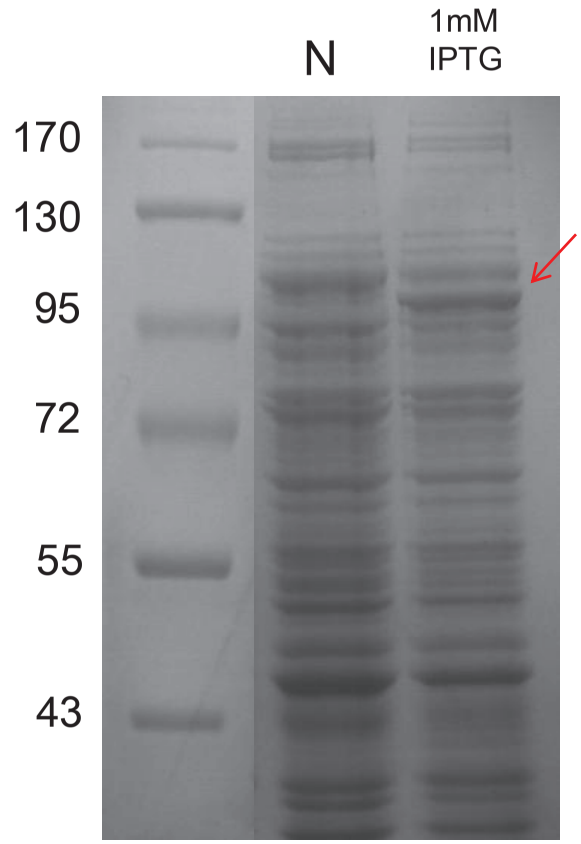
Lipo-TcdA-rRBD and TcdA-rRBD construction



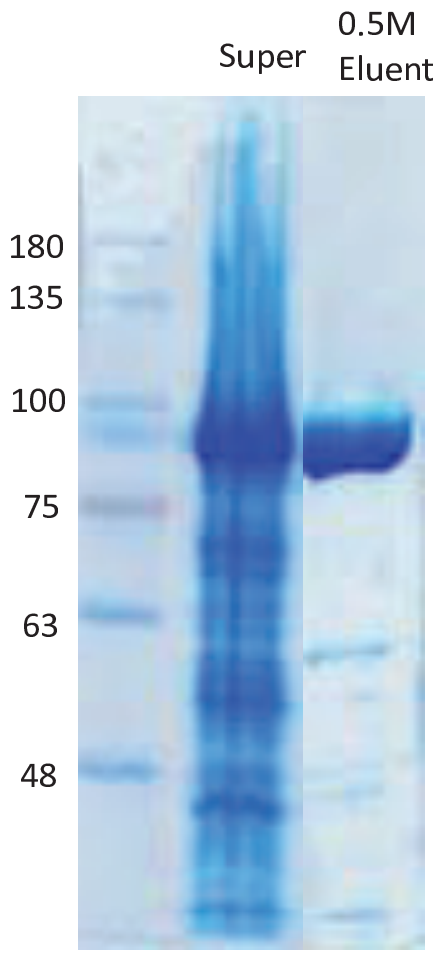


rTcdA-RBD expression and purification

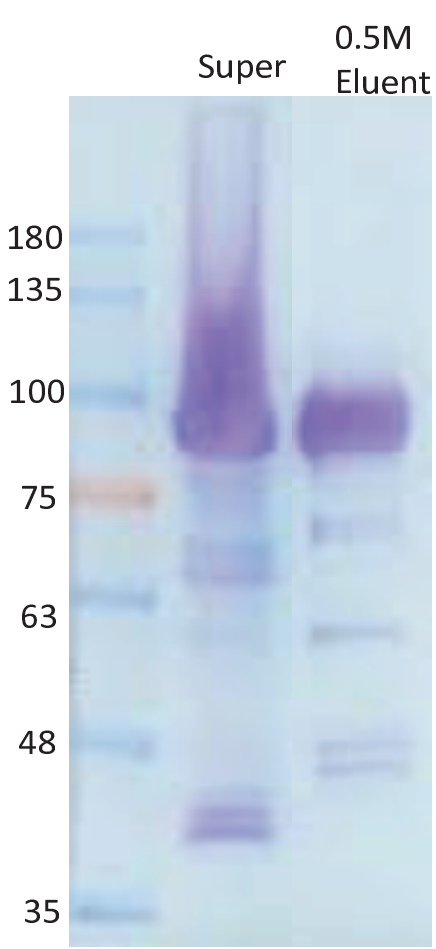
8% SDS-PAGE



8% SDS-PAGE

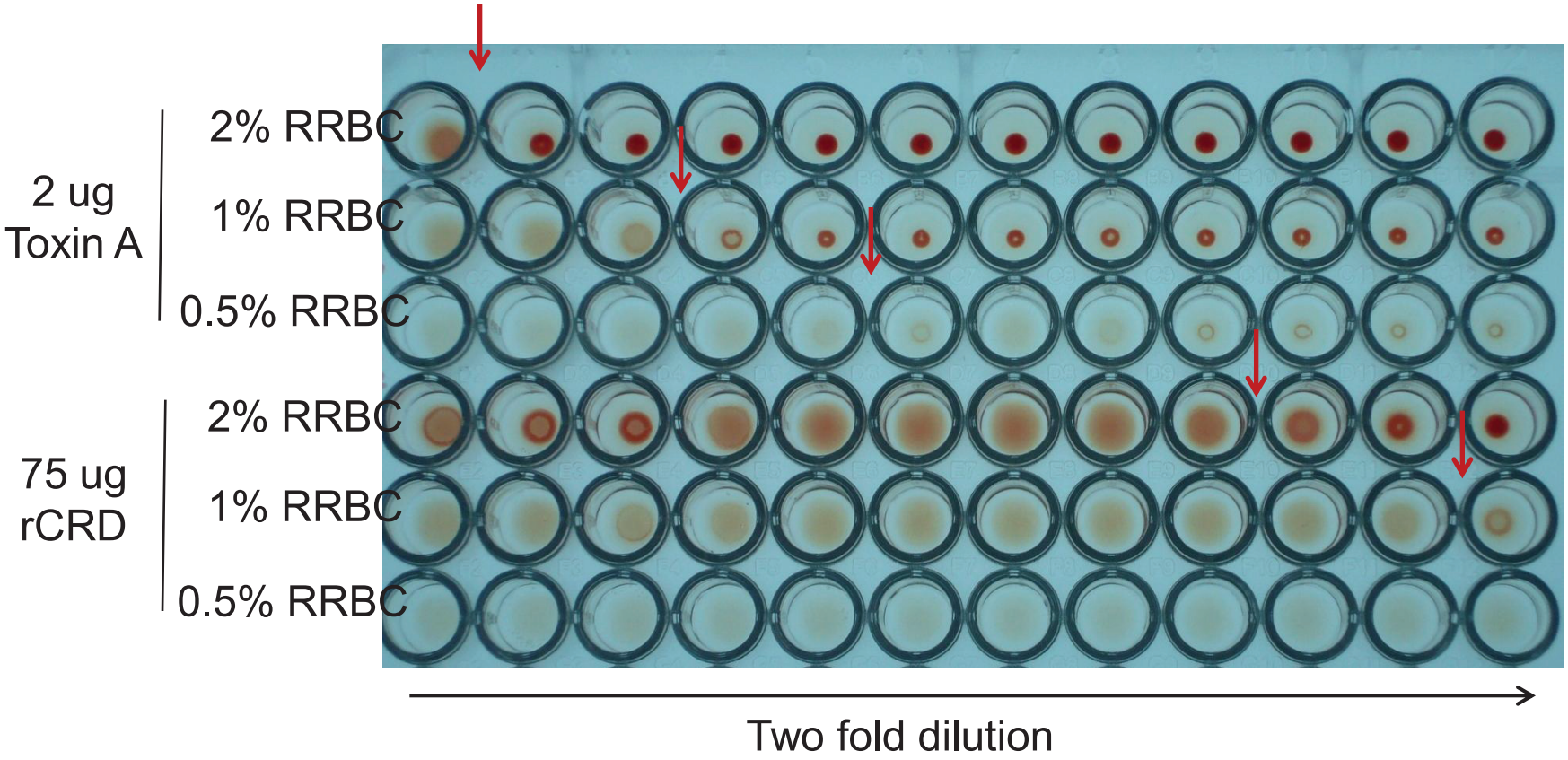


Western blot



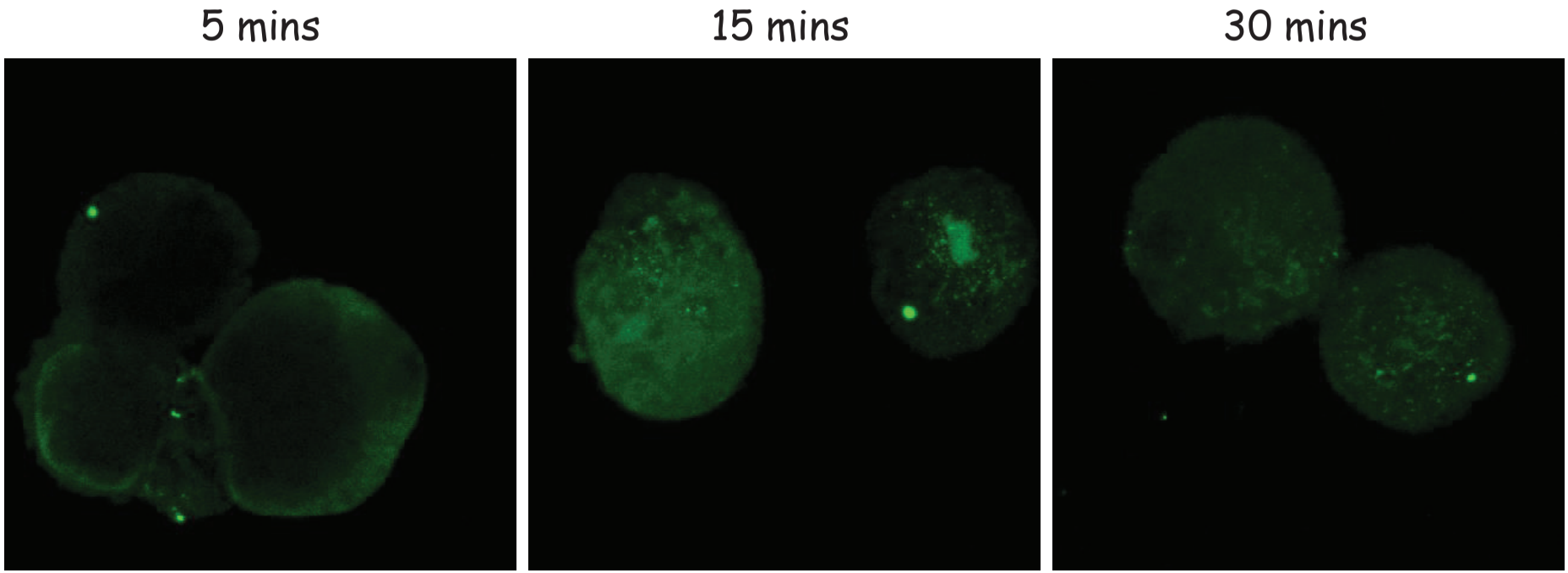
LB medium, 20 ℃ induction, overnight

HA activity of Toxin A and rRBD in different concentration of rabbit RBC



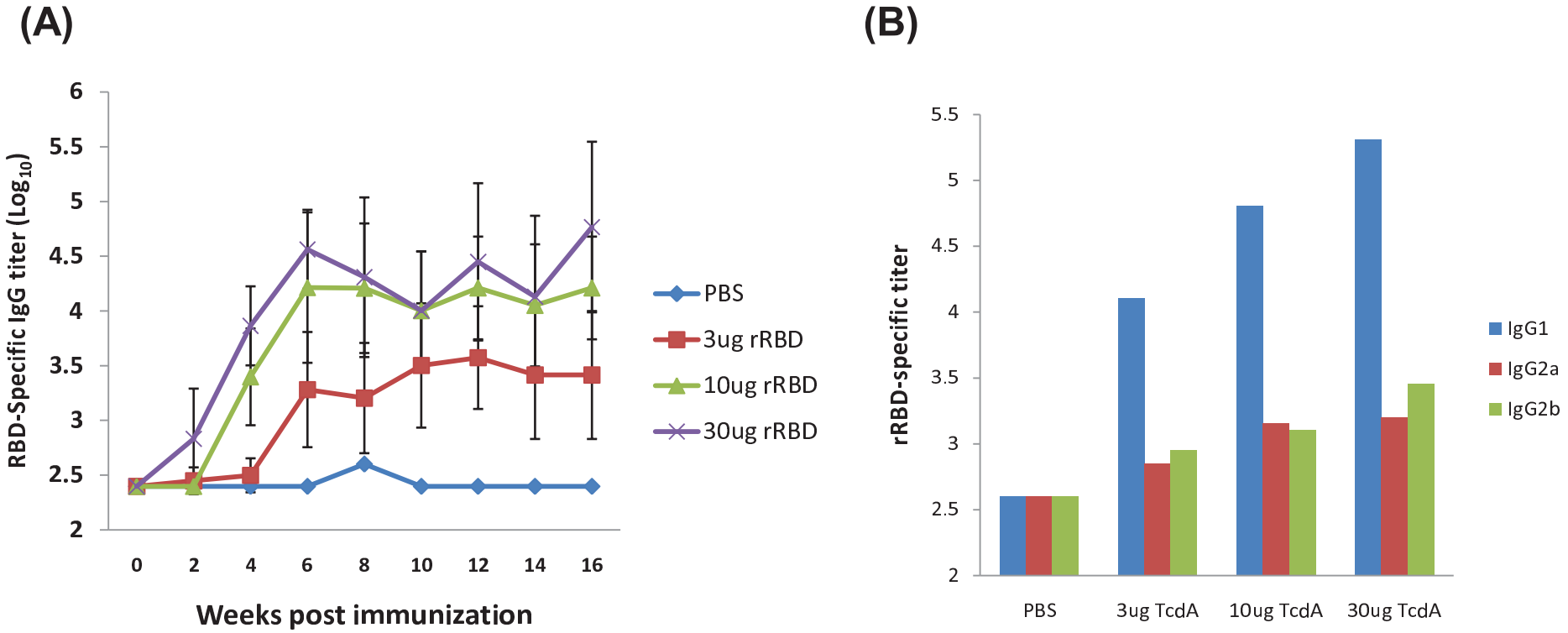
Results had shown that rRBD had high HA activity than those obtained by Toxin A in rabbit RBC assay

Assessing rRBD localization by confocal microscope



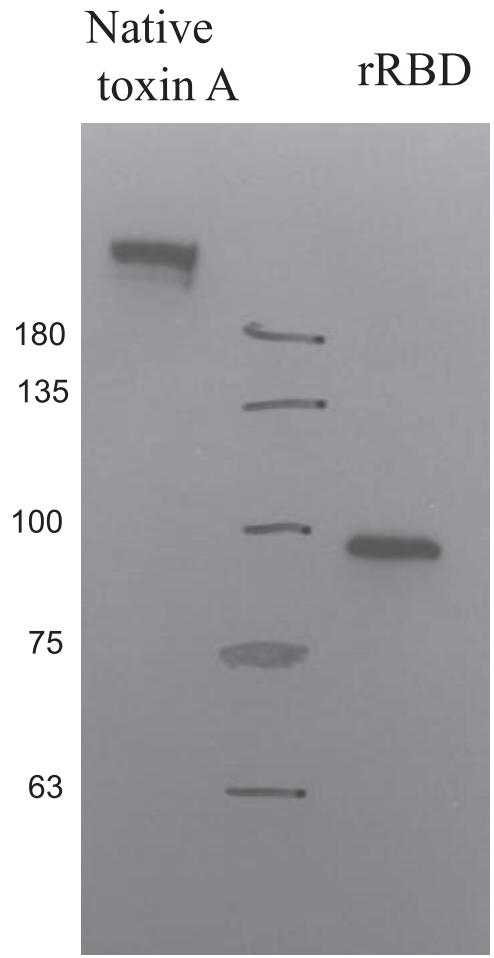
Immunogenicity of TcdA rRBD

➢To evaluate **immunogenicity and biological function of rRBD**

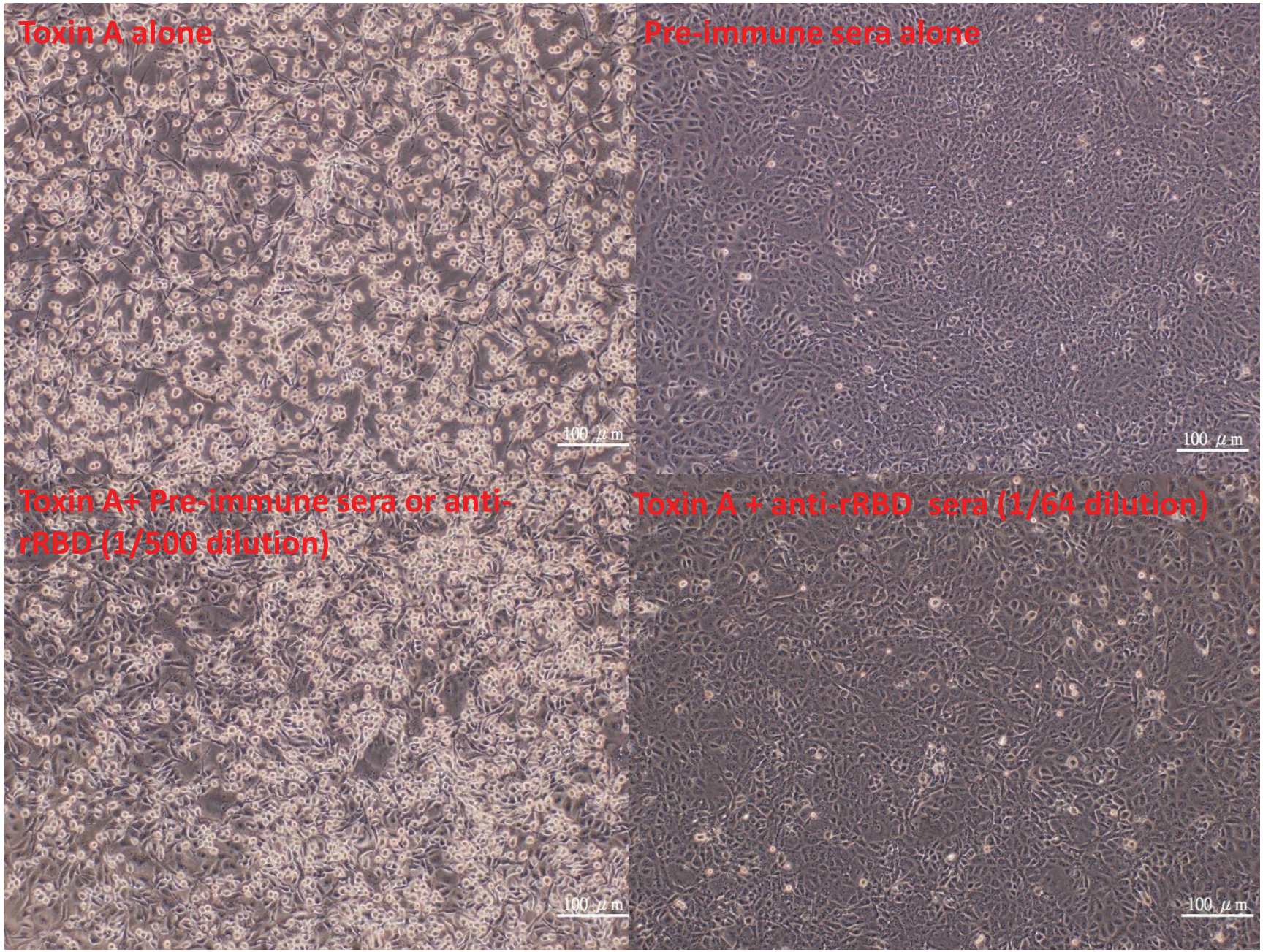


Systemic antibody responses after BALB/c mice immunization with TcdA rRBD . (A) rRBD-specific IgG titer was continually monitored to 16th week by serum titer ELISA. (B) Isotype IgG were detected at 8th week mouse serum.

Mouse anti-RBD antibody recognized *C. difficile* toxin A



Mouse anti-rlipo-RBD sera could neutralize the toxicity of Toxin A in the Vero cell assay



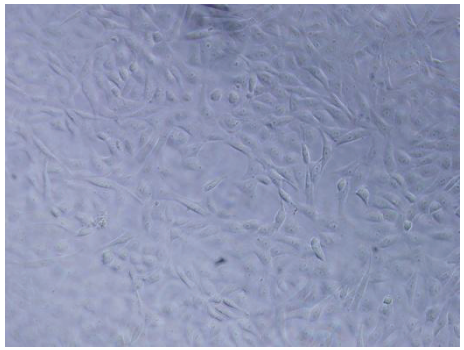
Anti-TcdA Neutralization titer

Neutralizing titer was defined as the reciprocal of the highest serum dilution that inhibited 100% cell rounding.

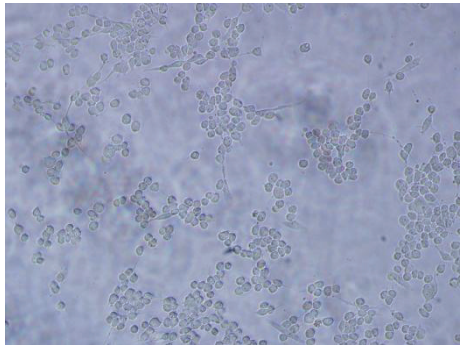
> 50% cell rounding

Serum Antitoxin Antibodies Mediate Systemic and Mucosal Protection from *Clostridium difficile* Disease in Hamsters. Paul J. Giannasca *et al.* 1999. *Infect Immun* 67(2): 527–538.

Medium only

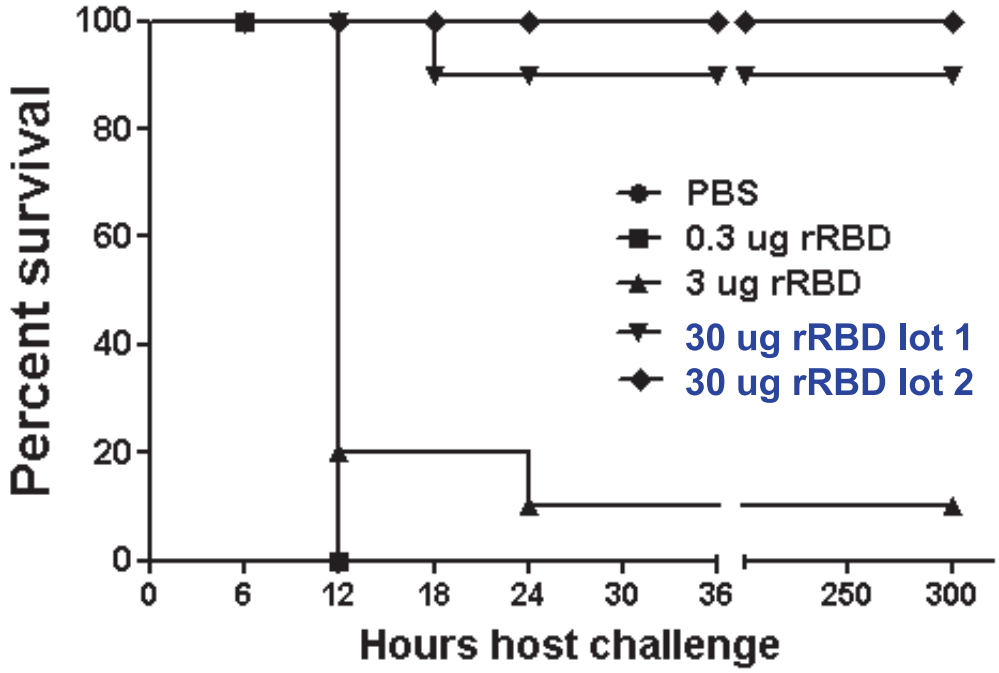


2.5 ng/ml tcdA



Immunization		neutralization titer
PBS		< 4
rRBD Lot 1	3 ug	8
	10 ug	64
	30 ug	64
rRBD Lot 2	30 ug	256

Protection against Toxin A toxicity in dose-response



Immunization	Percent survival (%)
PBS	0%
0.3 ug rRBD	0%
3 ug rRBD	20%
30 ug rRBD lot1	90%
30 ug rRBD lot 2	100%

Toxin A challenge dosage: 150 ng (5X LD50)

Design of a consensus sequence encoding tcdA rRBD and its fragments

A high homology and repetitive sequence: **35 short-term repetitive units,** (http://www.ebi.ac.uk/Tools/pfa/radar/)

No. of Repeats	Total Score	Length	Diagonal	BW-From	BW-To	Level
35	1146.90	18	18	302	319	1

55-	72	(39.10/15.30)	VTG.WQTINGKKYYFDINT
76-	93	(30.96/10.60)	STS.YKIINGKHFYFNNNG
97-	114	(28.01/ 8.89)	LGV.FKGPDGFEYFAPANT
127-	143	(29.31/ 9.64)	QSK.FLTLNGKKYYFD.ND
147-	164	(33.70/12.18)	VTG.WRIINNEKYYFNPNN
168-	185	(29.26/ 9.61)	AVG.LQVIDNNKYYFNPDT
189-	206	(31.51/10.91)	SKG.WQTVNGSRYYFDTDT
210-	227	(31.50/10.91)	FNG.YKTIDGKHFYFDSDC
231-	248	(25.19/ 7.25)	IGV.FSGSNGFEYFAPANT
261-	278	(31.84/11.10)	QSK.FLTLNGKKYYFDNNS
281-	298	(39.20/15.36)	VTG.WQTIDSKKYYFNTNT
302-	319	(41.82/16.88)	ATG.WQTIDGKKYYFNTNT
323-	340	(41.82/16.88)	ATG.WQTIDGKKYYFNTNT
344-	361	(31.63/10.98)	STG.YTIINGKYFYFNTDG
395-	412	(31.32/10.80)	QNK.FLTLNGKKYYFGSDS
415-	432	(40.42/16.07)	ITG.WQTIDGKKYYFNPNN
436-	453	(24.55/ 6.88)	ATH.LCTINNDKYYFSYDG
456-	473	(24.24/ 6.70)	QNG.YITIERNNFYFDANN
478-	496	(28.76/ 9.32)	VTGvFKGPNGFEYFAPANT
509-	526	(32.80/11.66)	QNK.FLTLNGKKYYFDNDS
529-	546	(37.17/14.19)	VTG.WQTIDSKKYYFNLNT
550-	567	(37.68/14.48)	VTG.WQTIDGEKYYFNLNT
571-	588	(40.46/16.09)	ATG.WQTIDGKRYYFNTNT
592-	609	(33.21/11.89)	STG.YTIINGKHFYFNTDG
613-	630	(29.80/ 9.92)	IGV.FKGPDGFEYFAPANT
643-	660	(31.32/10.80)	QNK.FLTLNGKKYYFGSDS
663-	680	(36.63/13.88)	VTG.LRTIDGKKYYFNTNT
684-	701	(42.11/17.04)	VTG.WQTINGKKYYFNTNT
705-	722	(30.09/10.09)	STG.YTIISGKHFYFNTDG
726-	743	(29.80/ 9.92)	IGV.FKGPDGFEYFAPANT
776-	793	(34.04/12.38)	ATG.WATIDGNRYYFEPNT
797-	813	(28.89/ 9.40)	ANG.YKTIDNKNFYFR.NG
817-	834	(29.99/10.03)	IGV.FKGPNGFEYFAPANT
847-	864	(24.88/ 7.07)	QNR.FLHLLGKIYYFGNNS
867-	884	(33.91/12.30)	VTG.WQTINSKVYYFMPDT

rRBD
911 aa

104 kDa

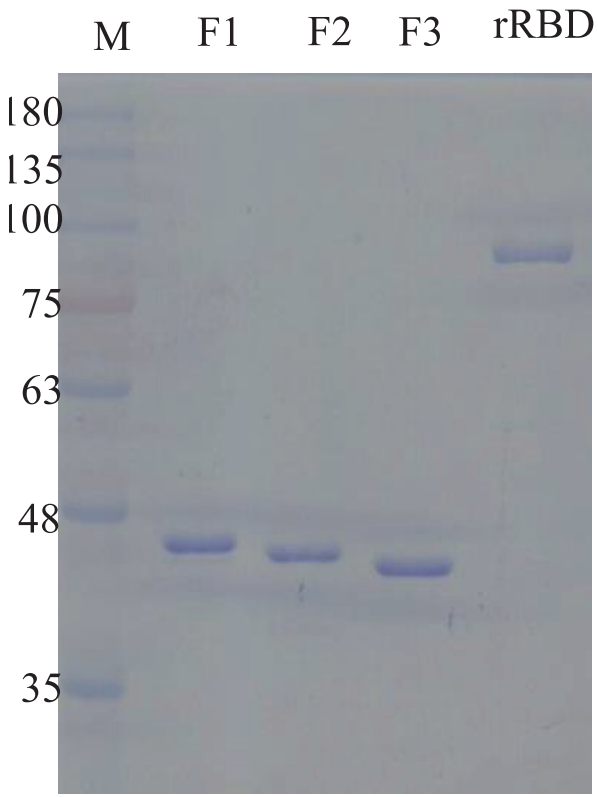
f1 46.8 kDa

f2 43.8 kDa

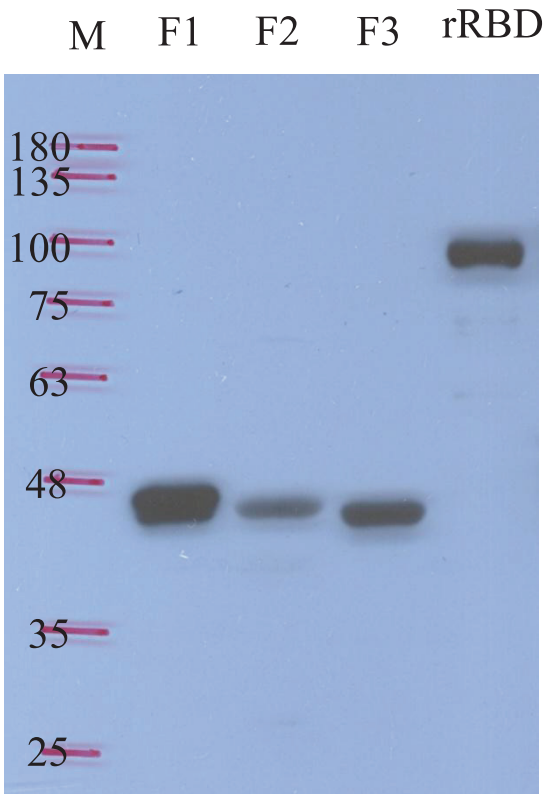
f3 43.7 kDa

Purification of recombinant TcdA RBD and its fragments

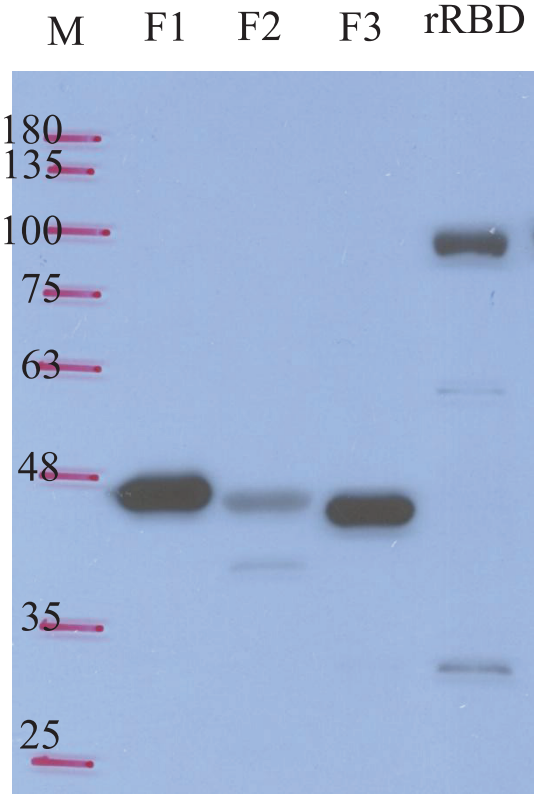
(A) **SDS-PAGE**



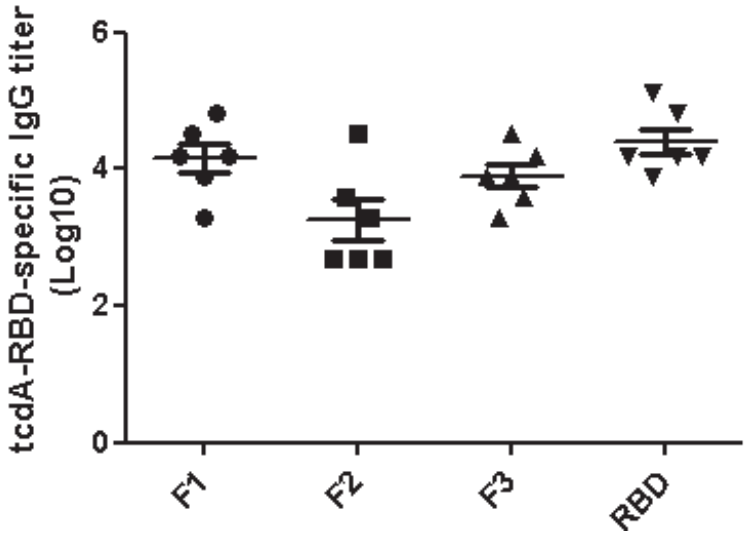
(B) **Western blot**
TcdA-specific Ab



(C) **Western blot**
Anti-HIS tag



Mouse Immunogenicity of TcdA RBD Fragments



Functional antibody response against toxin A toxicity

Immunization	Toxin A neutralization titer
F1	8
F2	4
F3	16
TcdA rRBD	256

TcdA RBD fragments could elicit functional antibody against TcdA toxicity.

Evaluation of dendritic cell maturation promoted by rRBD and activity of its fragments

DC maturation markers

CD40

CD80

CD86

MHC-II

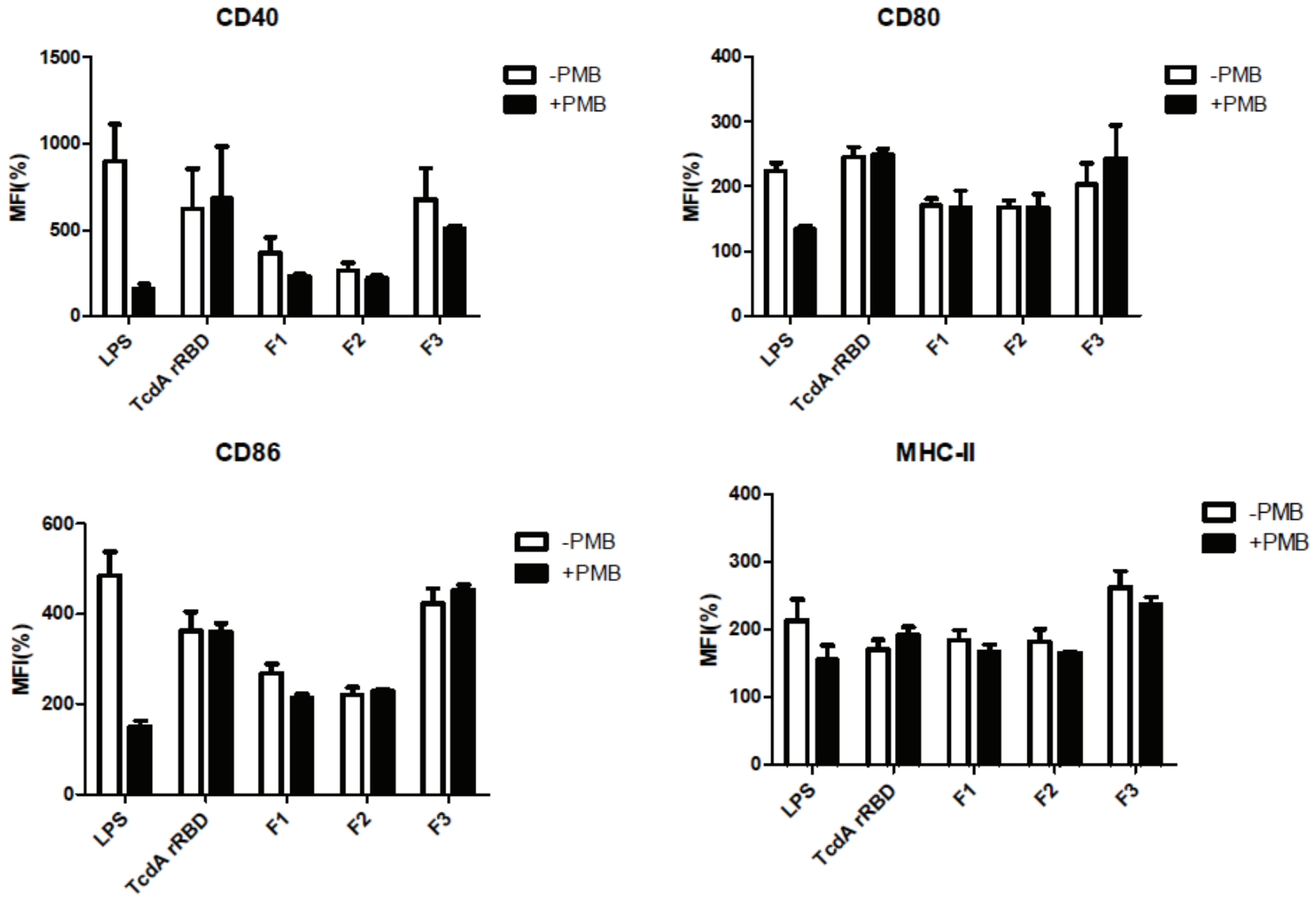
Cytokines

IL-6

IL-12

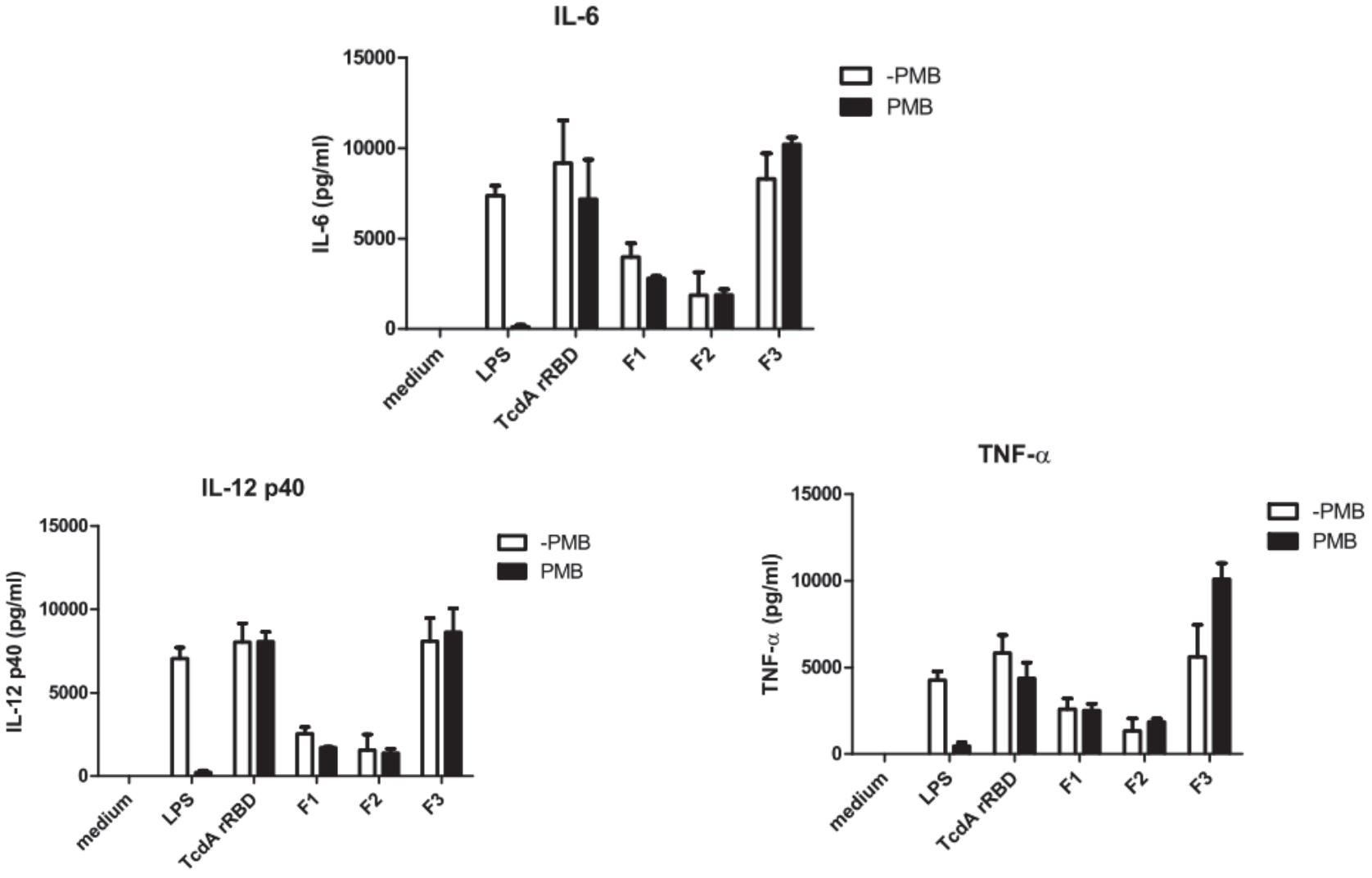
TNF- α

DC surface markers up-regulated by rRBD fragments

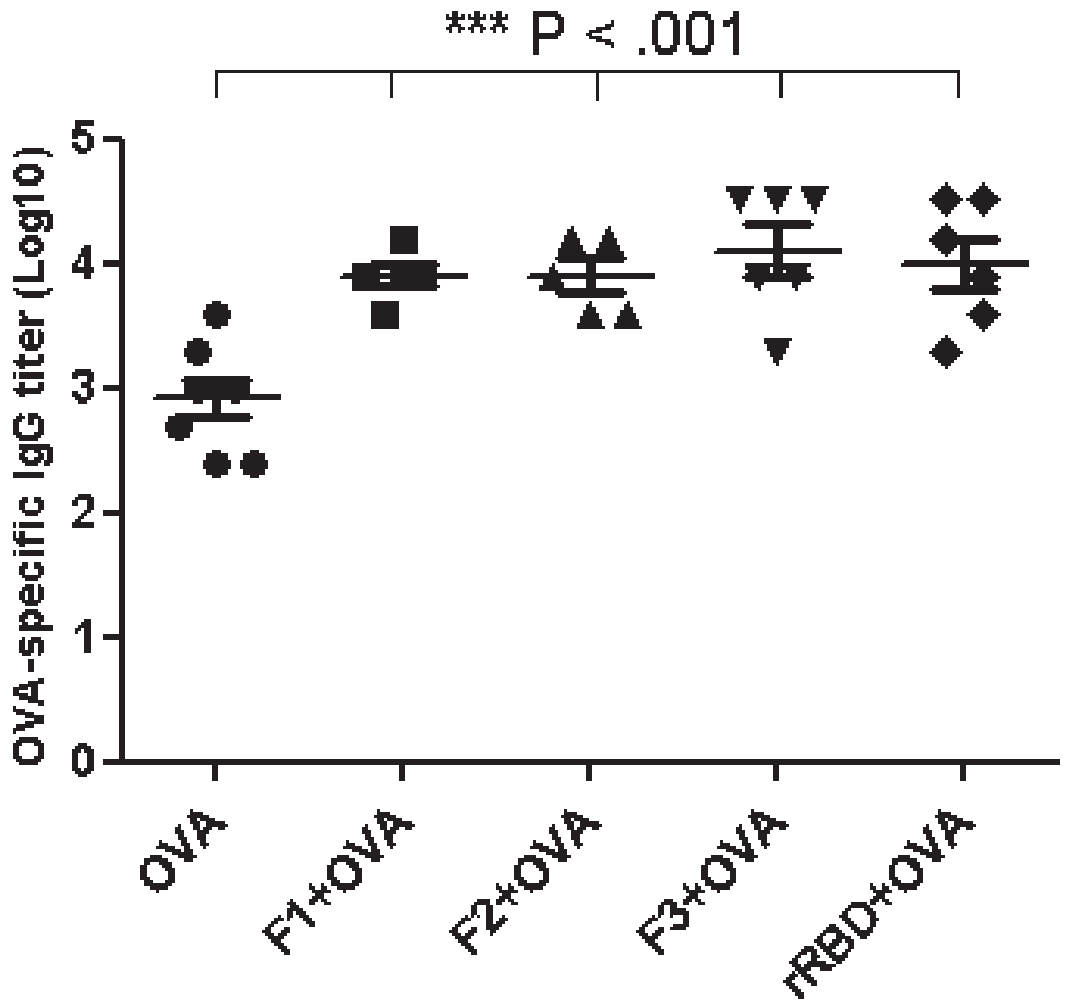


The mean fluorescence intensity(MFI) for cells culture in medium was defined as 100%

Cytokines secretion of DC stimulated by rRBD fragments



Evaluation of adjuvant effect of truncated RBD fragment



cdA rRBD fragments co-administration can enhance systemic OVA-specific IgG titer by intramuscular injection.

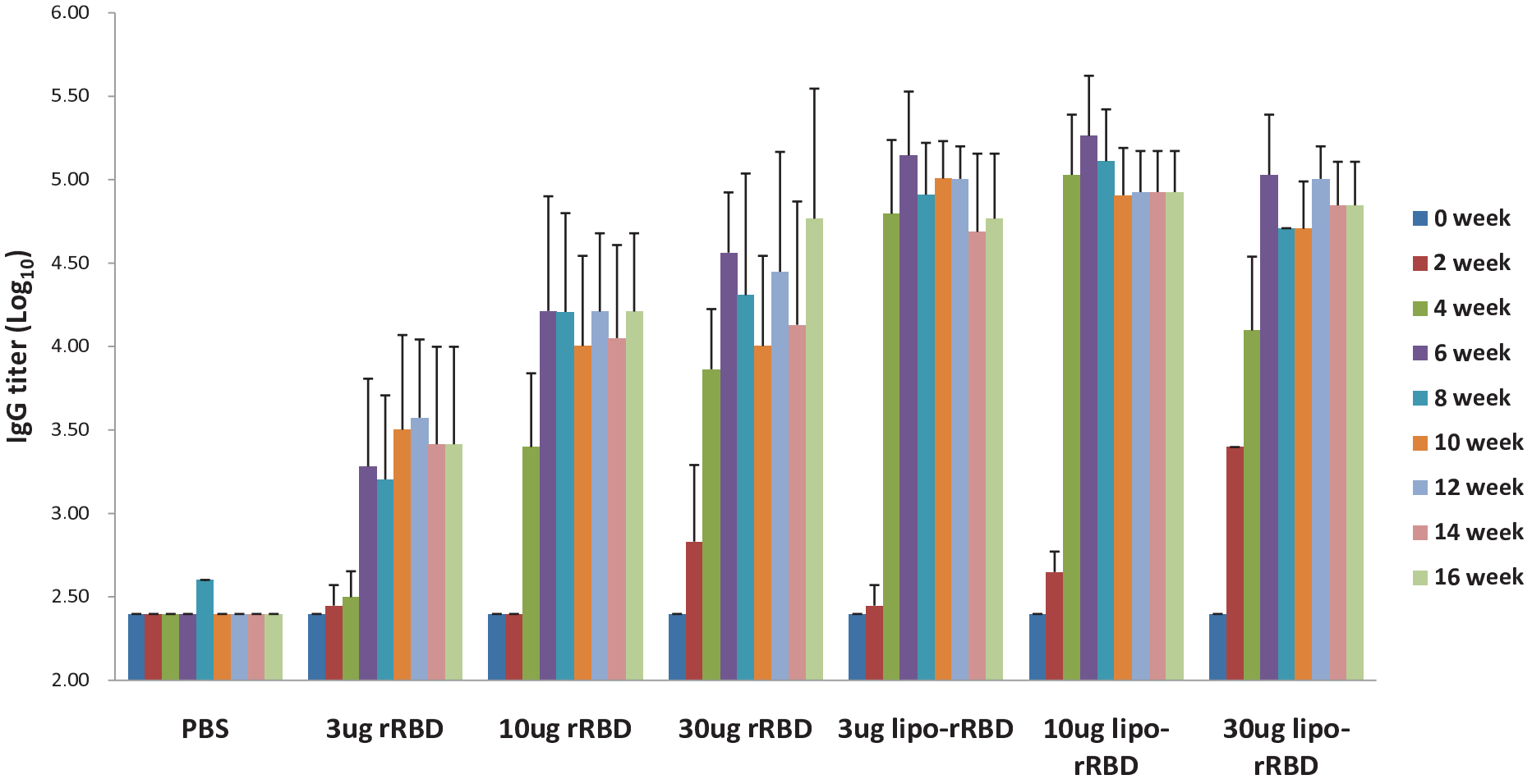
Summary

rRBD can

- agglutinate rabbit red blood cell (HA activity)
- bind and enter into the Vero cell
- alone elicit antibody responses that could inhibit TcdA toxicity
- at 30 ug dose elicit immune responses fully protecting mice from TcdA challenge
- up-regulate the immune effector molecules expression and stimulate cytokines release from dendritic cells
- function as an adjuvant (TLR-like agonist) to enhance anti-OVA antibody responses

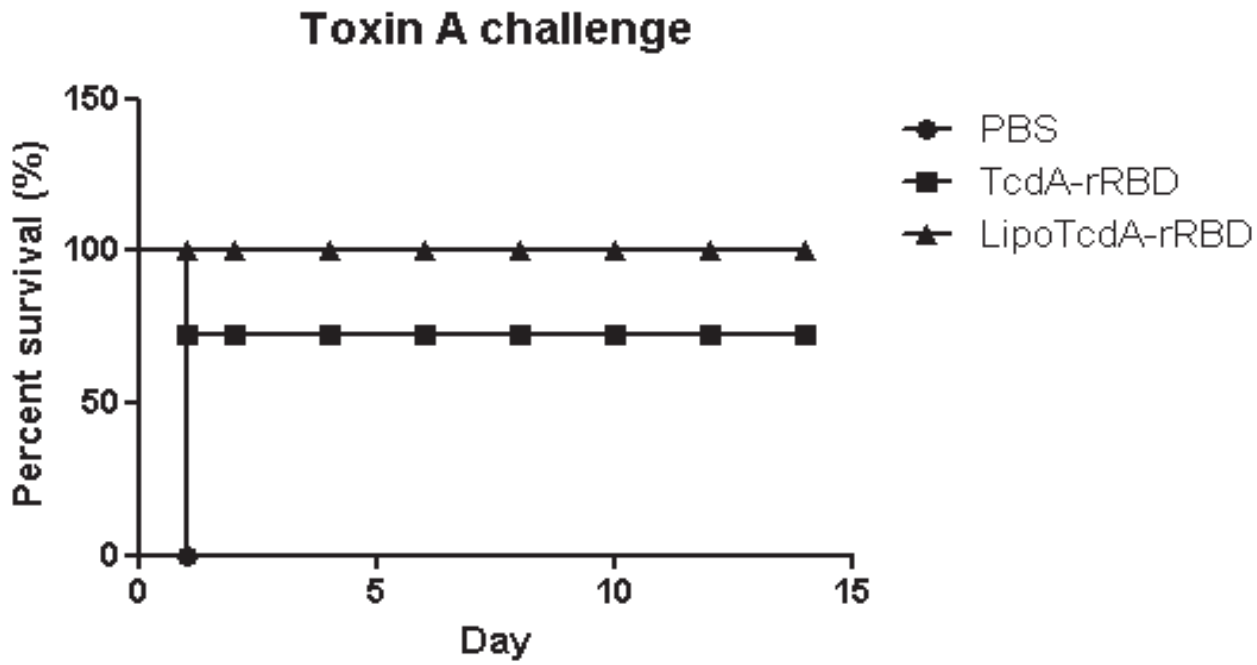
All biological functions of rRBD are located at the C-terminal 320 amino acids

Mouse IgG elicited against rlipo-TcdA RBD or rRBD alone



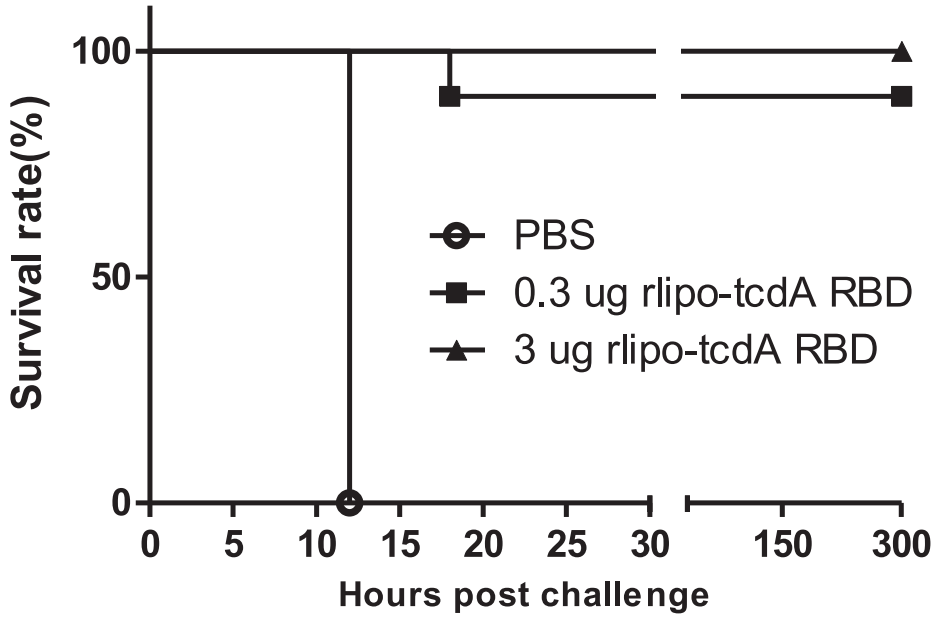
Results had shown that rlipo-RBD alone was highly immunogenic even at one dose and 10X more potent than rRBD formulated in alum

Clostridium difficile Toxin A challenge



	Percent Survival				
	0 hr	15 hr	24 hr	48 hr	Day 10
PBS	6/6 (100%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
30 ug TcdA-RBD	10/10 (100%)	7/10 (70%)	7/10 (70%)	7/10 (70%)	7/10 (70%)
30 ug rlipo-TcdA-RBD	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

Clostridium difficile Toxin A challenge



	Percent Survival				
	0 hr	15 hr	24 hr	48 hr	Day 10
PBS	6/6 (100%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)
0.3 ug rlipo-TcdA-RBD	10/10 (100%)	10/10 (100%)	9/10 (90%)	9/10 (90%)	9/10 (90%)
3 ug rlipo-TcdA-RBD	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)	10/10 (100%)

謝

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STACK:

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