



Immunological Toolbox Euro VetVacc Workshop

22/05/2018

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Mwangi



Introduction and background

Two meetings in 2015/2016 highlighted the continued importance of reagent development for veterinary species and the unsuitability of short term funding streams for sustained efforts.

Roslin and Pirbright were encouraged to fund toolbox activities through their Core Capability Grants.

Bids were successful and Roslin and Pirbright are working on a joint effort to coordinate and develop immune reagents.

The nature of CCG funding makes it imperative that we demonstrate wider support for research.

What is the immunological toolbox?

“Remove barriers to VetVacc research”

Three parts:

1. Pirbright CCG funded activities to sequence our antibody repository and manufacture recombinant antibodies
2. Roslin to generate new antibodies
3. GCRF funded website linked to the above as a source of information- as broad as possible
4. An international effort to consolidate all activities both past and present as part of the above

Two steering group meetings and two workshops held already

21-22nd August 2017

Two day international workshop at Pirbright focussed on previous efforts and priorities for sustainable funding

Agreed that many international efforts could be coordinated and sustainable funding focussed on the toolbox website

17th January 2018

One day workshop at Stirling- focussed on specific priorities and future funding (leveraged by current activities)

Updated Priorities

Secure current antibodies through sequencing

Create and maintain a website of available reagents and associated data (more later).

- *Facilitate information exchange and collaboration*

Generate new antibodies based on community demand.

- *Agreed priorities were T cell subset markers and B cell subset markers.*
- *Both antibodies against new antigens and recombinant antibodies*

Facilitate screening of uncharacterised hybridomas

The immunological toolbox activities at Pirbright: recombinant antibodies

To translate our current hybridoma stocks into transfectable gene blocks. Build in the option of class switching, catalogue current stocks

- Sequencing and expression
- Making Fab fragments
- Class switching
- Species switching; mouse-bovine, mouse-chicken or mouse-pig hybrids.
- Scale up of products from above protocols to milligrams quantities

Benefits of recombinant antibodies

- Secure reagents for the future as they will exist as sequences
- Reliable, long-term supply (rAbs antibodies are not susceptible to cell-line drift)
- Reduce cost of LN storage (not getting rid of all the vials!)
- Flexibility in production methods to potentially reduce cost or increase scale of manufacturing
- Make these antibodies simple to share without shipping cells or even supernatant
- Open the possibility of engineering antibodies to better suit research needs
- Consistent performance across lots (many hybridomas are not monoclonal)
- Highly specific and sensitive antibodies (no lot-to-lot variation, thus allowing for peak specificity and performance)

Benefits of recombinant antibodies

- Lack of an Fc effector functionality (e.g. Fabs to eliminate both cellular responses against the target)
- Flexible labelling and purification options, e.g. rAbs containing a Sortase recognition motif (LPXTG) to covalently add fluorophores, enzymatic substrates (HRP, AP...etc), in a directed and reproducible manner
- Or 6xHis tag (for nickel-based purification systems) and an avidin tag sequence for enzymatic biotin conjugation using the biotin ligase, BirA.



Our recombinant antibody pipeline

Collaboration with Prof Ray Owens & Dr Jo Nettleship at OPPF

Validated a sequencing protocol at Pirbright for mouse hybridomas, cattle heterohybridomas and cattle B cells

Vector backbone designed for cattle and mouse to allow ligation of commercially generated antibody gene blocks

This allows the following

- **Sequencing mAbs** ✓
- **Making Fab fragments from rAbs** ✓

Transfection and purification validated at OPPF

- **Scaling up rAbs** ✓

Recombinant antibody pipeline



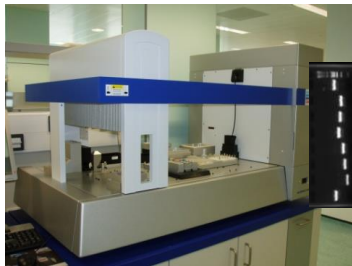
PCR



Ligation independent
cloning and transformation



Synthetic gene



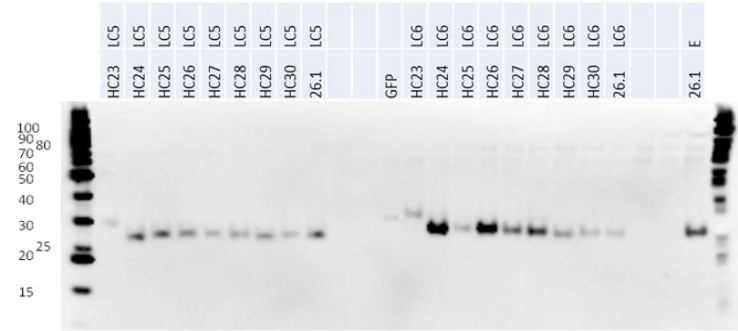
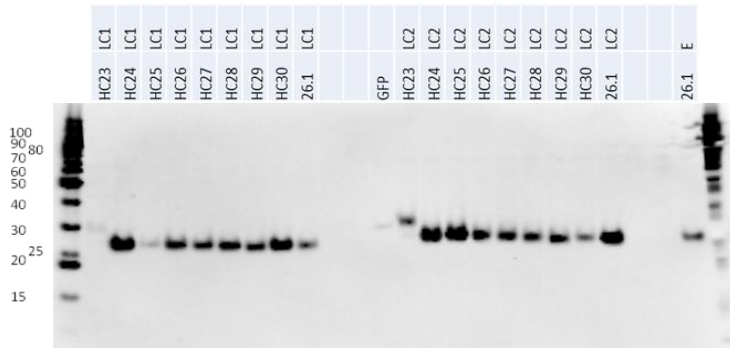
96 well miniprep and
PCR verification



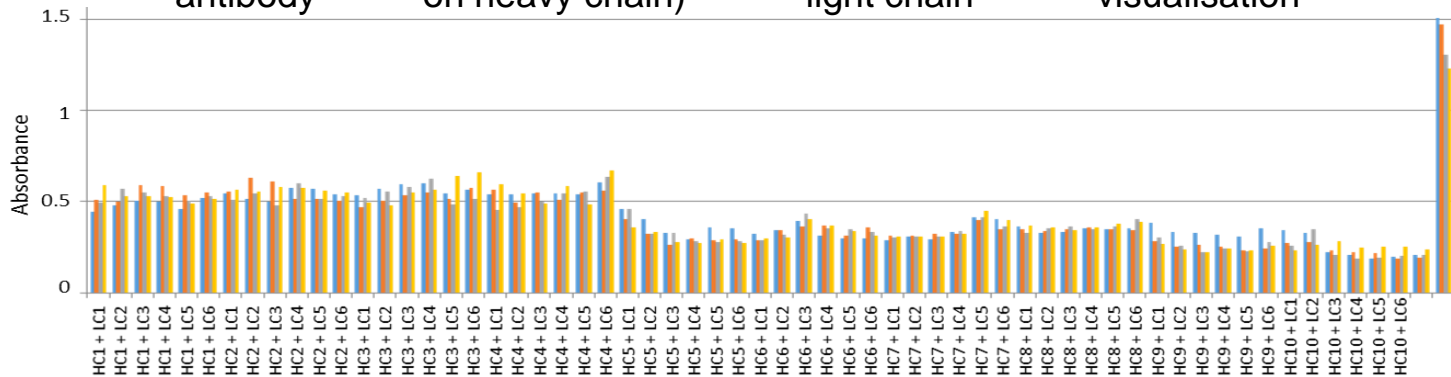
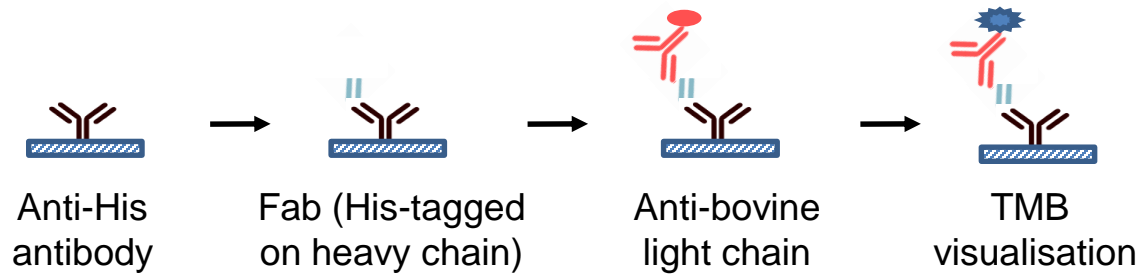
Small scale transient
expression screening

96 well expression analysis

Western blot analysis (Anti-His)



Assembly ELISA



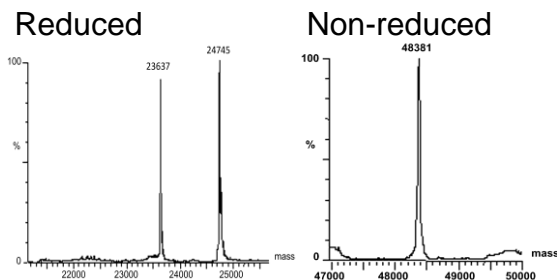
Protein production



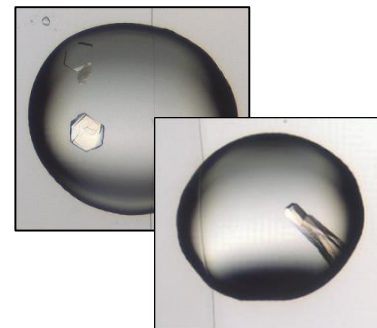
Expression mammalian cells



Protein purification



Quality assurance



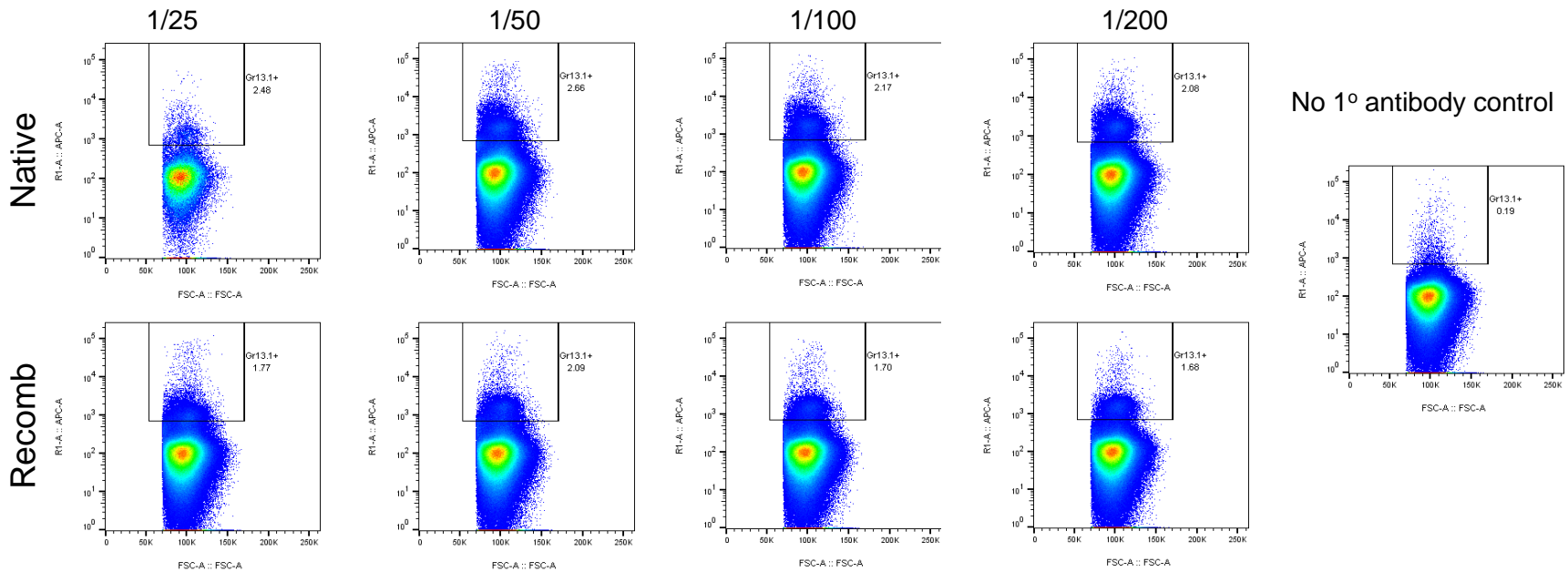
Crystallization

Nettleship et al. Methods Mol. Biol. (2009) 498: 245-263.

Nettleship et al. Methods Mol Biol. (2015) 1258:209-22.

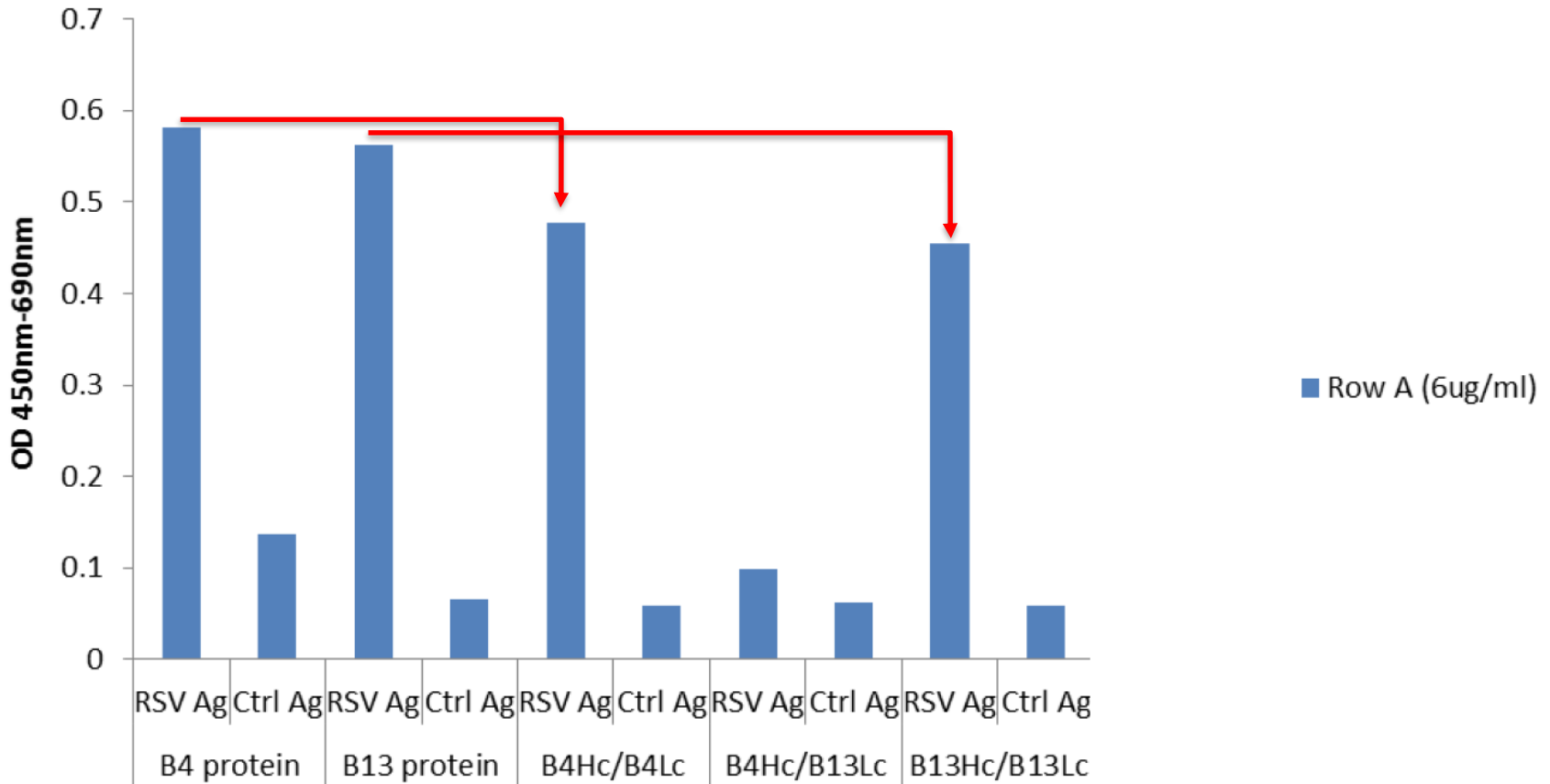
Gr13.1 recombinant mAb

- Staining of fresh PBMC with native vs recombinant Gr13.1 antibody



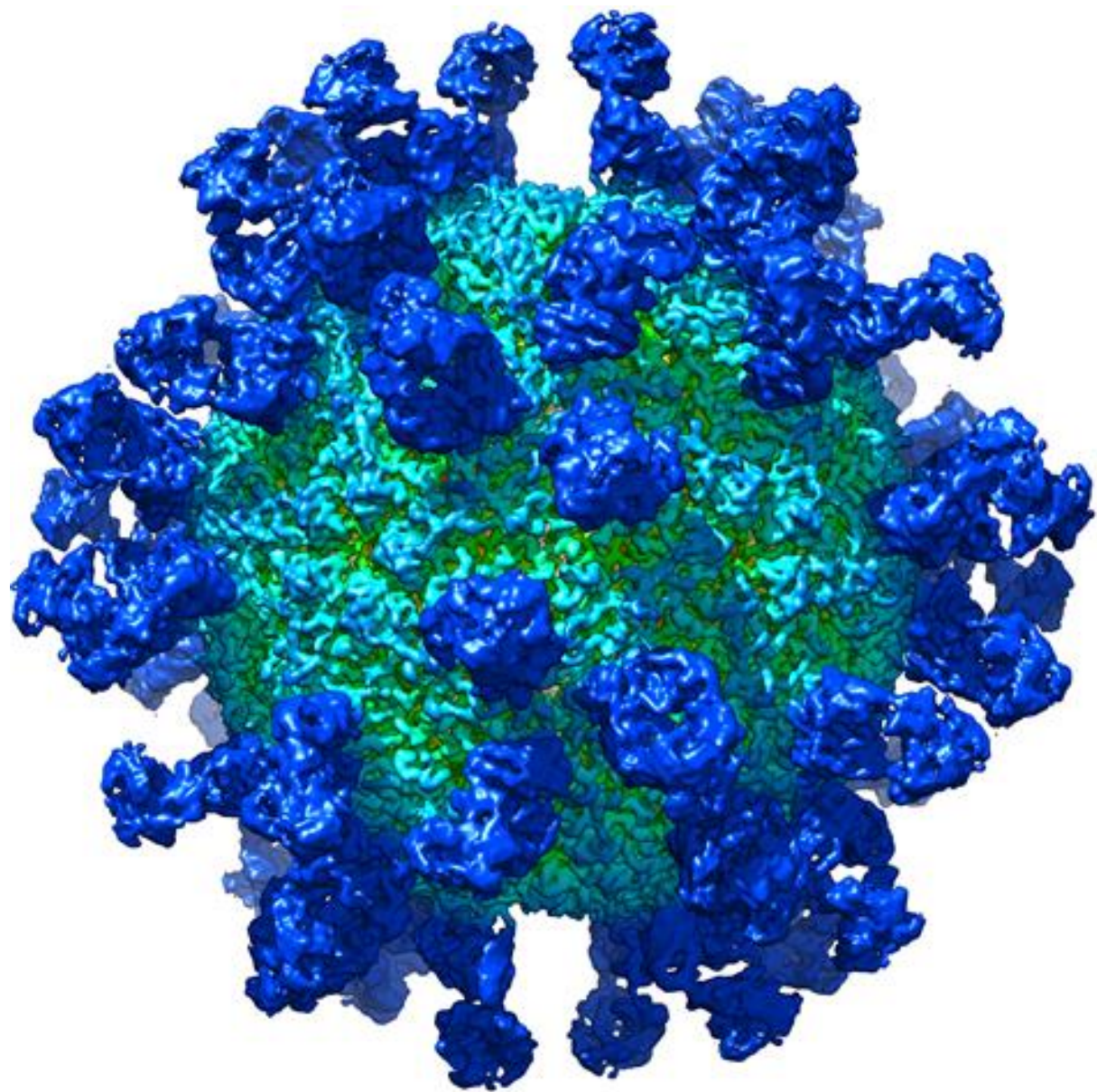
Anti-RSV recombinant mAbs

RSV ELISA

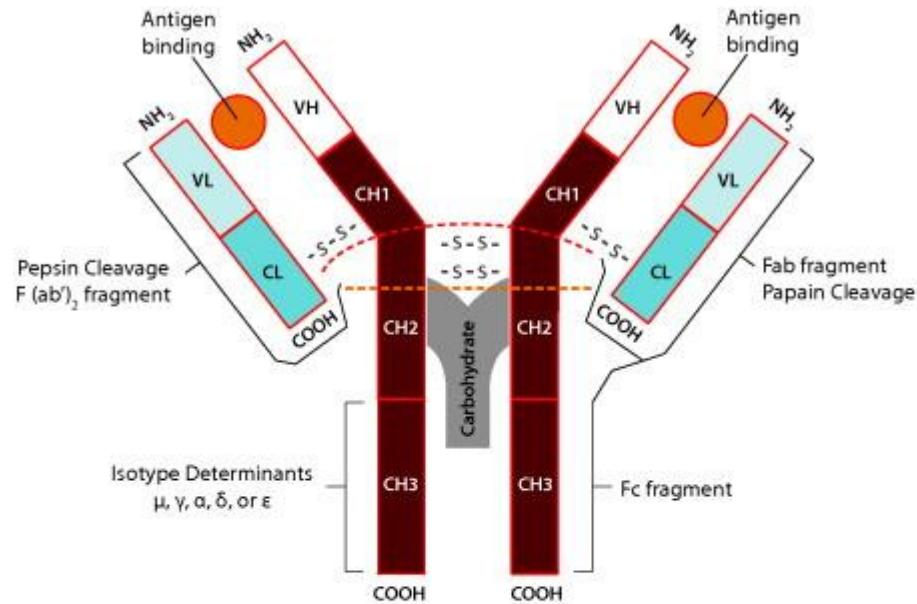


Anti FMDV SAT mAbs

- Predicted differences in binding patterns based on sequence information
- Cryo-electron microscopy of the Fabs bound to FMDV capsids confirmed differences in binding pattern



Antibody structure



- Heavy chain constant region
- Heavy chain variable region
- Light chain constant region
- Light chain variable region
- Carbohydrate
- Antigen binding site
- S-S- Disulfide bonds
- Papain cleavage site
- Pepsin cleavage site

IgG H Chain Subclasses



Human	$\gamma 1$	$\gamma 2$	$\gamma 3$	$\gamma 4$		
Complement fixation	weak	weak	strong	no		
Fc receptor binding	strong	weak	strong	weak		
Placental transfer	strong	weak	strong	strong		
Mouse	$\gamma 1$ (46 %)	$\gamma 2a$ (24%)	$\gamma 2b$ (27%)	$\gamma 3$ (2%)		
Bovine	$\gamma 1$	$\gamma 2$	$\gamma 3$			
Porcine	$\gamma 1$	$\gamma 3$	$\gamma 5.1$	$\gamma 5.2$	$\gamma 6.1$	$\gamma 6.2$
Chicken	IgY					

L chain usage

The ratio of kappa to lambda found in the Ig population varies by species

Species	% k	% λ
Mouse	99	1
Rat	99	1
Rabbit	90	10
Human	67	33
Pig	50	50
Goat	1	99
Sheep	1	99
Bovine	1	99
Horse	1	99
Chicken		up to 95?

Make Mouse k; Pig k and λ ; Bovine λ ; Chicken λ

Antibody expression vectors available at Pirbright

	Species/IgG	Name	ID		
			To linearise	Fwd extension	Rev extension
1	Mouse IgG1	pNeoSec-MmFc-IgG1	Kpn1 to Pst1	TGGGTTGCGTAGCT	GGGTGTCGTTTTGGC
2	Mouse IgG2a	pNeoSec-MmFc-IgG2a	Kpn1 to Pst1	TGGGTTGCGTAGCT	GGCTGTTGTTTTGGC
3	Mouse IgG2b	pNeoSec-MmFc-IgG2b	Kpn1 to Pst1	TGGGTTGCGTAGCT	GGGTGTTGTTTTGGC
4	Mouse LC-k	pNeoSec-MmLC-k	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGCAGCATCAGCCCG
5	Bovine IgG1	pNeoSec-BovFc-IgG1	Kpn1 to Pst1	TGGGTTGCGTAGCT	GCTGTGGTGGAGGC
6	Bovine IgG2	pNeoSec-BovFc-IgG2	Kpn1 to Pst1	TGGGTTGCGTAGCT	GATGCCAGAGGGTAG
7	Bovine IgG3	pNeoSec-BovFc-IgG3	Kpn1 to Pst1	TGGGTTGCGTAGCT	GAACTCAGAGGGTAG
8	Bovine LC-λ	pNeoSec-BovLC-λ	Kpn1 to Pst1	TGGGTTGCGTAGCT	GGACTTGGGCTGACC
9	Chicken IgY	pNeoSec-GgY	Kpn1 to Pst1	TGGGTTGCGTAGCT	GCGATGTGGGGCTCGC
10	Chicken LC-λ	pNeoSec-GgLC-λ	Kpn1 to Pst1	TGGGTTGCGTAGCT	GGCCACCTTGGGCTG
11	Swine LC-k	pNeoSec-SsLC-k	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGGCTTGGCATCAGC
12	Swine LC-λ	pNeoSec-SsLC-λ	Kpn1 to Pst1	TGGGTTGCGTAGCT	AGCGGCCTTGGGCTG
13	Swine IgG1	pNeoSec-SsFc-IgG1	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGGGGCCGTCTTGGG
14	Swine IgG3	pNeoSec-SsFc-IgG3	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGGAGCTGTGTTGTA
15	Swine IgG5.1	pNeoSec-SsFc-IgG5.1	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGGGGCCGTCTTGGG
16	Swine IgG5.2	pNeoSec-SsFc-IgG5.2	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGGGGCCGTCTTGGG
17	Swine IgG6.1	pNeoSec-SsFc-IgG6.1	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGGGGCCGTCTTGGG
18	Swine IgG6.2	pNeoSec-SsFc-IgG6.2	Kpn1 to Pst1	TGGGTTGCGTAGCT	TGGGGCCGTCTTGGG
19	Mouse IgG1 Fab	pNeoSec-MmFc IgG Fab	Kpn1 to Pst1	TGGGTTGCGTAGCT	GGGTGTCGTTTTGGC

Completed

Work in progress

- Mouse IgG1, IgG2a, IgG2b and Mouse LC-k used for cloning last month
- Bovine, chicken and pig vectors yet to be tested

List of hybridomas for sequencing

		Antibody	Other names	Isotype	Antigen
1	Chicken	1 AV 91	BF3	IgG1	Chicken CTLA-4
2		2 AV71	AF12	IgG1	Chicken CD40L
3		3 AV29	EG1	IgG2b	Chicken CD4
4		4 AV36	IE11	IgG1	Chicken CD3
5		5 AV7	AV7	IgG1	Chicken CD28
6		6 AV14	IB8	IgG2b	Chicken CD8a
7		7 AV37	AD4a	IgG2a	Chicken CD30
8		8 AV82	DC7	IgG2a	Chicken CD80
9		10 AV20	FE6	IgG1	Chicken Bu1 CHB6
10		11 2C10/D2	PD1 1.1	IgG1	Chicken PD1
11	Bovine	14 CC58	DA3	IgG1	Bovine CD8
12		15 CC-G33	GF3	IgG1	Bovine CD14
13		16 CC-108	CD4a	IgG1	Bovine MHCII
14		17 CC39	EB3	IgG1	Bovine WC1
15		18 CC15	BB1	IgG2a	Bovine WC1
16		20 CC219	EC4	IgG1	Bovine CD28
17		Porcine	22 PPT23	FY2P1C5	IgG1
18	23 PPT3		CD3	IgG1	Porcine CD3
19	25 PPT20		95FB4E11	IgG1	Porcine CD8.1aa
20	Viruses	27 FD7	FD7	IgG1	MDV Meq
21		28 IB11	IB11	IgG2a	FMDV
22		29 BD1	BD1	IgG2a	MDV pp38
23		36 CG12	CG12	IgG2a	Flu HA
24		38 JF8	JF8	IgG2a	Flu HA

Ongoing work

- Making porcine and Fab expression vectors
- Developing protocols for class switching (IgG3 and IgM to IgG1) as there is no need for IgM and IgG3 expression system

IgG3

CTGGTCACTGTCTCTGCCGCTACAACAACAGCC

L V T V S A A T T T A

IgG1(c)

GCCAAAACGACACCC

A K T T P

IgG3 to IgG1 class switch primer

CTGGTCACTGTCTCTGCCGCCAAAACGACACCC

L V T V S A A K T T P

- Developing protocols for isotype switching IgG1 to IgG2a/b for functional studies
- Developing protocols for species switching (start with porcine)
- Catalogue current stocks and clone/sequence of more hybridomas

New toolbox website

Leveraged money from the BBSRC Tools and Resources Fund (GCRF).

Basic design of a simple relational database backend that allows complex searches from the front end.

Should be easy for multiple curators to add and edit content, with a developer site and a live site- **link to Joan Lunney's species toolbox groups.**

This is not just about antibodies, all the way from genome variation through to PCR, isoforms, assays, expression etc

New toolbox website- Progress



- Likely bases around antigen/protein
- Database structure behind a web interface
- All fields searchable and linked across each field
- Capacity to have a 'star' rating for quality and/or validation
- Access agreement for some data based on an MTA model- no cost for academia- work ongoing
- Need to add in reagents for pathogens as well as host
- Species 'volunteers' to help curate have been identified
- Previous toolbox data considered high quality and the first data to translate into the new database
- First design will be pushed out for comment

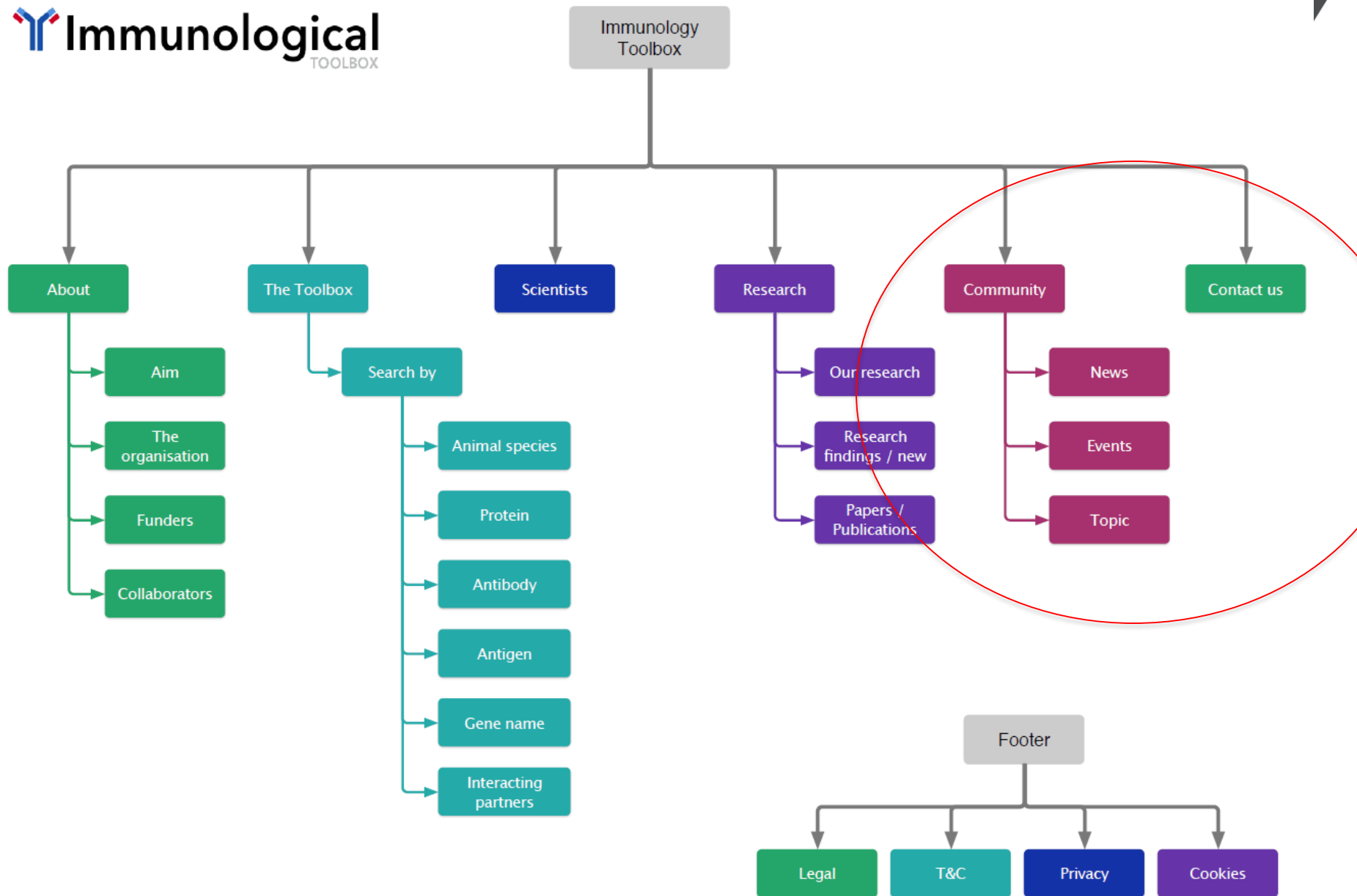
New toolbox website- key data fields



Protein	Epitope (can be multiple for the same protein)	Antibody
name	location on protein	Antibody sequence
animal species	isoform location	name
Expression patterns	structural confirmation	monoclonal/polyclonal
alternative names	protein sequence (if applicable)	subclass
Gene name	post translational modifications	species raised in
interacting partners		antigen raised against
functions		FACS plots
isoforms		blocking antibody?
		utility (FACS, ELISA, IH etc)
Avialable tools		
recombinant expression vectors		Avialable tools
recombinantly expressed antigens		DNA sequence
		Gene blocks
		transfection protocols
		staining protocols
		hybridomas
		supernatents

Capability to add pathogens into the database

Protein Sequence	cDNA sequence	Genomic DNA sequence
AA sequence	splice variants	DNA sequence
post translational modifications	exon/exon boundaries	location (genome)
splice variants		size
exon/intron boundaries	Potentially avialable tools	copy number variations
	siRNA	Single nucleotide polymorphisms
	primers	breed/line
	DNA probes	non-coding single nuc polymorphisms
	protocols	promoter sequences
		Avialable tools
		Single nucleotide polymorphism positions
		primers
		probes
		knock out phenotypes
		transgenic cassettes
		www.pirbright.ac.uk





Immunological Toolbox
Jayne Hope 22.05.18



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Immunological Toolbox - RI

- From 2017-2022 Core Capability Grant – funds 1 x FTE (2 posts)
- Fits within Institute Strategic Programme 2: Control of Infectious Diseases
 - Theme 3: Host responses underlying immunity
- Coordinated with the Pirbright Institute ISP
 - **New reagent and assay development at RI**

Immunological Toolbox - RI

Priorities will be guided by requirements of the veterinary immunology community but include:

- development of tools to study macrophage and **T cell** development and function;
- reagents and methods to dissect antibody responses of animals at the single **B cell** level to define the Ig repertoire and retrieve desirable antibody specificities;
- recombinant cytokines and chemokines;
- immunoassay development e.g. multiplex platforms for detection of cytokines

- Livestock species (ruminants, pigs, chickens)

- (pathogen specific reagents e.g. against FMDV antigens)

Immunological Toolbox – Defining Priorities

- Joint steering committee to define priorities based on requests and **input from network/community**
 - Steering committee members from RI (Eleanor Riley, Jayne Hope, Mark Stevens) and Pirbright (John Hammond, Simon Graham) plus external independent member (Gary Entrican)
 - Reagent request forms detailing requirements, community need, funding, commercialisation/distribution plans
 - Costed on an individual basis
 - New mAbs will be sequenced and all reagent information entered onto the database
 - (publications)



Immunological Toolbox

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Expanding the veterinary reagent portfolio



IMMUNOLOGICAL TOOLBOX

- Gene Cloning
- Recombinant Protein Production
- Monoclonal Antibody Production
- Conjugation service
- Assay Development
- Purification Service

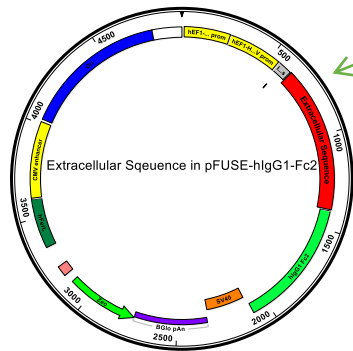
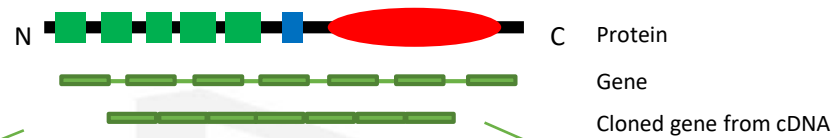
Expanding the veterinary reagent portfolio through the production and characterisation of desirable reagents.

- Services offered:
- Production of Recombinant Proteins
 - Immunogens for antibody production, growth factors, use within *in vivo* studies or as standalone reagents.
- Production of Monoclonal Antibodies
 - The immunogen can either be provided by the client or generated by the Toolbox. Screening can either be carried out by the client or by the facility. Screening reagents such as cell lines can also be produced if required. The Toolbox also has a library of cell and tissue samples from a range of veterinary species which is available for screening.
- Assay Development
 - Such as flow cytometry, bioimaging or ELISA.
- Conjugation service
 - The labelling of antibodies, recombinant proteins and growth factors (examples of available tags are: biotin, HRP, fluorescent conjugates such as FITC, PE, Alexa647, Alexa488).

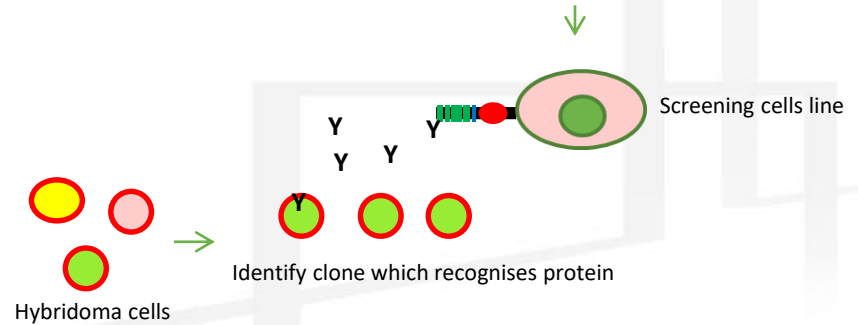
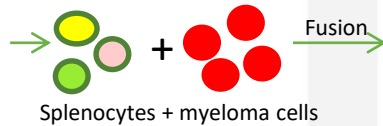
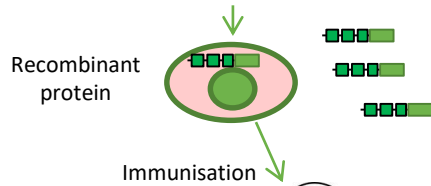
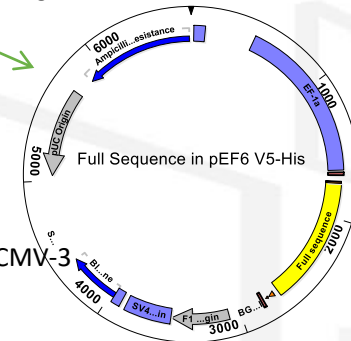
Work Flow – Monoclonal Antibody Production

Example: bovine CD107a (LAMP1)

- Marker Lysosomal Compartments
- Degranulation of CD8⁺ T cells + NK cells
- Collaboration with Tim Connelley

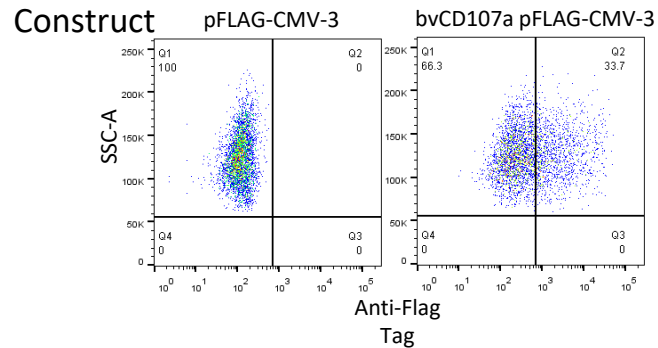


Screening plasmid
Tagged Functional protein.
Example: bvCD107a pFLAG-CMV-3

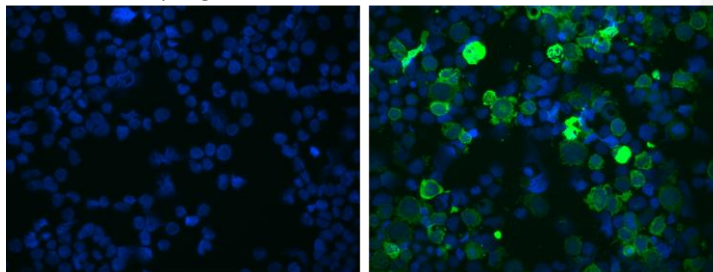


Screening construct and recombinant protein

Screening



Antigen Production

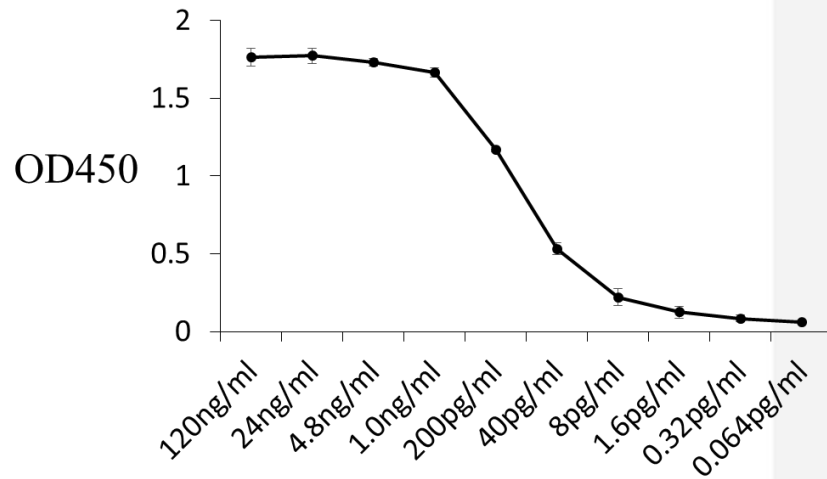


■ Anti-IgG2b-Alexa488
■ DAPI

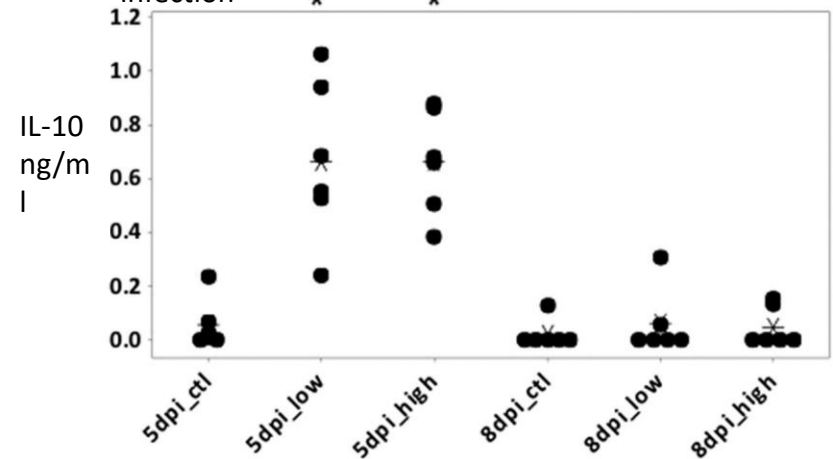
- CD107a (LAMP1)
 - Marker of lysosomal compartments
 - Marker of degranulation in NK and CD8⁺ T cells
- Screening construct
 - bvCD107a CHO cells
 - Full sequence bvCD107a
 - N terminal Flag tag
- Antigen
 - bvCD107a-mIgG2b Fc in HEK 293T cells
 - Recombinant bvCD107a
 - mIgG2b Fc tag

- Generation of chicken IL-10 ELISA
 - IL-10 is an anti-inflammatory cytokine which regulates nature and extend of inflammatory respond during infection
 - IL-10 antibodies and assay developed by Zhiguang Wu
 - Measured the expression of IL-10 in birds infected with *Eimeria tenella*

Capture ELISA assay to detect chicken IL-10

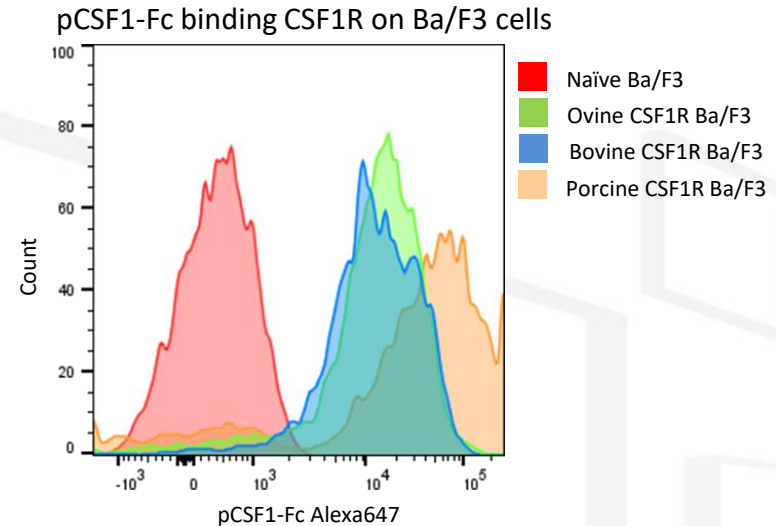


Detection of circulating IL-10 after *E. tenella* infection



Wu et al, 2016, Comp. Dev. Immunol

- Novel Application of recombinant protein
 - Recombinant Porcine CSF1-Fc (generated by Pfizer)
 - Conjugated with fluorescent tag (Alexa647)
- CSF1R targeted using the recombinant protein
 - Validated using CSF1R expressing Ba/F3 cells lines
 - Cell lines generated in house as screening tools



Acknowledgments



Toolbox Members (Roslin)

Anna Raper
Lindsey Waddell

Toolbox Steering Committee

Roslin Institute

Jayne Hope
Eleanor Riley

Pirbright

Simon Graham
John Hammond

Moredun Institute

Gary Entrican

Collaborators

Roslin Institute

Tim Connelley
Ruben Barroso

Zhiguang Wu

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QMRI

Clare Pridans

Mater Research Institute-UQ

David Hume

University of Massachusetts

Deborah Frenkel

Funding



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Immunological Toolbox - opportunities

- Project submission form available on IVVN website soon
- Request for information on reagent validation/testing/cross-reactivity/utility etc for entry into database
- Discussion groups: supported by IUIS-VIC

Pig – Joan Lunney/Wilhelm Gerner

Ruminant – Jayne Hope

Chicken – Bernd Kaspers

Fish – Carolina Tafalla-Pineiro

- International Society for Developmental and Comparative Immunology meeting (June 2018); Avian Immunology Research Group meeting (October 2018), European Veterinary Immunology Workshop (September 2018)
- Vetimm list (<https://list.umass.edu/mailman/listinfo/vetimm>)