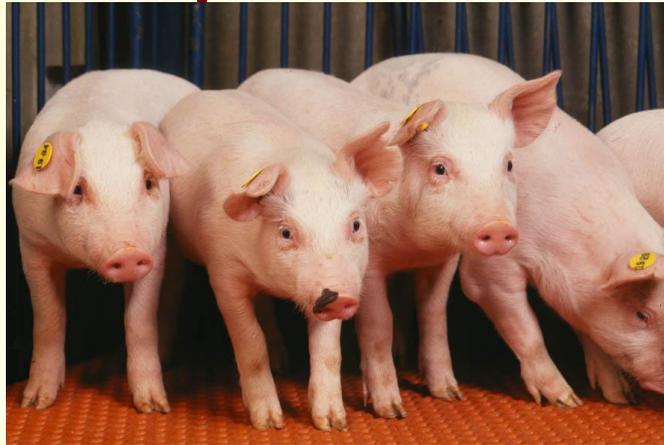


Biotherapeutics as alternatives to antibiotics: Effects of IFN α and G-CSF on innate and adaptive immunity in swine



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Antibiotic usage in food animals

- Therapeutic- treatment of diseased animal
- Prophylactic- disease prevention at times of stress when infection rates rise, such as shipping, weaning
- Metaphylactic- therapeutic and prophylactic herd use in face of an outbreak
- Growth promotion - accelerate growth of healthy animals
- **Regardless of personal opinions:**
 - Increasing concern regarding possible negative consequences
 - Increased regulation - likely to lead to ban of some antibiotics
- **Examination of alternatives to conventional antibiotic usage is warranted.**
 - Goal - improve animal health and production efficiency

What are the alternatives?

- **Healthy gut = happy pig**
 - Most proposed alternatives aimed at enhancing health of the gut microbiome
 - Prebiotics
 - Probiotics
 - Enzymes
 - Bacteriophages - more for treatment of specific disease
- **Immune modulation?**
 - Let the host immune system do the work...



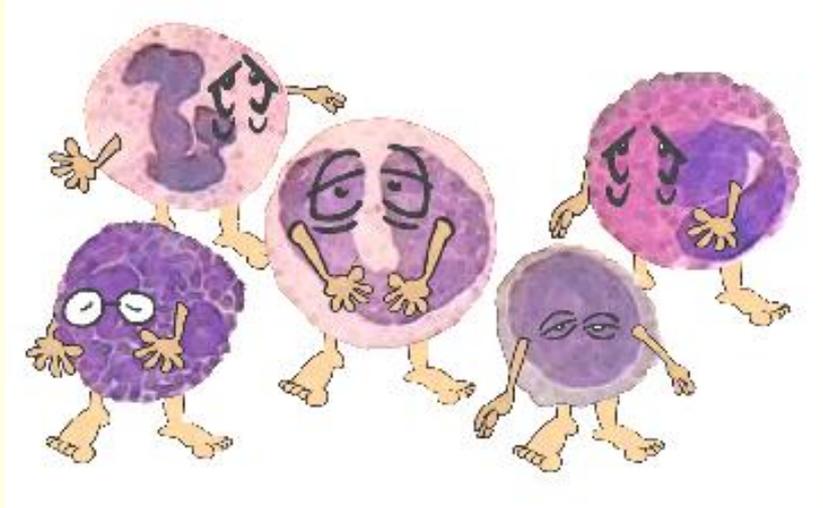
Biotherapeutics

Use of biological response modifiers for treatment to stimulate or restore the ability of the immune system to fight infection and disease.

- Immune specific mechanism instead of direct antibacterial effect
- Potential candidates - endogenous cytokines
 - IFN- α , G-CSF
- Work to restore dysfunctional or impaired immune capacity
 - Otherwise, potential for immunopathology
 - More is not always better
- Times of immune dysfunction...

Times of immune dysfunction...

- “Stress” - environmental, physiologic, disease induced
 - Periparturient/neonatal
 - Weaning
 - Transport
 - Disease - (2° infection)



- Often times of increased exposure with mixing of animals

Key points

- Financial return for producer and industry
- Ease of use and practical to implement
- Defined time period for management use
 - Critical periods of peak disease incidence include the neonatal period, weaning and transportation.
- Goal is to enhance disease resistance
- Well-designed, appropriately delivered immune modulators may work well.

Immune suppression  Normal immune function

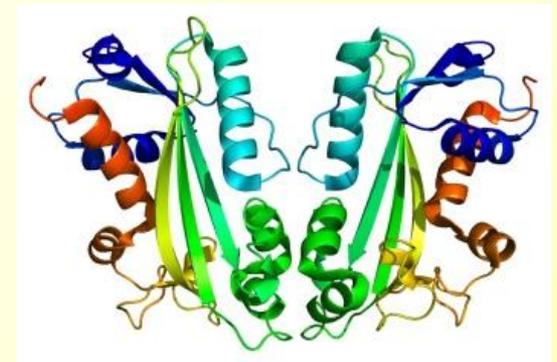
Pathogen Success

Immunity Success



Cytokines as modulators

- **Cytokines offer advantages**
 - Immune specific method of action
 - Food safety advantage to being a digestible protein
 - Should reduce therapeutic antibiotic usage
 - Should improve performance by reducing disease
 - Safety - metabolized by the same pathways as the natural protein
- **Delivery**
 - Route
 - Recombinant protein - Short half life
 - PEGylated, mutated
 - Adenovirus vector
- **Can we prevent disease by delivering immune modulating cytokines at specific times in production cycle?**

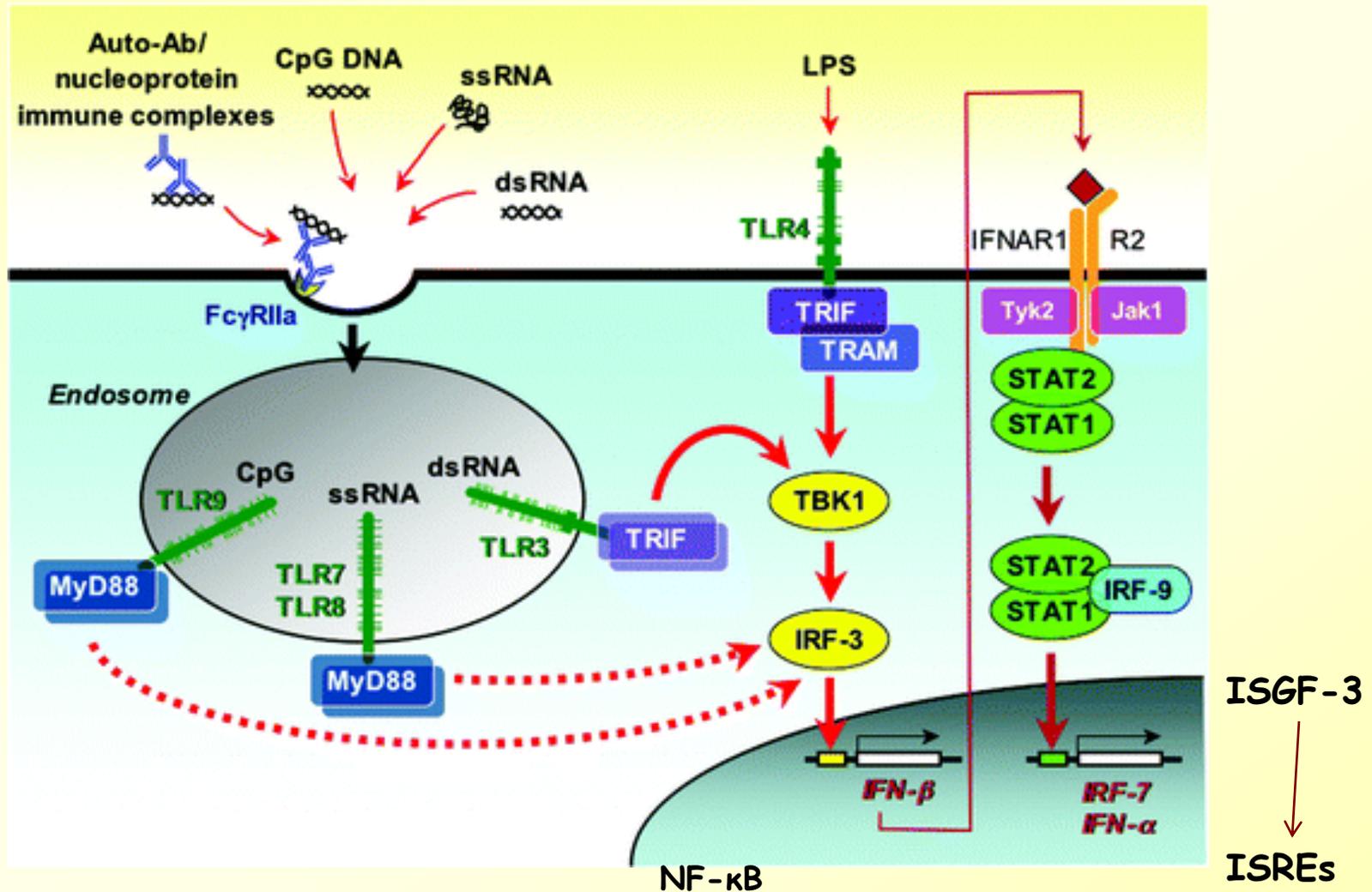


Type I IFN

- **Type I interferons**, such as IFN α and IFN β , have an important role in the innate and adaptive immune response.
- **Innate antiviral (PKR, Mx)**
- **Role in adaptive**
 - Incite NK cell activity
 - Induce the maturation of DC into Ag presenting cells
 - Induce macrophage development and maturation
 - With IL-6 promote B cell differentiation to plasma cells
- **Viruses immunosuppress host - secondary bacterial infections**
- **Therapeutic use of Type I interferons**
 - Hepatitis B and C, MS, cancers (melanoma, leukemia)



TLR induction of type I IFN



Adenovirus vectors

- Recombinant, replication-defective human adenovirus type 5 (Ad5)
- Allows animals to produce IFN α endogenously for a period of time

Group	Inoculum	Antiviral activity for day p.i. ^b					
		0	1	2	3	4	5
1	10 ⁹ PFU of Ad5-Blue	<25	<25	<25	<25	<25	<25
2	10 ⁸ PFU of Ad5-pIFN α	<25	133	58	25	<25	<25
3	10 ⁹ PFU of Ad5-pIFN α	<25	800	400	267	25	25

PRRSV



- Porcine reproductive and respiratory syndrome virus (PRRSV) is a widely disseminated virus that causes reproductive and respiratory disease in swine and predisposes to other respiratory pathogens.
- PRRSV is a member of the Arteriviridae family (+ sense ssRNA) and primarily infect cells of the monocyte/macrophage lineage. Highly mutable.
- Infection with PRRSV characterized by prolonged viral persistence.
- Vaccines fail to provide disease control, especially against genetically unrelated strains.

Immune dysfunction and PRRSV

- Innate and adaptive immunity to PRRSV reduced or delayed.
 - PRRSV inhibits IFN α production and induces low levels of IFN α compared to other viruses (SIV, PRCV) that infect the respiratory system.
 - Delayed effective adaptive immune response
 - Rapid non-neutralizing antibody response
 - Delayed neutralizing antibody response and CMI response (4-8 weeks)
- **Immunosuppress host**
 - Increased secondary bacterial infections

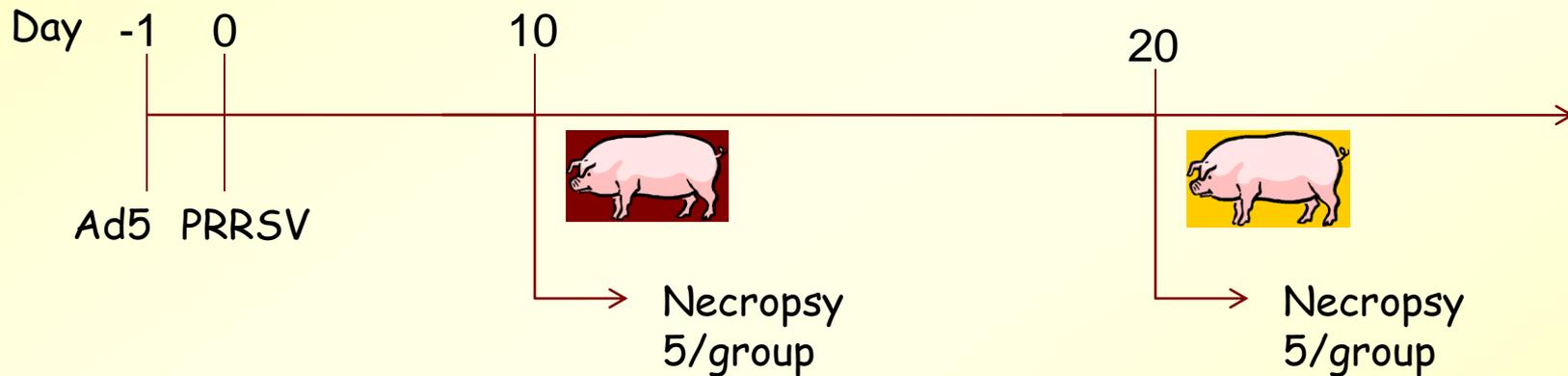
Experiments

- We ran an initial experiment using a replication defective human adenovirus 5 expressing porcine IFN α to determine if the presence of interferon would reduce or eliminate PRRSV infection.
- A follow-up experiment examined whether the presence of IFN α alters the immune response to PRRSV.

Exp. 1 - Experimental Design

10 pigs/group

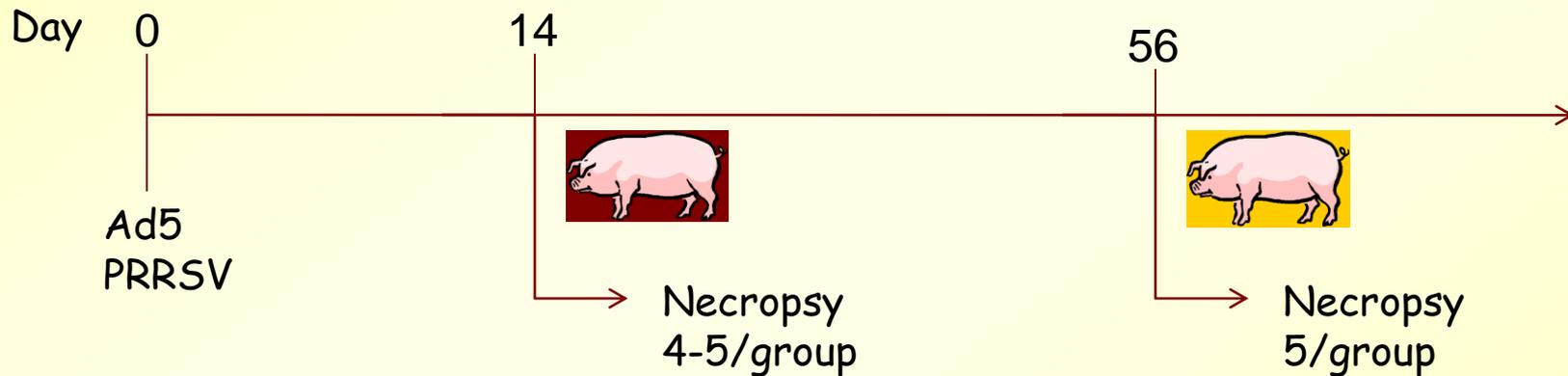
Ad-IFN α
Ad-IFN α /PRRSV
Ad-null/PRRSV



- Rectal temperatures were taken daily
- Sera for IFN α levels, viremia, seroconversion.
- Lung lesions

Exp. 2-Experimental Design

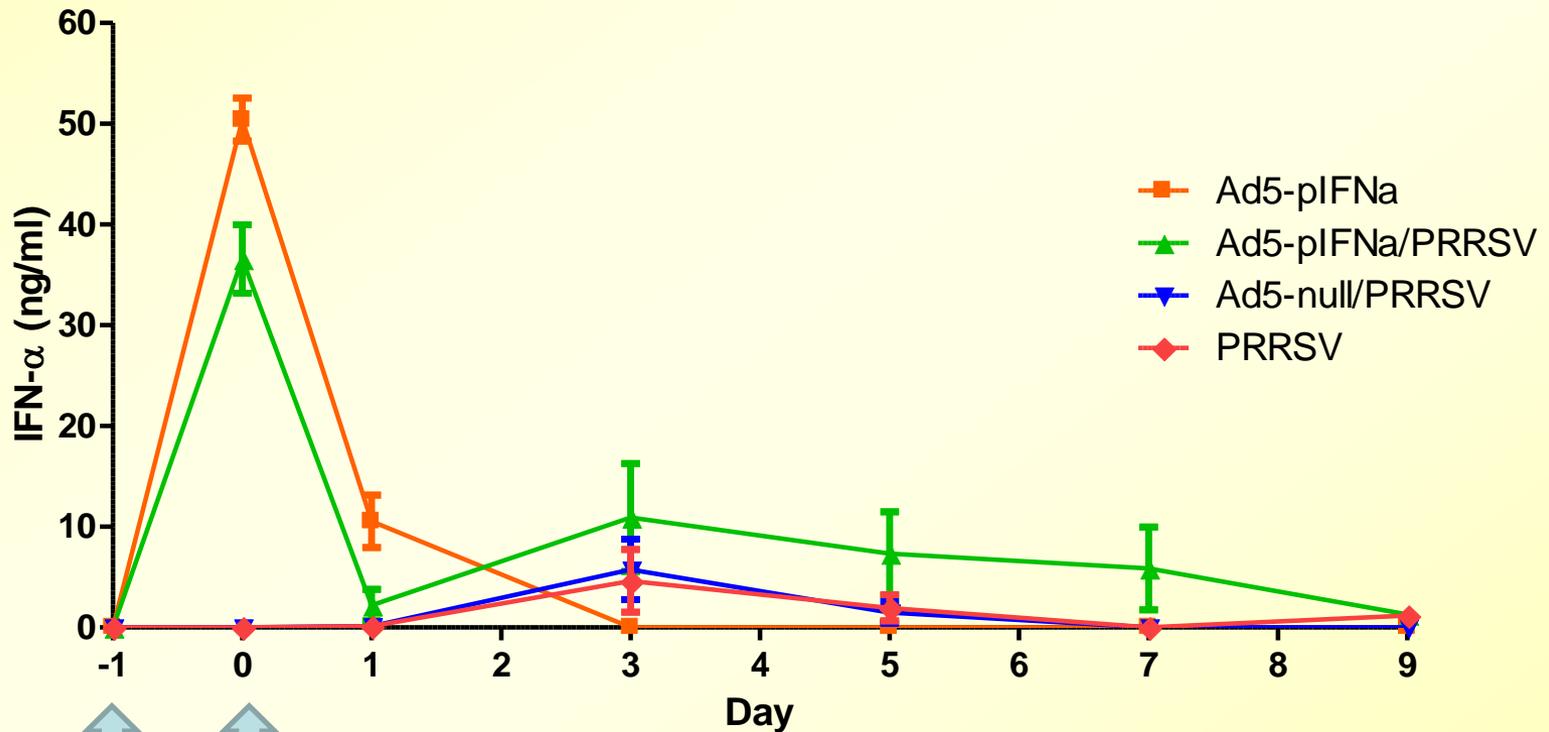
9-10 pigs/group



- Sera for IFN α levels, viremia, seroconversion by ELISA and neutralization
- PBMC for ELISPOT
- BALF at day 14 for cytokine levels

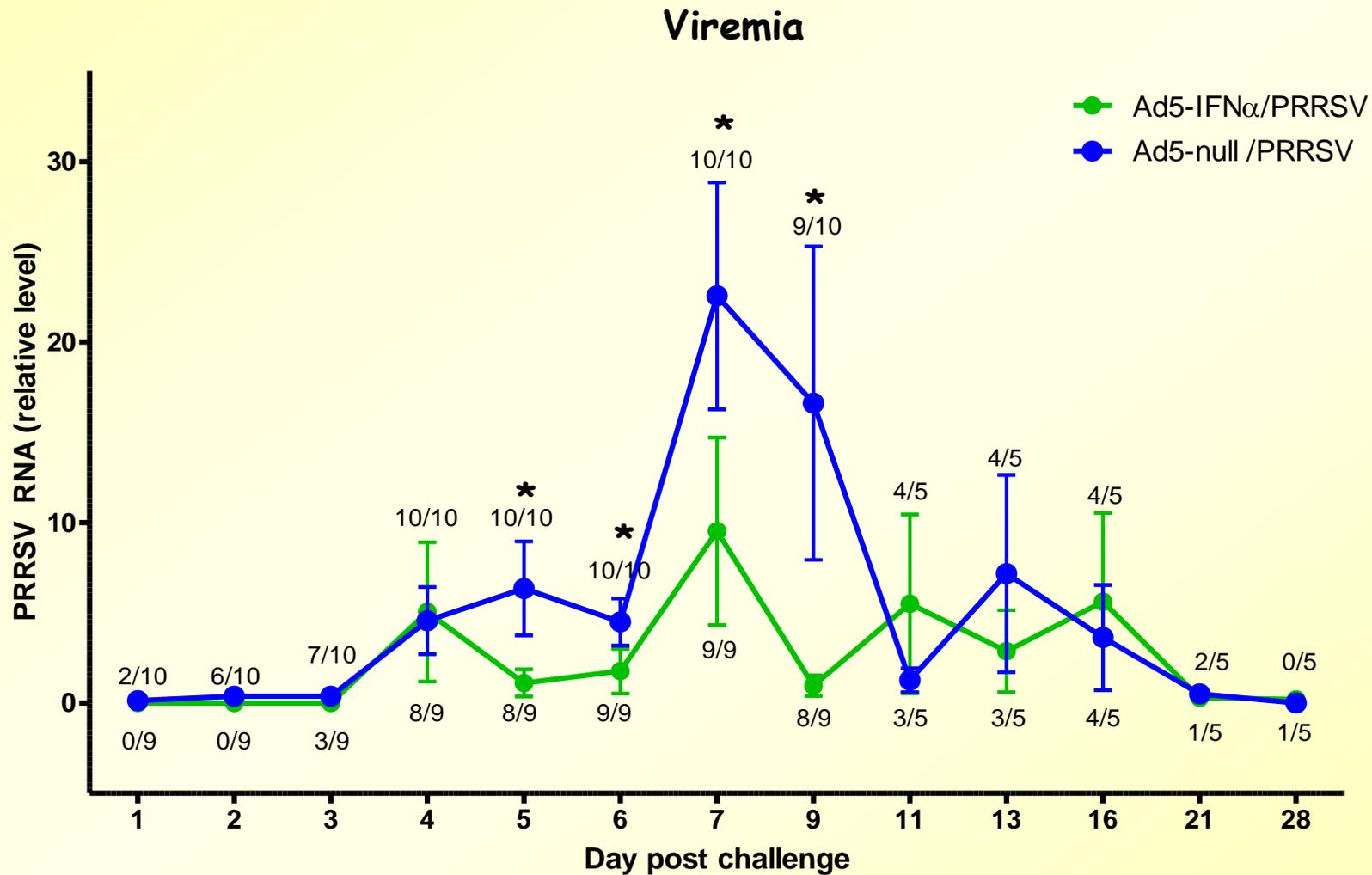
IFN α levels

IFN α serum levels



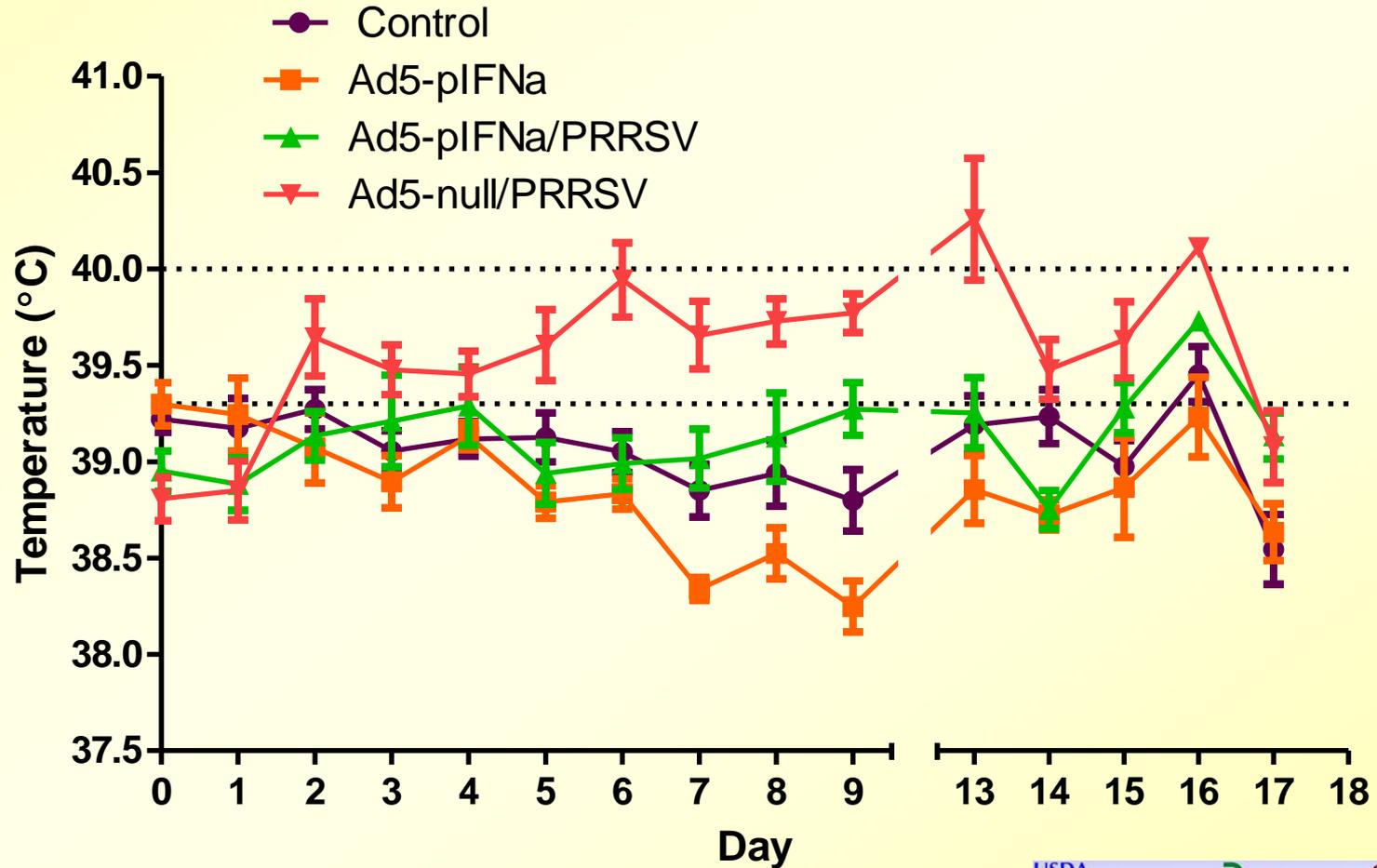
IFN α PRRSV

Viremia



Febrile response

Rectal Temperature

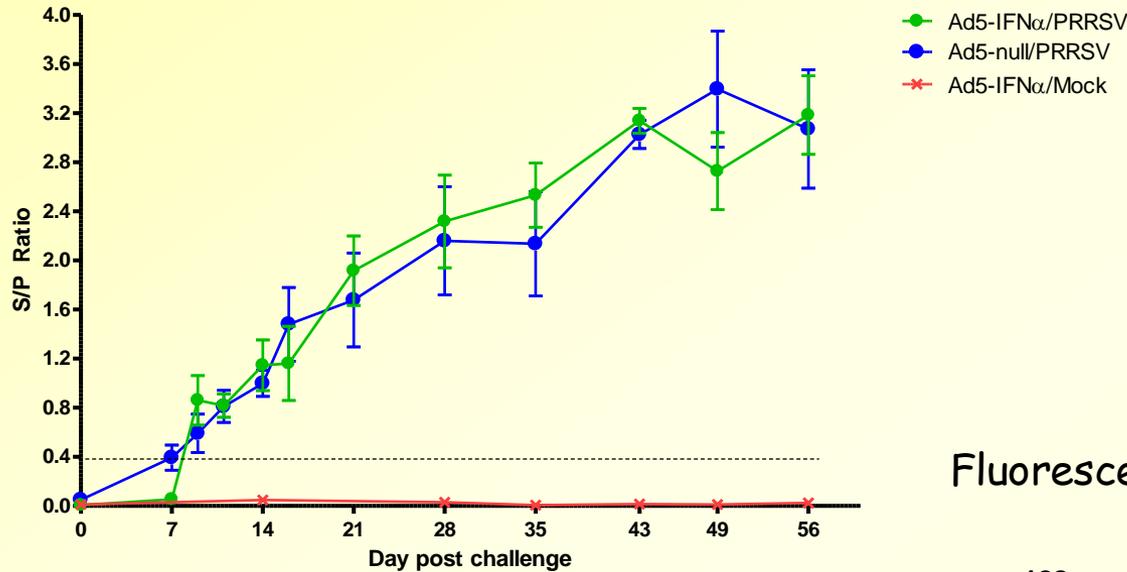


Exp. 1 - Macroscopic lung lesions

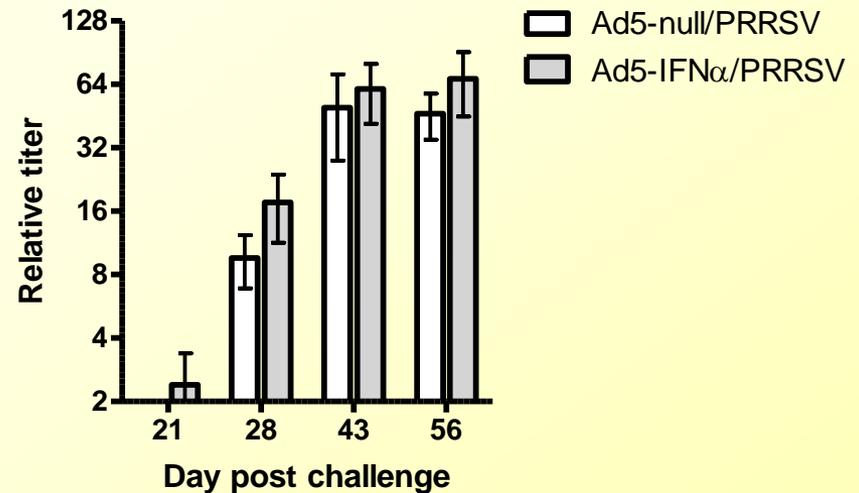
Group	Day 10	Day 20
Control	0 ^a	0.2(0-1) ^a
Ad5-pIFN α	0 ^a	0.5(0-2.5) ^a
Ad5-pIFN α /PRRSV	28.6(10-49) ^b	34.7(3.5-56) ^b
Ad5-null/PRRSV	65.8(21-100) ^c	66.6(23-93) ^c

Humoral response

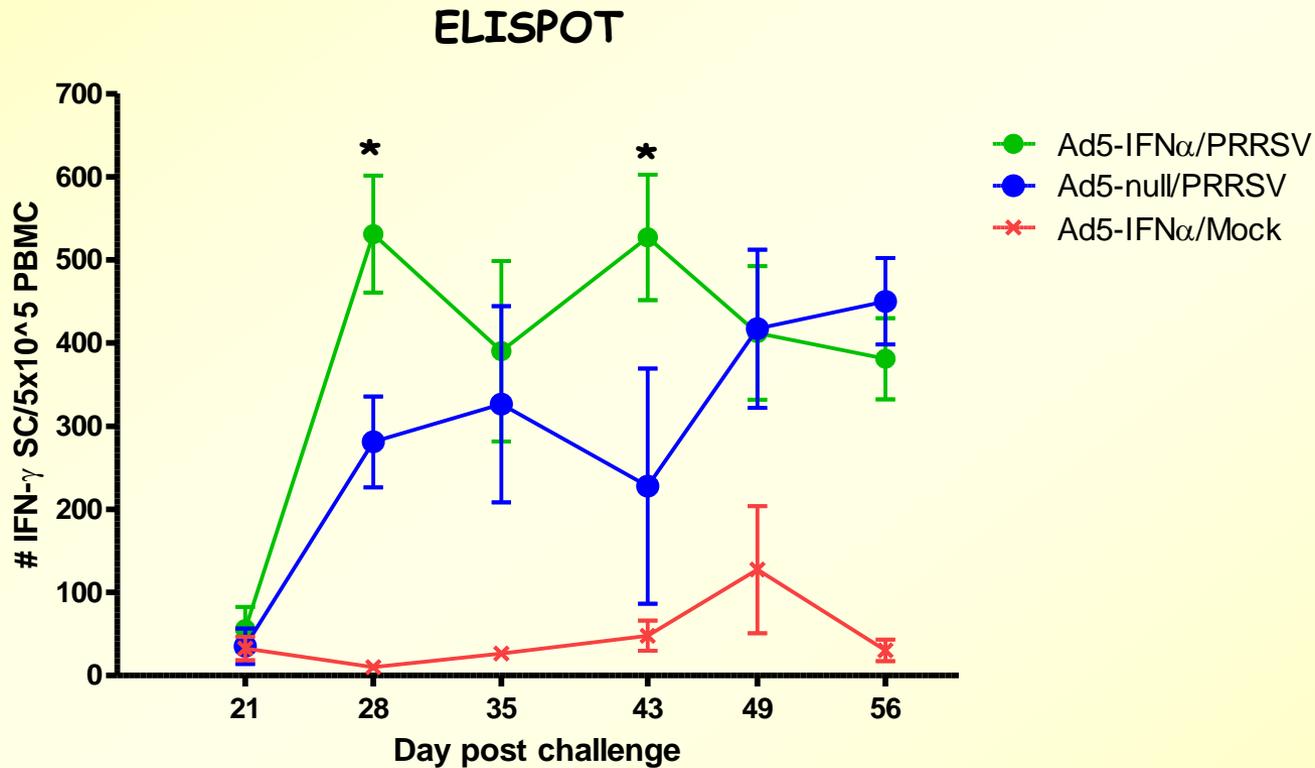
Seroconversion (IDEXX ELISA)



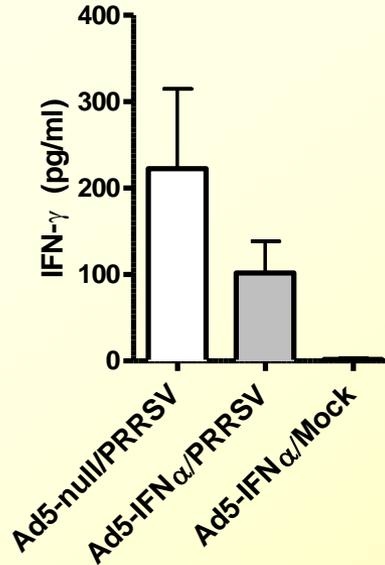
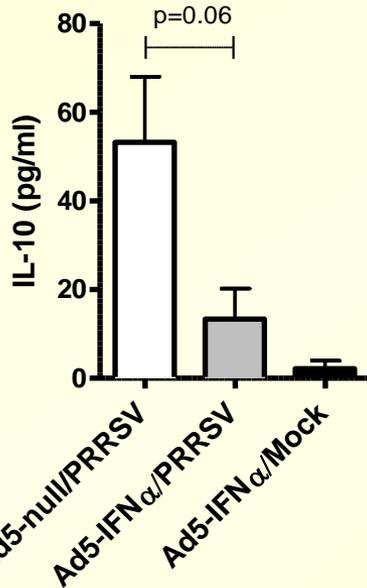
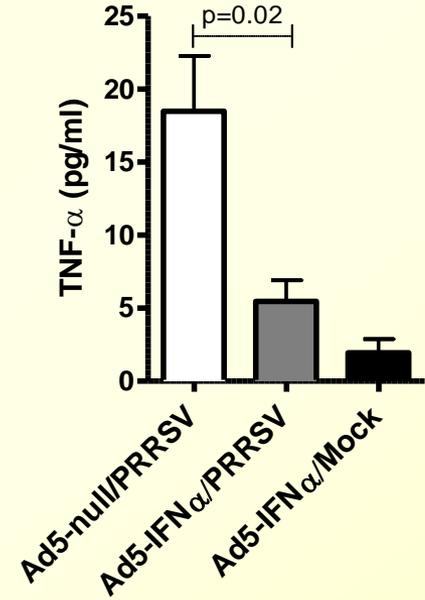
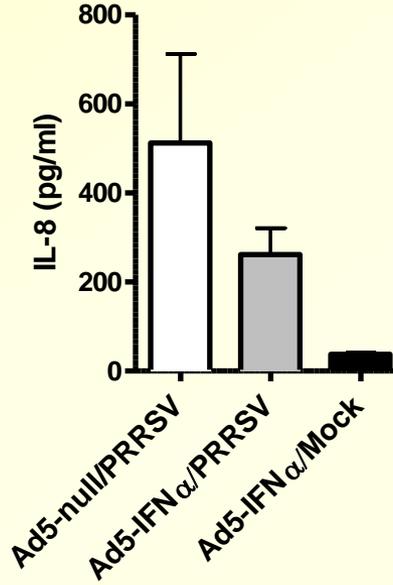
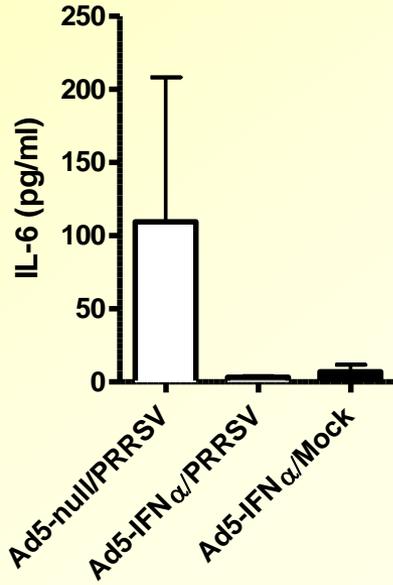
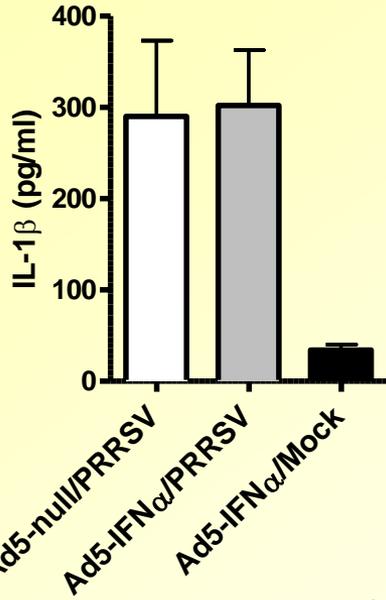
Fluorescent focus neutralization assay



Cell-mediated response



Cytokine levels in BALF d14



Summary

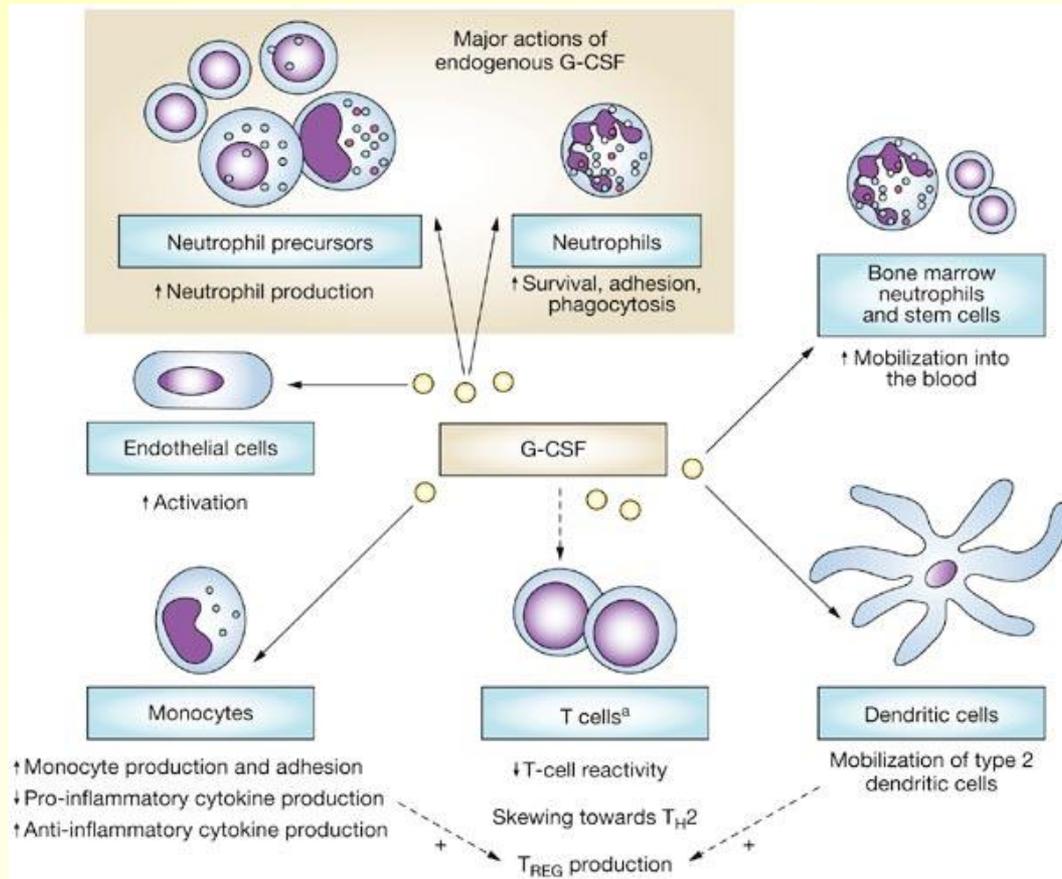
- Presence of IFN α :
 - delayed/reduced viremia
 - decreased febrile response
 - decreased lung lesions observed
 - slight delay in seroconversion, but then not much difference - maybe slight trend for quicker neutralizing response
 - greater number of IFN γ secreting cells early (cell mediated response)
 - reduction in cytokine levels in the BALF

PMN's & G-CSF



- **PMN are critical for acute inflammatory response**
 - Professional phagocytes
 - Critical for many infectious diseases
 - Most PMN enter the gut, exit via digestive tract
- **Granulocyte Colony Stimulatory Factor**
 - Responsible for mobilization of PMNs from bone marrow
 - Increases bone marrow production of PMNs
 - Stimulates proliferation, differentiation, survival & function of PMN
- **Recombinant human G-CSF**
 - Approved for use in humans to treat neutropenia

Figure 5 The major biologic actions of G-CSF



Eyles JL *et al.* (2006) Granulocyte colony-stimulating factor and neutrophils—forgotten mediators of inflammatory disease *Nat Clin Pract Rheumatol* **2**: 500–510 doi:10.1038/ncprheum0291

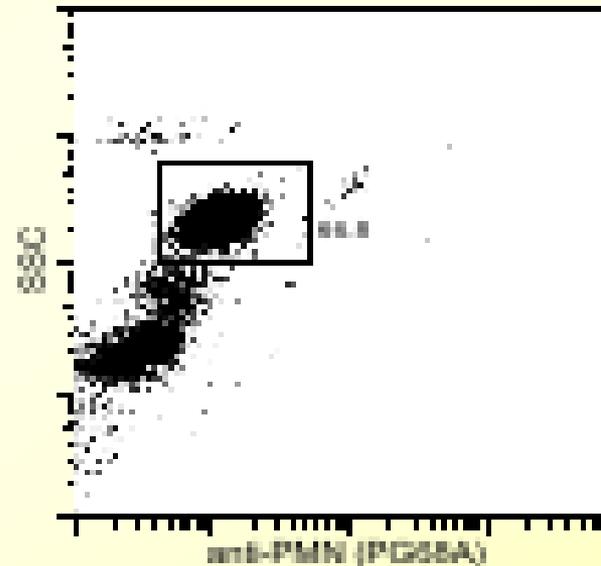
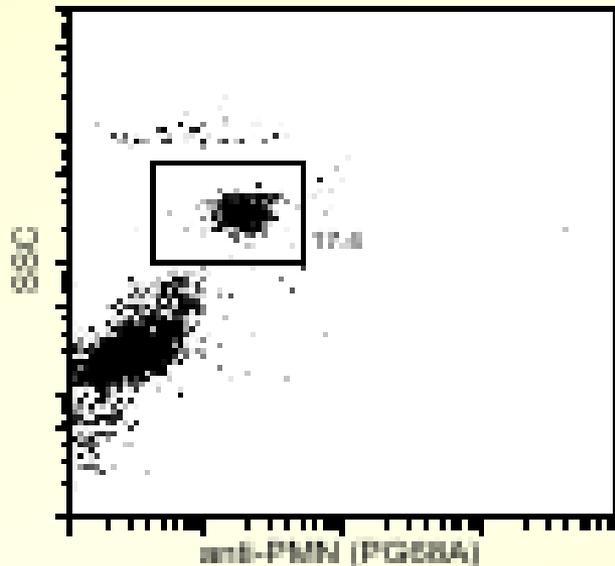
rhG-CSF

- Neulasta® (pegfilgrastim) - pegylated rhG-CSF
 - Increase half-life
- Neulasta is a leukocyte growth factor indicated to decrease the incidence of infection, as manifested by febrile neutropenia, in patients with non-myeloid malignancies receiving myelosuppressive anti-cancer drugs associated with a clinically significant incidence of febrile neutropenia.
- Dose - 6mg in 0.6mL syringe administered subcutaneously once per chemotherapy cycle.
- Estimate:
 - 150lb person ~ 68kg
 - Dose = 88ug/kg

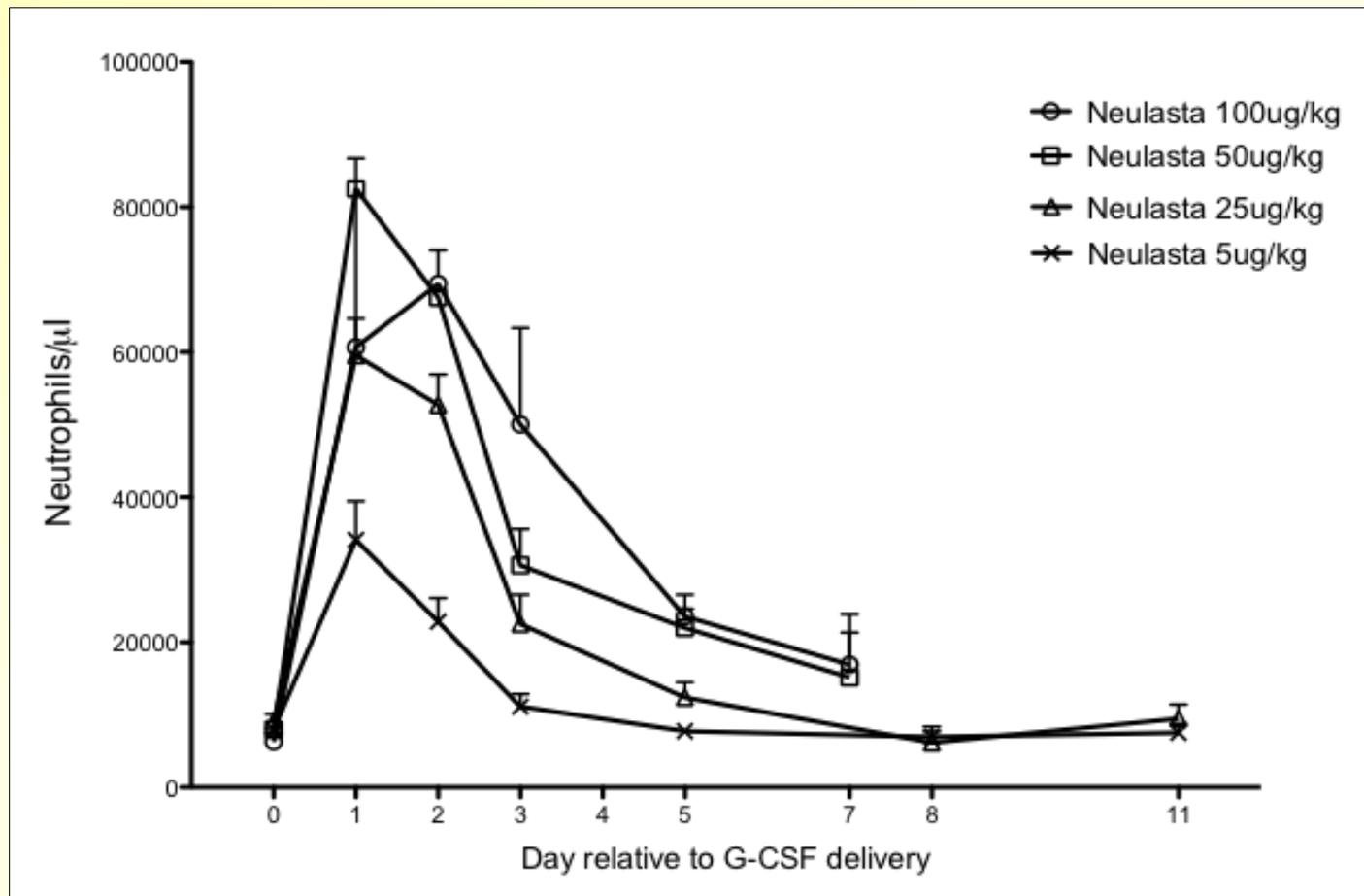


Does Neulasta increase pig PMNs?

- 2-5 pigs per group - dose curve
- Subcutaneous injection on day 0
- Bleed daily for 5 days, then every other day
- Enumerate PMNs using flow cytometric assay

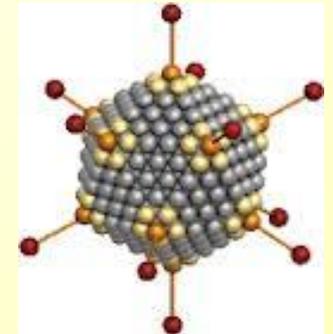


Neulasta® in pigs



Different delivery method?

- **rhG-CSF increased PMN counts in pigs**
 - Short-lived
 - Peak within a day, return to baseline within a week
 - Desire longer-lasting effects
- **Adenovirus gene delivery - sustained effects?**
 - Encode porcine G-CSF in Ad5 vector
 - Ability to generate altered pG-CSF constructs to increase potency via:
 - Receptor binding
 - Ligand half-life
 - Histidine switching
 - Sarkar et al., 2002; Sarkar et al., 2003



Porcine G-CSF constructs

- Generate porcine G-CSF mutant

10 20 30 40 50 60
 -----|-----|-----|-----|-----|-----|

GCSF_[Sus_scrofa] MKLMALQLLLWHIALWMVPEAAPLSPASSLPQSFLKCLEQVRKIQADGAELQERL---C
 GCSF_mutagenized_[Sus_scrofa]
 GCSF_[Homo_sapiens]S..T.Q..T..G.....G...A.....VSE.

70 80 90 100 110 120
 -----|-----|-----|-----|-----|-----|

GCSF_[Sus_scrofa] ATHKLCHPQELVLLGHSLGLPQASLSSCSSQALQLTGCLNQLHGGLVLYQGLLQALAGIS
 GCSF_mutagenized_[Sus_scrofa]
 GCSF_[Homo_sapiens]W.P....P.....A.....S..F.....E...

130 140 150 160 170 180
 -----|-----|-----|-----|-----|-----|

GCSF_[Sus_scrofa] PELAPALDILQLDVTDLATNIWLQMEDLRMAPASLPTQGTVPTFTSAFQRRAGGVLVVSQ
 GCSF_mutagenized_[Sus_scrofa]H..H.....
 GCSF_[Homo_sapiens] ...G.T..T....A.F..T..Q....G...LQ...A..A.A.....A.H

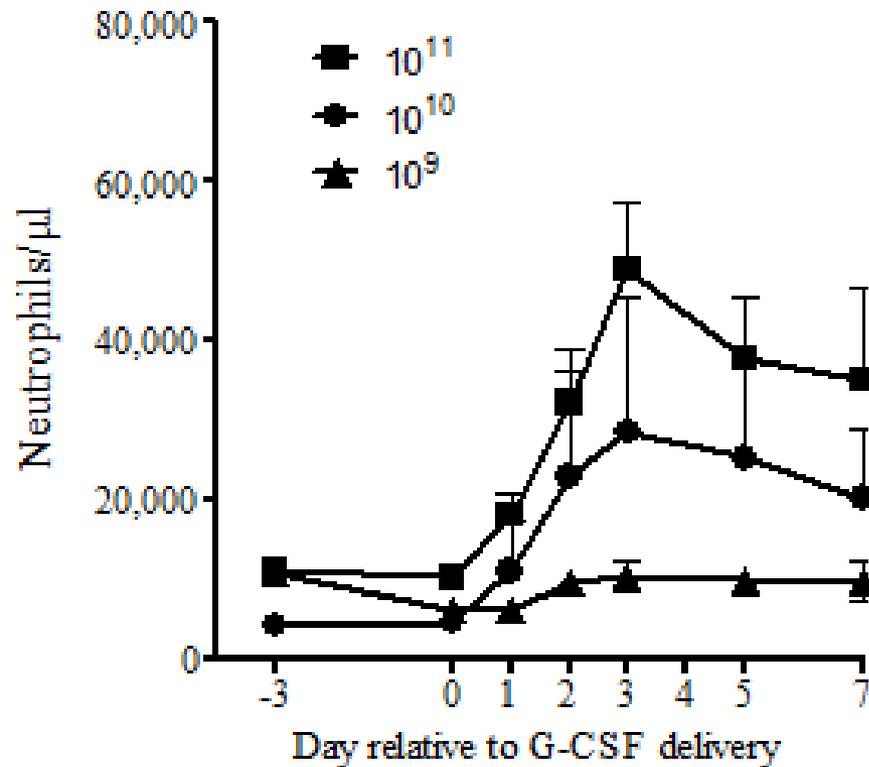
190 200
 -----|-----|

GCSF_[Sus_scrofa] LQSFLELAYRVLRYLAEP
 GCSF_mutagenized_[Sus_scrofa]
 GCSF_[Homo_sapiens]

Sarkar et al., 2002

Sarkar et al., 2003

Ad5 dose curve



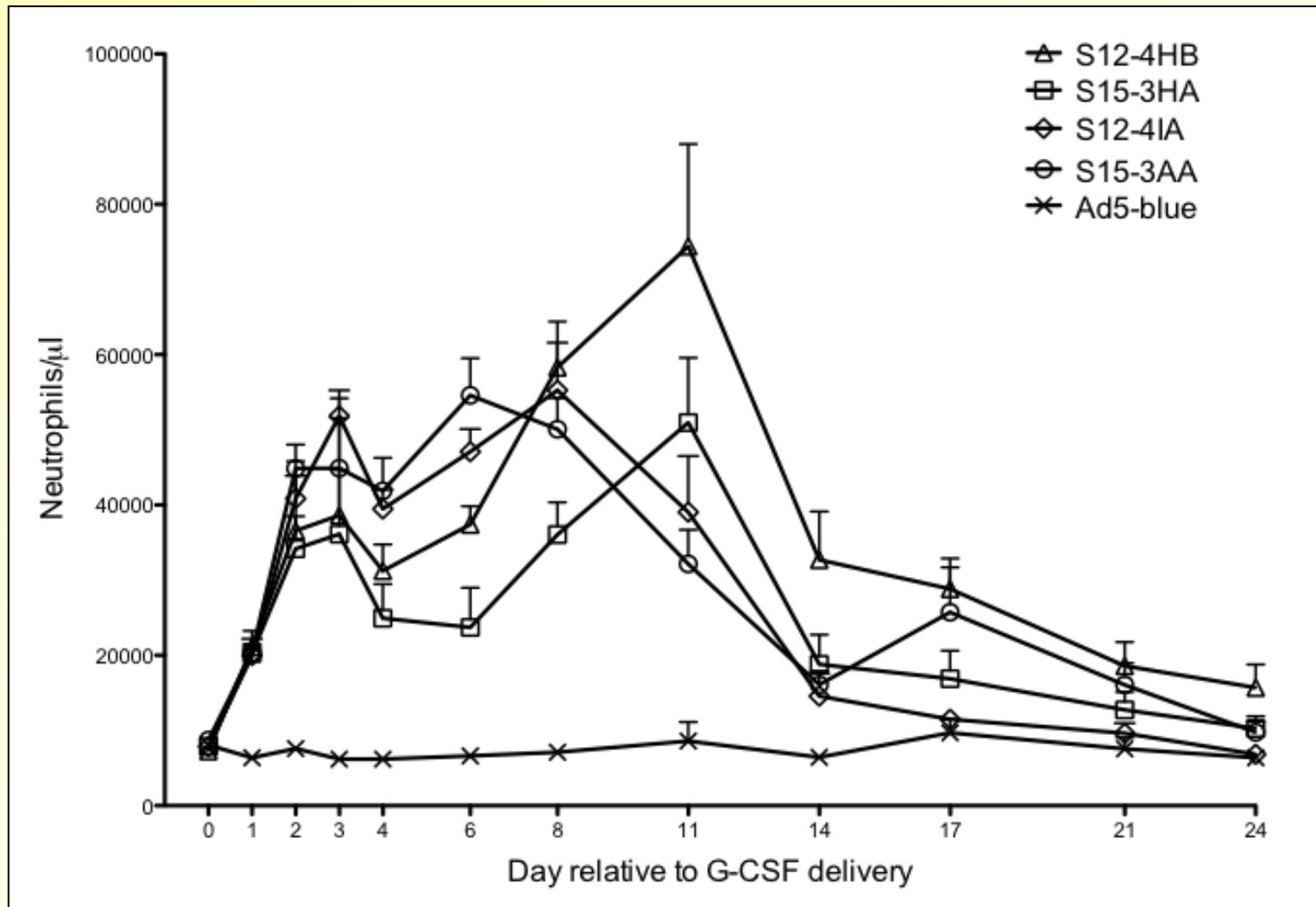
Ad5-pGCSF Experiment

- 4 recombinants to test
 - Same histidine changes, different recombination event

Group	Treatment		Route
1	Ad5-mutG-CSF @ 10^{10} PFU	S15-3HA	IM
2	Ad5-mutG-CSF @ 10^{10} PFU	S12-4HB	IM
3	Ad5-mutG-CSF @ 10^{10} PFU	S12-4IA	IM
4	Ad5-mutG-CSF @ 10^{10} PFU	S15-3AA	IM
5	Ad5-blue (empty)		IM

5 pigs per group

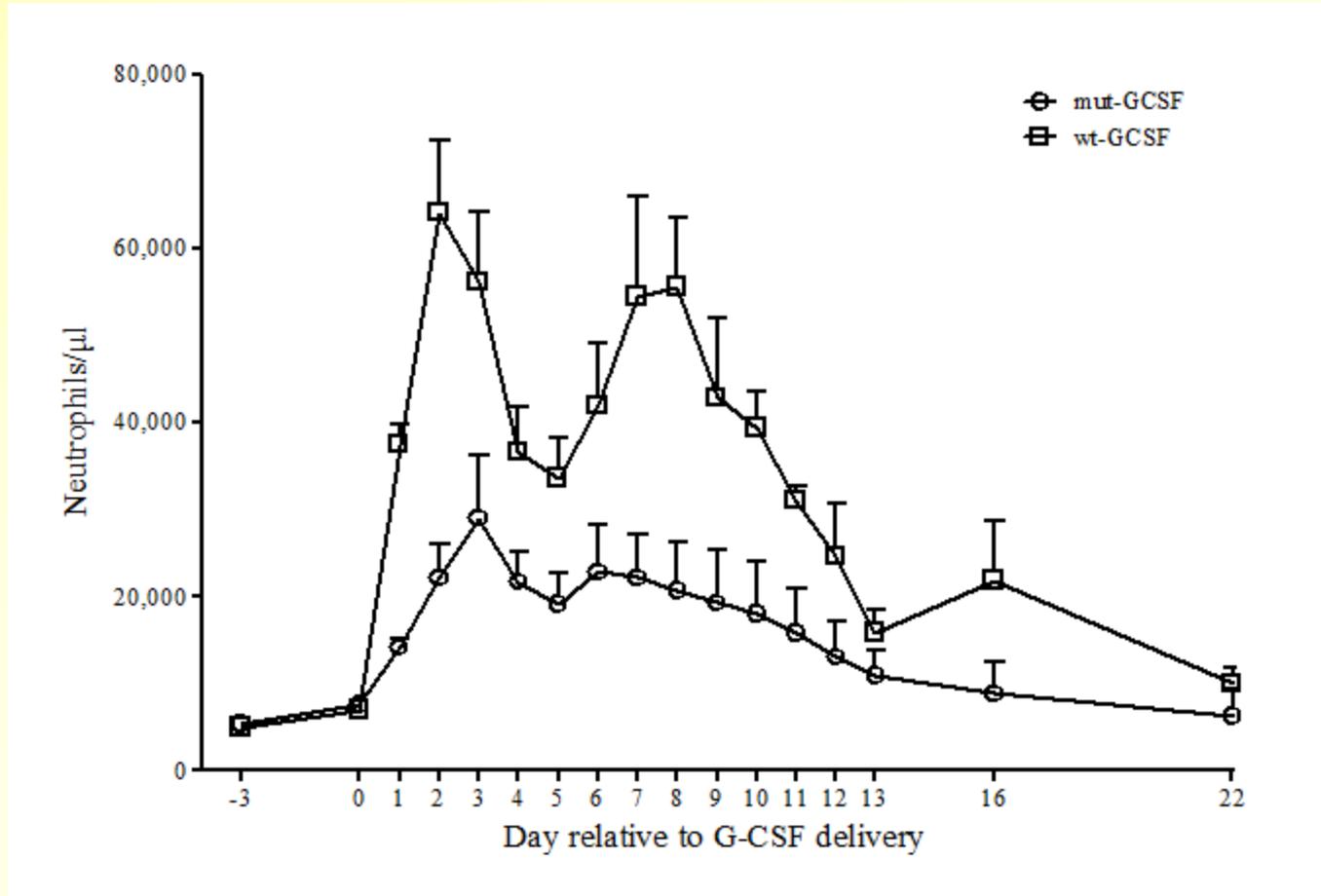
Ad5-pGCSF



Ad5-pGCSF

- **Construct dependent**
 - Magnitude of response
 - Day response peaked
- **Biphasic response**
 - More prominent in S15-3HA & S12-4HB
- **Elevated neutrophil counts for extended length of time**
 - 24 days following single Ad5-pGCSF dose - 2x the # cells compared to Ad5-blue
- **Increased duration over Neulasta® injection**

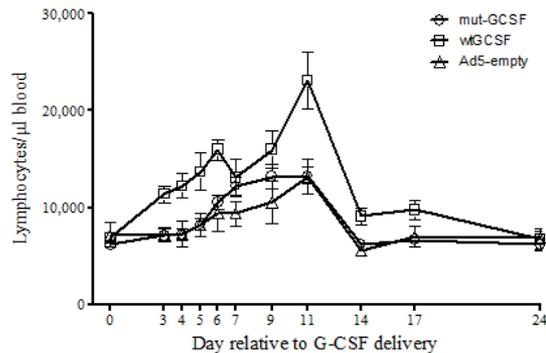
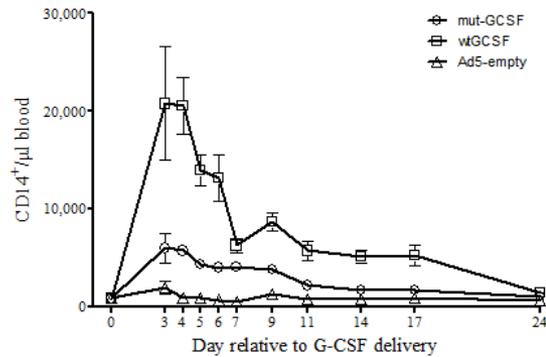
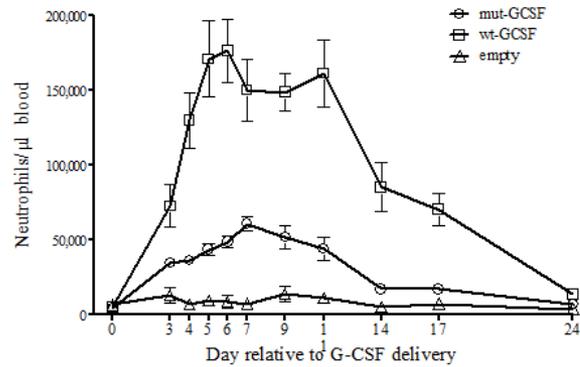
Mutated vs Wild Type pG-CSF



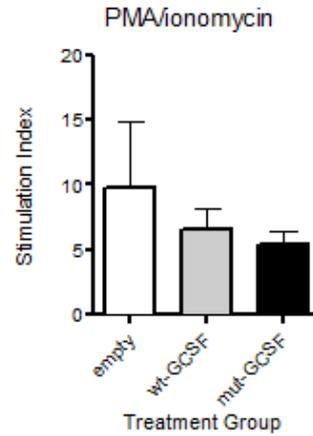
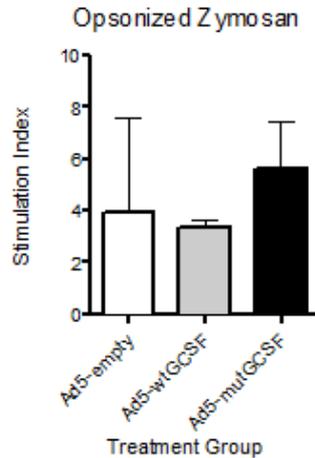
Detecting G-CSF

- No reagents for evaluating porcine G-CSF protein levels
- Use anti-human G-CSF ELISA
 - Sera samples collected at same time as PB
- Positive signal in sera from pigs treated with Neulasta
- No signal in sera from pigs treated with Ad5-pGCSF constructs (porcine G-CSF)
 - Difficult to determine kinetics of G-CSF expression versus long-term effects in the bone marrow with Ad5 delivery.

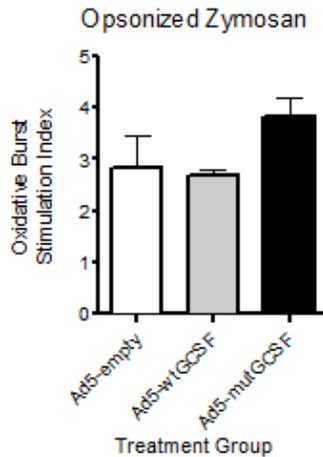
PMN, Monocytes, Lymphocytes



PMN functional assays



NET assay



Oxidative burst assay

Innate immune response

- **Sera cytokine response**
 - No changes in IL-1 β , TNF- α , IL-6 or IFN- γ
 - Days 0-5 tested
 - Compared to pigs given Ad5-blue
 - Little to no cytokine detected
- **No obvious clinical signs in pigs through course of experiment**

Does it protect from disease?

- Studies with mastitis in dairy cows....

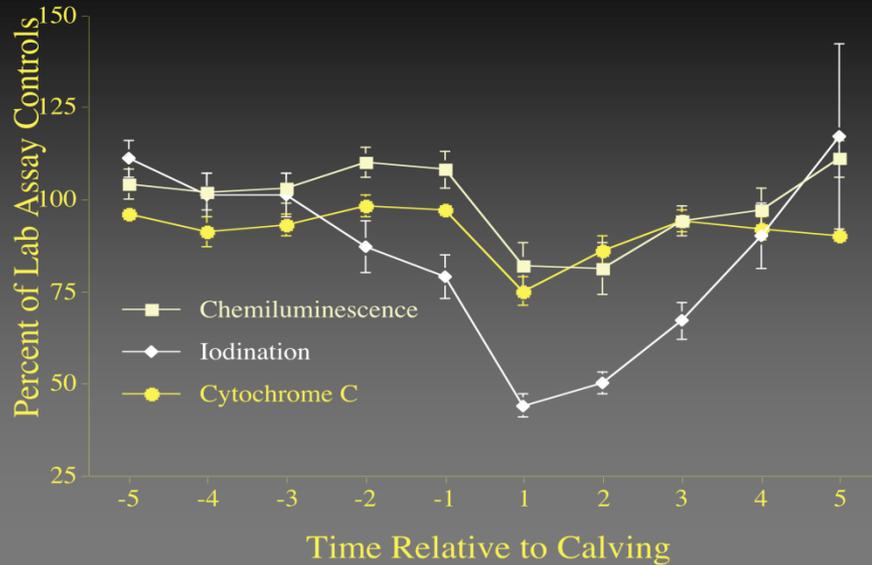
PMNs and mastitis

- PMNs are critical for fighting many postpartum infectious diseases including mastitis, retained placenta, metritis, etc.
- Neutrophils are predominant (97%) cell type found in mastitic milk
- Normal milk PMNs are impaired relative to blood PMNs
 - Decreased phagocytosis
 - Decreased bactericidal activity
- Based on high incidence of mastitis in first month of lactation, researchers assessed phagocytosis relative to parturition
 - Neutrophil phagocytic uptake of pathogens altered for 3 weeks postpartum

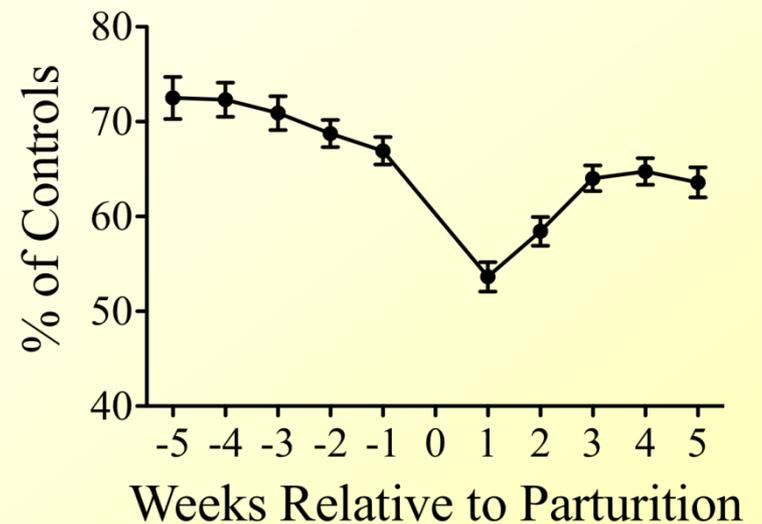


Periparturient immunosuppression

Neutrophil Phagocytosis-associated Oxidative Metabolism



PMN Iodination (n = 137 Holsteins)



G-CSF as a biotherapeutic for mastitis

Can we tweak the circulating neutrophil pool to provide a larger reservoir of phagocytic cells to arrive earlier in the mammary gland to combat a pathogen?

- Primary role of G-CSF - provide PMN from bone marrow
- Literature reported G-CSF activates a critical PMN adhesion molecule (CD62L) that would predict faster response to disease.
- Also reported G-CSF enhances FcR ability to trigger cytotoxic activity of PMN
- Other studies report range of effects on PMN functions -
 - *One benefit of G-CSF therapy may lie in enhanced number and survival of PMN in the body.*

G-CSF as a biotherapeutic for mastitis

- **Reduced experimental *S. aureus* mastitis by 46.7%**
 - Nickerson SC, et al., 1989. J Dairy Sci 72:3286-3294.
- **Reduced experimental *Klebsiella* mastitis**
 - Infection Status:
 - Controls - culture positive for 7 days
 - G-CSF - cleared infection by 6 hours
 - Clinical Symptoms:
 - Controls - fever and abnormal milk for 5 days
 - G-CSF - no fever at 12 hours and milk normal in appearance by 24 hours
 - Kehrli ME, Jr., et al., 1991. J Dairy Sci 74:4399-4412.

G-CSF as a biotherapeutic for mastitis

- Cows were administered rHuG-CSF (1 mg/kg, s.i.d. X5, SC) starting 3 d postpartum and challenged 3 d later with 30 cfu *E. coli* in one quarter and monitored 14 d
 - rHuG-CSF increased circulating neutrophils as expected
 - rHuG-CSF provided protection against coliform mastitis
 - 50% reduction in number of new infections
 - faster bacterial clearance rates
 - reduced clinical severity scores
 - improved milk production and feed consumption

Prophylactic G-CSF versus experimental *E. coli* mastitis in early lactation

	Hours to clearance of <i>E. coli</i>
Saline	93.1±30.5
rhuG-CSF	30.0±14.5

- Kehrli ME, Jr. (1997) Efficacy of granulocyte-colony stimulatory factor alone or in combination with granulocyte macrophage-CSF against *Escherichia coli* bovine mastitis. In: 78th Conference of Research Workers in Animal Disease, Chicago, IL, p P88
- Kehrli ME, Jr. (1998) Efficacy of granulocyte-colony stimulatory factor as an immunomodulator to prevent *Escherichia coli* mastitis during early lactation. In: 37th Annual Meeting National Mastitis Council, Inc. National Mastitis Council, Inc., St. Louis, MO, pp 336-338

Future work

- **Disease prevention**
 - Our group focuses on respiratory disease
 - Protection provided at times of stress
 - Piglets at weaning
- **Studies planned with swine challenge models -**
Streptococcus suis, *Bordetella bronchiseptica*,
Pasteurella multocida, *Haemophilus parasuis*



Acknowledgements

Crystal Loving
Tracy Nicholson
Karen Register
Marcus Kehrli, Jr.
Howard Lehmkuhl
Laura Miller
Eric Nelson
Randy Sacco
Sarah Schlink
Kelly Lager
Marvin Grubman
Doug Brough
Dadomar ETTYREDDY
Darrel Bayles

Steven Kellner
Zahra Olson
Bruce Pesch
Kim Driftmier
Gwen Nordholm
Sarah Shore
Ann Vorwald
Lea Ann Hobbs
Deb Adolphson
Sarah Pohl
David Michael
Theresa Waters

Jason Huegel
Jason Crabtree
Tyler Standley



Questions?