Swine Toolkit plans and progress for US Veterinary Immune Reagent Network

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Abstract

The US Veterinary Immune Reagent Network (VIRN) (http://www.umass.edu/vetimm/) was established to address the lack of sufficient immunological reagents specific for ruminants, swine, poultry, equine and aquaculture species with a goal of 20 reagents per species group. As a result of this US Network plans are underway to produce sets of reagents: recombinant cytokines and chemokines; monoclonal antibodies (mAb) to them and their receptors; and mAb that identify the major leukocyte subsets, CD antigens, T cell receptor (TCR) and Toll-like receptors (TLR). These are needed to evaluate changes during disease and following vaccination. For the US Swine Toolkit efforts our first focus was on developing a list of available swine reagents so that our priority list for new reagents could be developed. A priority list and development plan for swine has been developed (and is regularly updated) based on 1) importance for swine immune studies; 2) priority for other toolkit efforts ; 3) availability of swine sequence information; and 4) likelihood of developing reagent. Since many swine cytokine and CD reagents are available the priority focused on anti-TCRαβ, chemokines and their receptors, efforts are underway to produce bioactive IFNA, IL7, IL15 and IL13 and relevant mAb. Because no anti-CD45RO could be produced from traditional efforts a peptide immunization protocol is being tested. Before making anti-TLRs efforts are underway to test for cross reaction of known anti-human reagents. Our goal is to produce reagents that function in ELISA, ELISpot and flow cytometric applications as well as in fixed tissue sections. Products developed in this proposal will be openly available to collaborators and be made commercially available using non-exclusive licenses. We expect our efforts to benefit a large group of researchers including veterinary immunologists, pathologists and microbiologists and scientists using the swine as a biomedical model for humans.
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U.S. Veterinary Immune Reagent Network Team

Overall Background: This is a multi-species immune reagent grant from USDA CSREES for development of a US Veterinary Immunological Reagents Network, which will support immunological reagents specific for ruminants, swine, poultry, equine and aquaculture species to advance veterinary immunology and disease control.

Swine Plans: The emphases for swine will be on developing and characterizing bioactive immune proteins, cloned cytokine and chemokine proteins, as well as monoclonal antibodies (mAbs) to these proteins and their receptors. Additional mAb will be produced to swine cell subset proteins, the CD antigens, and toll-like receptor (TLR) proteins. Examples of our current approaches for each type of reagent is given on this poster as well as the current priority list. A separate anti-Ig effort for swine is underway with Drs. Butler and Muyldermans (see their poster).

Expected Applications: These reagents will be used to: (1) evaluate changes during disease and following vaccination and (2) give scientists the ability to manipulate these cell populations to evaluate their roles in protective immunity as well as in immunopathology.

Swine T Cell Receptors (TCRs)
Example of strategy for expression and mAb production

1) Bioinformatics (BARC).
The TCRs consist of an alpha and beta chain with variable and constant regions, hinge, transmembrane region and cytosolic tail. For our expression and hybridoma production we are targeting the constant region, the hinge and the area before the transmembrane region. The hinge involves a cysteine disulfide link. The strategy is to produce mAb only to either the alpha or the beta chain constant region, and not the conformational complex (which would require difficult expression strategies). Originally a clone was requested from Saalmuller and Uenishi. Fortunately sequence information for swine TCR alpha/beta molecules was available; there were some sequence differences probably due to allelic differences. Primers were designed in collaboration with Dr. D Zarlenga at BARC.

2) **cDNA preparation** (BARC)**:** cDNA was produced from pooled immune cDNAs generated from control and infected minipigs.
3) **PCR product production** (UMass)**:** PCR product produced, cloned and sequence validated.
4) **Mammalian expression (Cornell):** Reamplification with restriction sites, insertion into equine IgG1 heavy chain expression vector, stable transfection and clone selection; followed by protein production and purification.

Final Swine TCR sequence after reamplification at Cornell:

```
Swine TCR alpha
       1 atcaagaacc ctgaccccgc cgtgtaccag ctgaaaggcc ccaaatctaa caacatcagt
      61 gtatgcctat acactgattt tcaaatgaat acaacaaaag actcggagcc cggcgtgttc
     121 agcttgagca ggactgtgtt caactcaaac acagctgtgc tagacatggg ggcctttggt
     181 tccaagagca acgggctcgt ggcttggagc aaaagcaccg attttgaatg tcaaagcacc
     241 ttccagcagg aattctatcc taactcagga atttcctgtg atgccaagtt ggtagagaaa
     301 agctttgaaa cggatatgaa cctcaacctc caaaacctgt cagtgatggg gttccgcatc
     361 atcctcctga aaatggttgg gtttaacctg ctcatgacac tgcggctgtg gtccagctga

Swine TCR Beta
       1 gaggacctgc agcaggtgag accacccaag gtggccgtgt ttgaaccatc ggaagcggag
      61 atctcccgga cccagaaggc cactctcgtg tgcctggcca caggcttcta ccccgaccac
     121 gtggagctga gctggtgggt gaacgggaag caggtccaga gcggggtcag cacggacctt
     181 cagccctaca gggaggaccc cagccgcaat gactccagct actgtctgag cagccggctg
     241 agggtcaccg ctttcttctg gcacaacccc cgcaaccact tccgctgcca agtccagttc
     301 tatgggctca cggaggacga cgagtgggag tacaactgga ccaagcccat cacccagaac
     361 atcagtgcgg aggcctgggg cagagcagac tgtggcttca gctctgcgtc ctatcagcaa
     421 ggggtcctgt ctgccaccct cctctatgag atcctgctgg ggaaggccac cctgtacgct
     481 gtgctggtca gcgccctggt gctgatggcc acggtaaaga aaaaggattc ctgagaccag
```

The purple is the coding region for the constant region that will be inserted into the mammalian expression vector. The turquoise box is the cystine for the disulfide link at the hinge. The green is the start of the transmembrane region. The green boxes are nucleotide differences from the Japanese clones. The black is the cytosolic area. Cornell forward and reverse primer sites are indicated by italics.

5) **Hybridoma production** (UMass). Planned.
6) **Screening** (UMass and BARC): The initial screen will be run at UMass against the recombinant protein. The functional test will be at BARC against pig T cells by flow cytometry.

Members of the U.S. Veterinary Immune Reagent Network Team (In order from left to right):
Dr. Joan Lunney (USDA ARS BARC; swine), Dr. Peter Johnson (USDA CSREES), Dr. Bettina Wagner (Cornell; equine and expression), Dr. Cyril Gay (USDA ARS), Dr. Cynthia Baldwin (Univ. Massachusetts; PI, bovine, hybridomas), Dr. Calvin Keeler (Univ. Delaware; chicken), Joanna LaBresh (Kingfisher; expression), Dr. David Horohov (Univ. Kentucky, equine), Dr. Norman Miller (Univ. Mississippi; catfish), Dr. John Hansen (USGS, trout).

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Summary of Current Swine VIRN Toolkit Plans for reagent Development

	Type Reagent	Common Name	Gene Symbol	Swine Unigene clone	U Mass - Clone and Sequence	Protein expression	mAb Production	Planned BioAssay
1	cell surface	TCR alpha	TRA@	L21158	U completed	C in process		FCM
2	cell surface	TCR beta	TRB@	AB079521	U completed	C in process		FCM
3	cell surface	CD45RO	PTPRC	AY444871	peptide*	N/A	7/16/2007	CD45 cell based ELISA
4	cell surface	CD213A1	IL13Ra1	AY266142	plasmid**	C in process		FCM
5	cell surface	CD124	IL4Ra	AY266143	plasmid**	C in process		FCM
6	cytokine	IL-13	IL13	NM_213803	U completed	K in process		IL-6 production
7	cytokine	IL-15	IL15	U58142	U completed	K in process		CTLL-2
8	cytokine	IL-7	IL 7	AB035380	U completed	K in process		T cell proliferation
9	cytokine	CTLA-8	IL17	AB040441	U completed	K in process		IL-6 production
10	chemokine	MCP-1	CCL2	X79416	U completed	K in process		Chemotaxis
11	chemokine	IP-10	CXCL10	AY789646	U completed	K in process		Chemotaxis
12	chemokine	MIP-1a	CCL3L1	AY643423	U completed	K in process		Chemotaxis
13	chemokine	RANTES	CCL5	AJ583704	U completed	K in process		Chemotaxis
14	chemokine	MIG	CXCL9	BP169836	U completed	K in process		Chemotaxis
15	chemokine	IP-9	CXCL11	BX914688	B in process			Chemotaxis
16	cytokine	IFNB	IFNB1	AY687281	B in process			antiviral activity
17	cytokine	IFNa	IFN1@	AY345969	U completed	K in process		antiviral activity
18	cell surface	CD197	CCR7	AB090356	B in process	N/A		FCM
19	cell surface	CD183 &182	CXCR3	AJ851240	B in process	N/A		FCM
20	cell surface	CD127	IL7Ra	BP157102	B in process	N/A		FCM
21	cell surface	CD101	IGSF2		B in process	N/A		FCM

B = BARC in silico gene discovery; cDNA preparation, primer design for U Mass
U = U Mass cloning into appropriate vector; confirmatory sequencing
C = Cornell mammilian stable expression with equine IgG or IL4 expression vectors
K = Kingfisher *Pichia* expression
* CD45RO peptide designed with, and transfected cells for ELISA provided by, W Schnitzlein and F Zuckerman-U IL
** Plasmid provided by D Zarlenga, BARC
FCM = Flow cytometry on T cells/PBMCs

Plans for producing mAb to swine CD45RO

Background: anti Swine-CD45 mAb
During the Third International Swine CD Workshop CD45 mAb were screened. A pan CD45 (K252.1E4) had been assigned. The result was seven mAbs (PG77A, PG96A, PG167A, PGB78A, 3C/9, MIL13, NHT 101) recognized the portion of the CD45 molecule encoded by the A exon (CD45RA), while one (MIL15, and later a 2nd MIL5) was specific for that portion encoded by the C exon (CD45RC). Zuckermann FA, Schnitzlein WM, Thacker E, Sinkora J, Haverson K. 2001. Veterinary Immunology and Immunopathology 80: 165-74. These results were based on work with stable transformed CHO adherent cell line produced that expresses CD45RO,CD45RC,CD45RAC and CD45RA. The technique was published by W. Schnitzlein and F. Zuckermann (1998) Veterinary Immunology and Immunopathology 60 389-401.

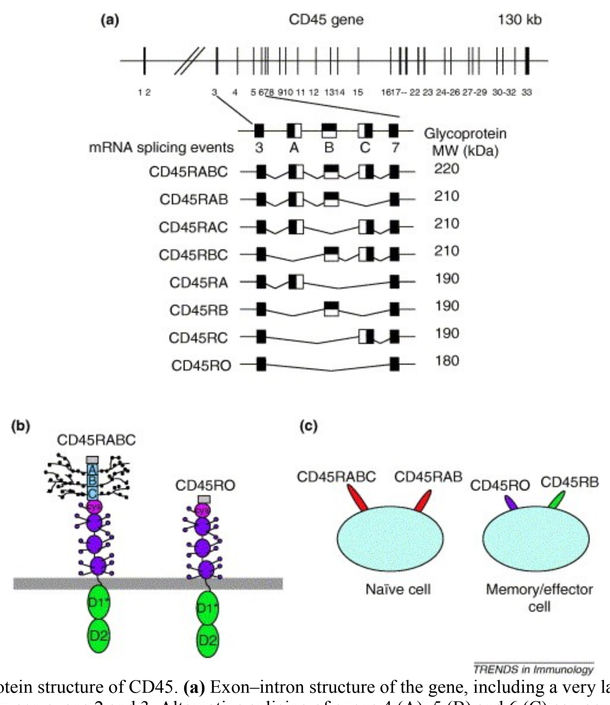
It should be noted that **no mAb recognizing CD45RO, the memory associated CD45 isoform**, were detected. Thus a collaborative effort is underway to use peptide immunizations to derive CD45RO specific mAb. Note the official gene symbol for CD45 is PTPRC and Name: protein tyrosine phosphatase, receptor type, C [***Homo sapiens***]

Immunization strategy:
Plan: Not to use very much of the 24 aa antigenic portion of the CD45R0 protein that is common to other splice forms. Aim for the beginning of the mature CD45R0protein N-terminal. Couple peptide to BSA and immunize mice for hybridoma production.

CHO cells
Stable transformed CHO adherent cell line were produced that expresses CD45RO,CD45RC,CD45RAC and CD45RA with cDNA ligated into pcDNA3 (Invitrogen) and selected for Geneticin (Invitrogen)-resistance; single cell clones were then screened for reactivity to common and restricted anti-CD45 antibodies. Parental CHO cell (CHO), CHO cells expressing either porcine CD45RO (CD5), CD45RA (CD505), or CD45RC (CD516) were grown under a reduced concentration of Genticin selection pressure to prevent them from reverting to original parental non expression. We have all 5 cell lines including the parental line as a non specific control.

Cell ELISA for CD45 Reactivity.
1. Cells are grown to semi-confluent monolayers of each cell type in 96 well plates [I X 10(5) cells/ml, 200ul per well; 24 hour). Media is removed.
2. 50ul of each test or control antibody diluted in PBS pH7 with 5% FCS (dilution buffer) is added for 1 hour at 37. Wash 1 X with dilution buffer at 37 for 5‘ and then 2 X at RT.
3. 50ul of goat anti mouse IgG H&L HRP labeled added, plates rocked at 37 for 1 hour. Same wash as above.
4. 100ul of Sure blue (KPL) is added and left at RT until color develops.

Gene and protein structure of CD45 in mammals.



Gene and protein structure of CD45. **(a)** Exon–intron structure of the gene, including a very large intron (~50 kb) between exons 2 and 3. Alternative splicing of exons 4 (A), 5 (B) and 6 (C) can generate eight isoforms. **(b)** The highest (CD45RABC) and lowest (CD45RO) MW isoforms are shown. Anti-CD45R (restricted) antibodies to the A, B and C exons (blue) detect any isoform containing the respective exon, whereas anti-CD45RO detects an isoform lacking the A, B and C exons. All isoforms have a common extracellular part derived from exons 7–15 that encodes a cysteine rich domains (cys, pink) and three fibronectin-like domains (purple), a transmembrane segment (exon 16) and intracytoplasmic phosphatase domains (green; D1* catalytically active and D2 inactive). O-glycosylation of the A, B and C exons (solid dark lines with filled circles) and N-glycosylation of the fibronectin-like domains (short lines and purple circles) are shown. **(c)** As well as detecting specific isoforms, anti-CD45R antibodies identify subsets of leucocytes. For example, anti-CD45RA antibodies identify naïve T cells expressing high MW isoforms (CD45RABC and CD45RAB), whereas anti-CD45RO antibodies identify memory cells expressing low MW (CD45RO and CD45RB) isoforms.

Tchilian, Beverley. 2006. Trends Immunol. 27:146-53.

Swine Chemokine
Example of strategy for expression, bioassay and mAb production

Swine CCL2 (MCP-1: monocyte chemoattractant protein-1)

1) Bioinformatics and cDNA prepared (BARC).
Swine sequence information available in Unigene under NCBI’s Entrez, www.ncbi.nlm.nih.gov/sites/entrez?db=unigene. Cloned in 1994 it has been given a PROVISIONAL REFSEQ status. Polyclonal antibody has been used successfully for immunostaining for this molecule. Homology with human is 80%, 23 AA signal sequence that is post translational cleaved by a protease. Apparently the Q that is left on the peptide and its modifications are important in the rat for various functions. The human, mouse and rat have all been successfully produced in E. coli. The bioassay works with varying degrees of success. The bacterial product works just as well as the mammalian when you take differences in weight into consideration. The Human and Rat native molecule has significant O-linked carbohydrates and sialic acid residues.

CCL2 nucleotide (CB475628 Plum Island clone:NM_214214 is Swine Unigene clone)

```
  1 cccgaccagg acccatcagc tcccacaccg aagcttgaat cctcatcctc cagcatgaag
 61 gtctctgcag ccctcctgtg cctgctgctc actgcagcca ccttctgcac ccaggtcctt
121 gcccagccag atgcaattaa ttctccagtc acctgctgct atacacttac cagtaagaag
181 atctcgatgc agcggctgat gagctacaga agagtcacca gcagcaagtg tcctaaagaa
241 gcagtgatct tcaagaccat cgcgggcaag gagatctgtg cagaacccaa gcagaagtgg
301 gtccaggact ccataagcca cctggacaag aaaaaccaaa ctccgaagcc ttgaagcctg
361 actccacccc cccgagaatc tgaagacaat tcatttcctt ctaacttgcc ctaaataccc
421 tcagatatta ttttattata attttaaaga gtatgaactt tattgatacg aaacatgatg
481 ccttaagtaa cattaatctt atttaagtta ttgatgtttg aagatcctcc atgactactg
541 gtgtttttatt tttaaacgga gacttgggca cattgctttc ctctgtgaat ctcagtcctc
601 cccctgggat gacggaaggg tccttgcaag gatggtgaac acaaaaactt tctgttttaa
661 attctaagcc attgttaaaa atgata
```

NM_214214
MKVSAALLCLLLTAATFCTQVLAQPDAINSPVTCCYTLTSKKISMQRLMSYRRV
TSSKCPKEAVIFKTIAGKEICAEPKQKWVQDSISHLDKKNQTPKP

The beginning of the sequence, at the end of the signal sequence, is in purple on both the nucleotide and the protein sequences. This removes the signal peptide that would normally be cleaved off in a mammalian system. The normal start and stop sites for the coding region are noted by the grey boxes. The underlined area is targeted for expression.

2) **cDNA preparation** (BARC)**:** cDNA was produced from pooled immune cDNAs generated from control and infected minipigs.
3) **PCR product production** (UMass)**:** Primer design, PCR product production, sequence validation, and reamplification with the same primers plus restriction sites for the yeast vector.
4) **Yeast expression** (Kingfisher)**:** Digestion and insertion into yeast *Pichia* vector, transformation, clone selection, and culture production and protein purification on HPLC for 20 mg final product. In process.
5) **Bioassay** (BARC) – Planned: chemotaxis assay with 2 day cultured monocytes.
6) **Hybridoma production** (UMass). Planned
7) **Screening** of hybridoma supernatants (UMass; BARC). Planned ELISA at UMass with recombinant protein and functional assays at BARC.

Chemokine Motifs

Intercrine chemokine family (CXC and CC chemokines).

Many low-molecular weight factors secreted by cells including fibroblasts, macrophages and endothelial cells, in response to a variety of stimuli such as growth factors, interferons, viral transformation and bacterial products, are structurally related. Most members of this family of proteins seem to have mitogenic, chemotactic or inflammatory activities. These small cytokines are also called intercrines or chemokines. They are cationic proteins of 70 to 100 amino acid residues that share four conserved cysteine residues involved in two disulphide bonds, as shown in the following schematic representation:

```
                    +-----------------------------------+
                    |                                   |
xxxxxxxxxxxxxxxxxxxxxxCxCxxxxxxxxxxxxxxxxxxxxxxxxCxxxxxxxxxxxxxCxxxxx
                       |                         |
                       +-------------------------+
```

'C': conserved cysteine involved in a disulphide bond (----).

These proteins can be sorted into two groups based on the spacing of the two amino-terminal cysteines. In the first group, the two cysteines are separated by a single residue (C-x-C), while in the second group, they are adjacent (C-C).

NCBI’s Blast alignment of pig CCL2 versus human CCL2 and pig CCL2versus Bovine CCL2

Pig(NM 214214) versus Bovine NM 174006.2 Protein level

```
Score =  147 bits (371),  Expect = 2e-34
 Identities = 84/99 (84%), Positives = 91/99 (91%), Gaps = 0/99 (0%)

Query  1   MKVSAALLCLLLTAATFCTQVLAQPDAINSPVTCCYTLTSKKISMQRLMSYRRVTSSKCP  60
           MKVSAALLCLLLT A F T+VLAQPDAINS V CCYT  SKKISMQRLM+YRRVTSSKCP
Sbjct  1   MKVSAALLCLLLTVAAFSTEVLAQPDAINSQVACCYTFNSKKISMQRLMNYRRVTSSKCP  60

Query  61  KEAVIFKTIAGKEICAEPKQKWVQDSISHLDKKNQTPKP  99
           KEAVIFKTI GKE+CA+PKQKWVQDSI++L+KKNQTPKP
Sbjct  61  KEAVIFKTILGKELCADPKQKWVQDSINYLNKKNQTPKP  99
```

Pig(NM_214214) versus Human NM_002982.3 Protien level

```
Score =  139 bits (350),  Expect = 5e-32
 Identities = 79/98 (80%), Positives = 87/98 (88%), Gaps = 0/98 (0%)

Query  1   MKVSAALLCLLLTAATFCTQVLAQPDAINSPVTCCYTLTSKKISMQRLMSYRRVTSSKCP  60
           MKVSAALLCLLL AATF  Q LAQPDAIN+PVTCCY  T++KIS+QRL SYRR+TSSKCP
Sbjct  1   MKVSAALLCLLLIAATFIPQGLAQPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCP  60

Query  61  KEAVIFKTIAGKEICAEPKQKWVQDSISHLDKKNQTPK  98
           KEAVIFKTI  KEICA+PKQKWVQDS+ HLDK+ QTPK
Sbjct  61  KEAVIFKTIVAKEICADPKQKWVQDSMDHLDKQTQTPK  98
```