REPORT

Report on participation of the ICMR International Fellow (ICMR-IF) in Training/Research abroad

1.	Name and designation of ICMR- IF	:	Dr Vishal Chander, Senior Scientist
2.	Address	:	Division of Virology, ICAR- Indian Veterinary Research Institute, Mukteshwar, Distt. Nainital, Uttarakhand Pin: 263 128 (India)
3.	Frontline area of research in which was carried out	:	Role of innate-like T cells in immunity to zoonotic swine influenza infections training/research
4.	Name & address of Professor	:	Dr John P. Driver, PhD, Associate Professor 1201 East Rollins St. Bond Life Sciences Center University of Missouri, Columbia, MO 65211
5.	Duration of fellowship with exact date	:	12 months (30.03.2023 to 22.03.2024)
6.	Highlights of work conducted	:	

(i) Technique/expertise acquired

- Single cell isolation from different tissues including Spleen, lymph nodes, lungs, Broncho-alveolar lung fluid.
- Flowcytometry
- FACS analyses
- ELISAs
- Elispot assay
- Fluorospot assay
- Immunofluorescent microscopy
- Immunohistochemistry
- In-situ hybridisation
- Single cell sequencing analyses
- Statistical analysis on the Graph Pad Prism
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ii) Research results, including any papers, prepared/submitted for publication

- Establish the contribution of CD1- and MR1-restricted ITC populations to influenza immunity using genetically modified pigs. The hypothesis included that lung-resident group 1 CD1- and MR1-restricted T cells play an important role in influenza immunity. Aim 1 was to determine the impact of different ITC (innate-like T cells) populations on IAV virulence and pathogenesis by challenging CD1- and MR1-knockout pigs with different strains of IAV. A range of innovative tools were used to characterize pulmonary leukocytes and their effector functions to determine which immune responses and cell types that are subject to ITC regulation during an IAV infection.

Methods and analysis

Influenza challenge: Each ITC population were respectively tested in one of three similar experiments as follows; 20 CD1A-/-B-/-, CD1D-/- or MR1-/- piglets (-/-) and 6 littermate control piglets (+/+) farrowed from the high-health breeding facility and were transferred to isolation rooms at 4 weeks of age, after being tested to confirm they were seronegative for H1N1 and H3N2 IAVs using hemagglutination inhibition (HAI) assays. Mixed sex piglets of both genotypes were divided into 2 groups of 10 animals each that were inoculated intranasally (i.n.) and intratracheally with a total of 10^6 $TCID_{50}$ of one of two different IAV isotypes: pandemic H1N1 SI A/California/04/2009 virus (CA04) or H3N2 influenza A/SW/TX/4199-2/98 virus (TX98) (Table 1). Both CA04 and TX98 cause disease in pigs. Six littermate control pigs were mock infected with MEM media (Mock) as controls. Five pigs from each infected group and three control pigs were euthanized by Pentobarbital Sodium IV injections and necropsied on days 2 and 5 post infection (p.i.) to analyze innate and adaptive immune responses that develop during these two phases of disease. The proposed number of animals were based on results from previous IAV challenge studies, as the animal number required to obtain statistically significant results for a power of 0.8 using an alpha level of 0.05, and an effect size between 0.3 to 0.5.

Table 1. Assignment of pig groups.

Group	Genotype	Challenge	N
1	+/+	Mock	6
2	-/-	CA04 H1N1	10
3	-/-	TX98 H3N2	10
4	+/+	CA04 H1N1	10
5	+/+	TX98 H3N2	10

Mutant (-/-) or wild-type (+/+) alleles of CD1A/B, CD1D or MR1 genes;10^6 $TCID_{50}$ CA04 or TX98

Analysis: Piglets were monitored daily for body weight, feed intake, and body temperature to evaluate the severity of disease signs. Nasal swabs were collected from inoculated pigs on days 1-5 p.i. to determine viral titers for each pig. At necropsy, tissues were assessed for how the absence of each type of ITC affects virus-induced respiratory lesions using viral titers and assessment of inflammatory responses within the nasal mucosa, nasal associated lymphoid tissue (NALT), bronchoalveolar lavage fluid (BALF), lung, spleen and draining tracheobronchial lymph nodes (TBLN). Briefly, macroscopic volume density of pneumonia was determined for each lung lobe by photodigitizing morphometry. The right lung was separated and fixed by airway fixative perfusion. Fixed lung tissue was stained with hematoxylin-eosin (H&E) and examined for the extent of inflammatory infiltrate, bronchitis/bronchiolitis, fibrin deposition, periarterial/bronchiolar hemorrhage, bronchiolar epithelium hyperplasia, smooth muscle thickening, and mucus production (as measured by PAS staining). Lung pathology was correlated with density of viral antigen by morphometric techniques.

To measure innate immune responses induced by infection, blood, nasal wash, BALF, and homogenized lungs were assayed for key inflammatory cytokines/chemokines associated with influenza infection, T cell trafficking, and neutrophilia (IFN-a, IFN-ab, IFN-g, IL-1a, IL-1b, IL-

1ra, TNF, IL-6, IL-15, IL-17, IL-23, GCSF, MIG, IP-10, RANTES, MCP-1, CCL2, CCL3, CCL4, CCL11, CCL19, CCL21) using specific porcine ELISA's, Millipore Porcine Cytokine Multiplex assay, and realtime PCR where appropriate. In addition, blood, NALT, BALF, spleen and TBLN were examined by flow cytometry for changes in the frequency and activation status of αβ and γδ T cells, NK cells, B cells, DCs, and myeloid-derived leukocytes. Throughout, correlation of distinct kinetics of inflammatory response in airway tissues with virus load, lung pathology and severity of disease symptoms in the presence and absence of each ITC population was carried out. Data was analyzed for statistical significance ($p<0.05$) by repeated measures using PROC MIXED of SAS (V9.4, SAS Institute Inc., Cary, NC). When main effects or interactions are significant, multiple comparisons was done using Tukey's test. For variables where residuals are not normally distributed, even after transformation, the non-parametric Kruskal-Wallis test were performed using PROC NPAR1WAY.

Flowcytometry results

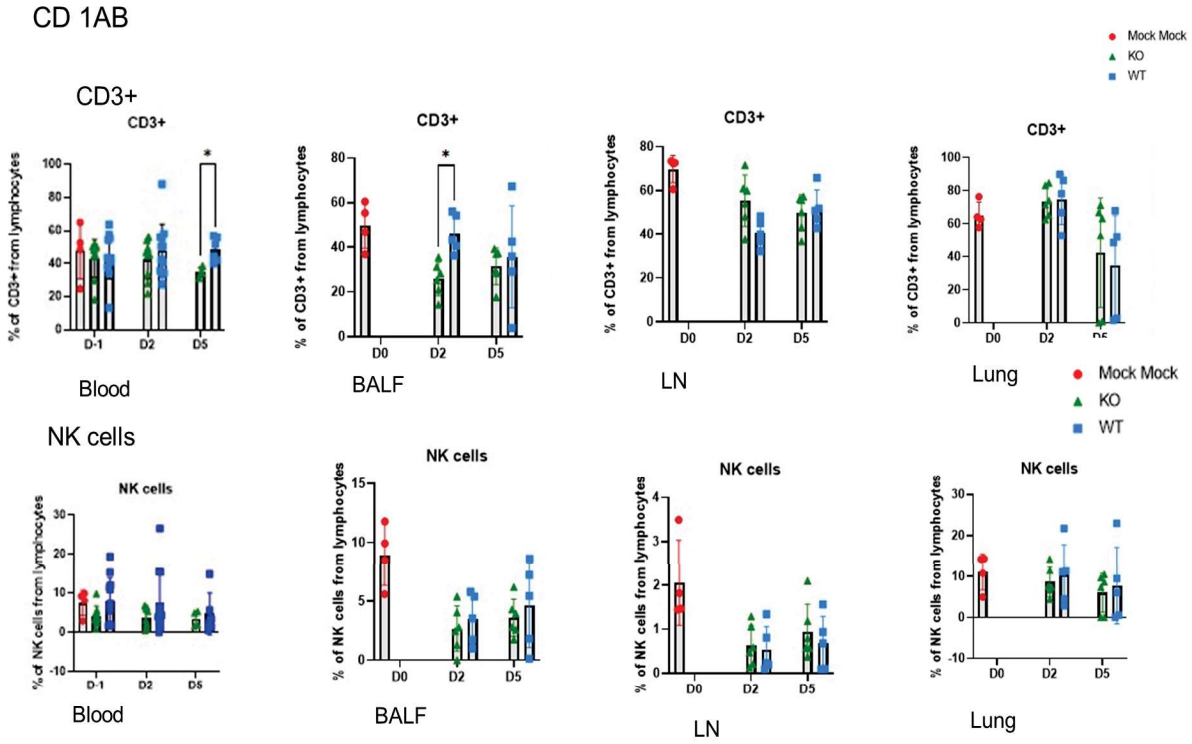

Fig 1: Flow cytometery analysis of immune cells on day 0,2 and 5 post-challenge; percentage of A) CD3+ cells and B) NK cells at day 0,2 and 5 post infection were analysed for Blood, BALF, LN (Lymph node) and Lung for the groups Mock, Knock out (KO) and Wild type (WT). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

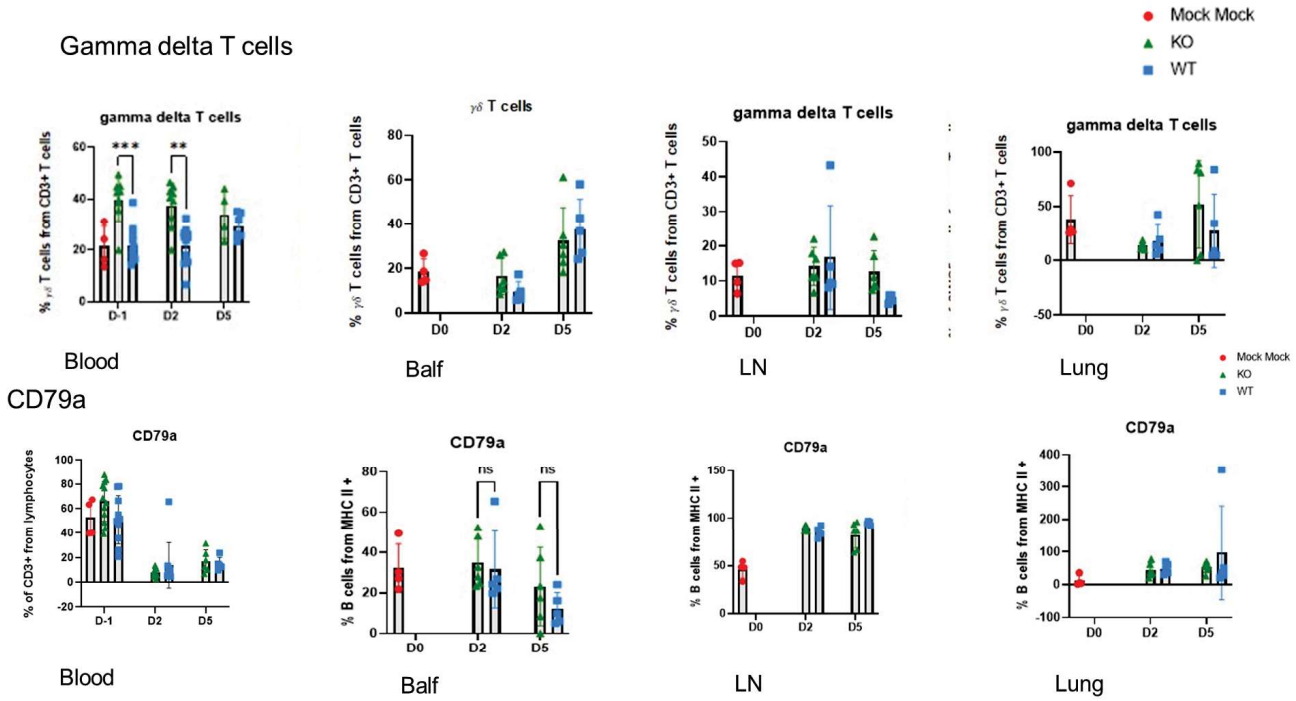

Fig 2: Flow cytometry analysis of immune cells on day 0,2 and 5 post-challenge; percentage of A) gamma delta cells and B) CD 79a cells at day 0,2 and 5 post infection were analysed for Blood, BALF, LN (Lymph node) and Lung for the groups Mock, Knock out (KO) and Wild type (WT). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

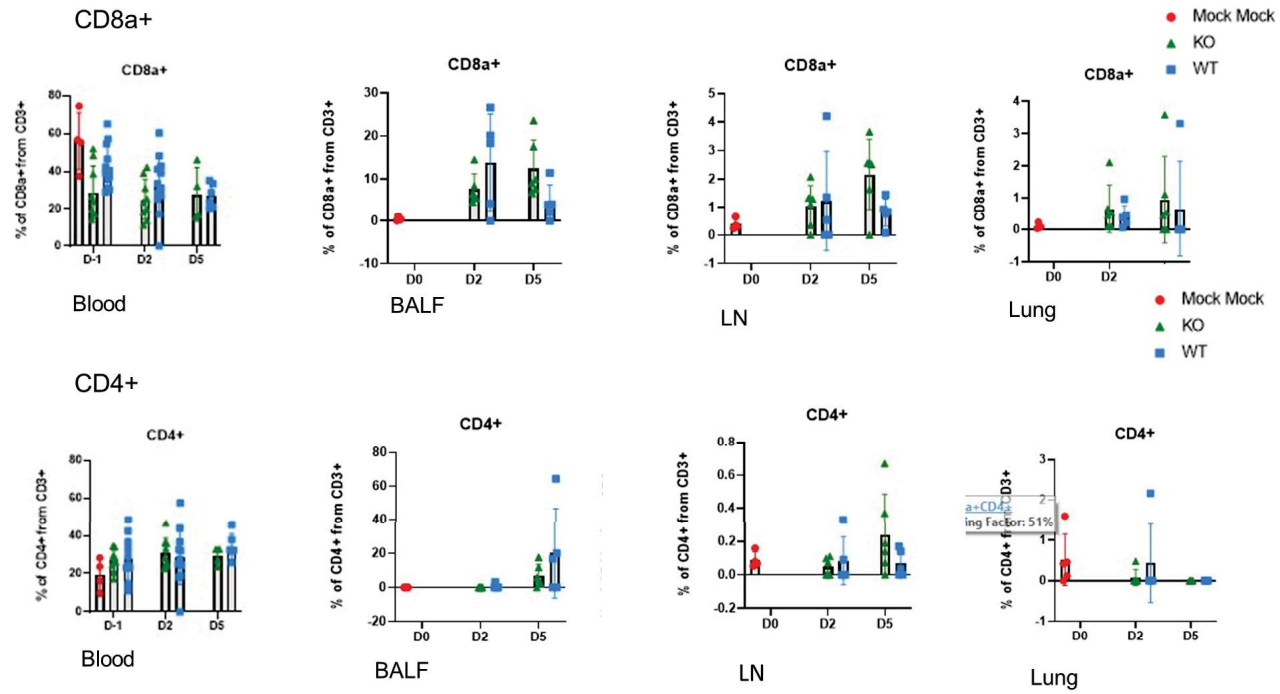

Fig 3: Flow cytometry analysis of immune cells on day 0,2 and 5 post-challenge; percentage of A) CD 8a+ cells and B) CD 4+ cells at day 0,2 and 5 post infection were analysed for Blood, BALF, LN (Lymph node) and Lung for the groups Mock, Knock out (KO) and Wild type (WT). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

CD1 D

CD 1D

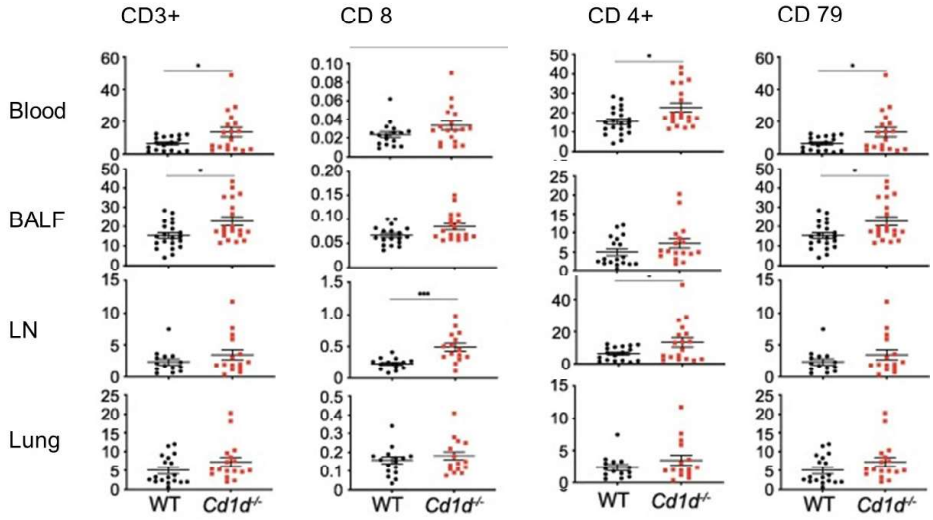

Fig 4: Flow cytometry analysis of immune cells for Wild type (WT) and CD1D + day 5 post infection were analysed for Blood, BALF, LN (Lymph node) and Lungs. The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

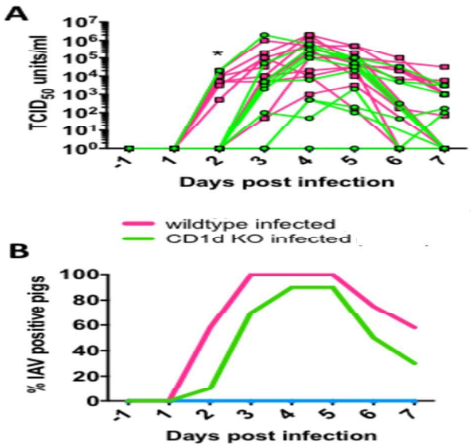

Fig 5: Role of porcine NKT cells in IAV infection. (A) Viral titers in nasal swabs of CD1d-KO and -intact pigs infected with pandemic H1N1. Each line represents an individual pig. (B) Proportion of CD1d-KO and -intact pigs shedding virus after CA04 challenge.

MR 1

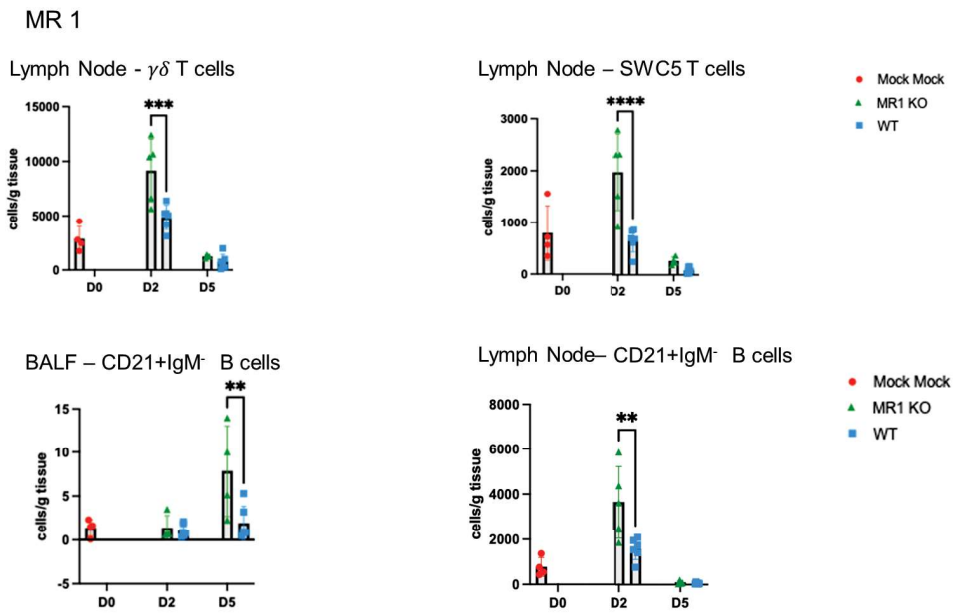

Fig 6: Flow cytometry analysis of immune cells on day 0, 2 and 5 post-challenge; percentage of LN A) gamma delta and SWC 5T cells and B) BALF and LN CD 21+IgM B cells were analysed for the groups Mock, Knock out (KO) and Wild type (WT). The *P* values between groups were determined by one-way ANOVA followed by Tukey's multiple comparisons post-hoc test ($p<0.05$).

Virus titer

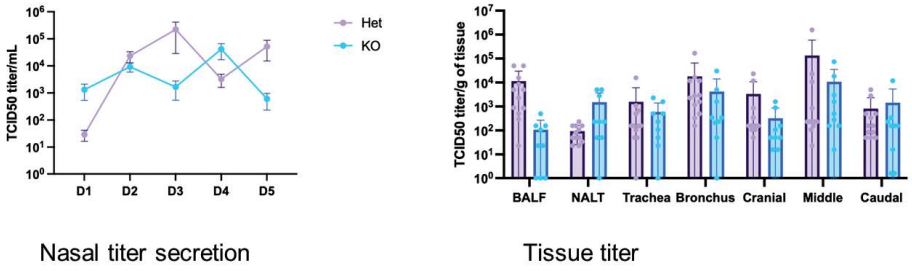

Fig 7: Role of porcine NKT cells in IAV infection. (A) Viral titers in nasal swabs of MR1-KO and WT pigs infected with pandemic H1N1. Each line represents an individual pig. (B) Proportion of MR1-KO and WT pigs shedding virus after CA04 challenge.

scRNAseq

Lung-resident leukocytes were profiled alongside their cellular gene expression using scRNAseq. CD45+ leukocytes were isolated from the lungs of three pigs per group at necropsy (day 2 & 5 p.i.) and analysed for flow cytometry, histology, and virology. Dissociated cells from collagenase digested lung samples were stained with an anti-CD45 Ab and sorted on the Sony SH88S cell sorter that is housed in a Biosafety Cabinet that is specifically approved to work at Biosafety Level 2 (BSL2). scRNAseq was performed, on CD45+ cells using the 10X Genomics system that is contained within a biosafety cabinet at the ICBR. Sequencing results were analyzed using Cell Ranger, which aligns reads and generates feature-barcode matrices. Count

matrices were analyzed using Seurat, after which cells were clustered and visualized using UMAP plots as shown in Fig. 8.

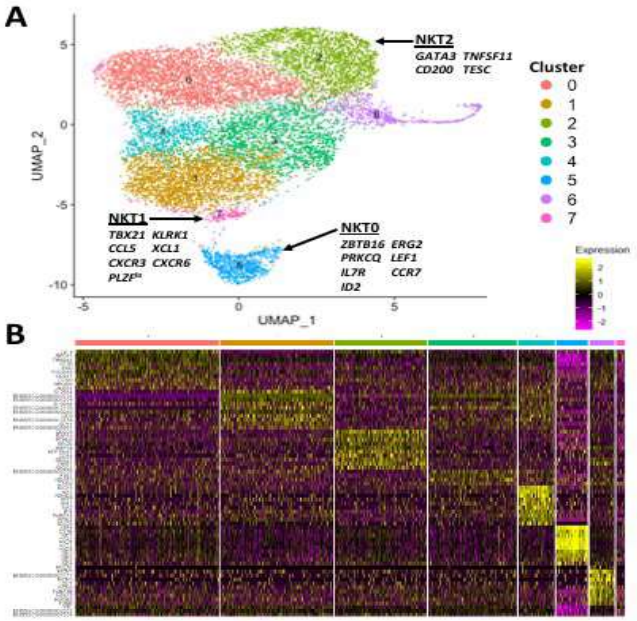

Fig 8. scRNAseq analysis of pig thymic NKT cell subsets. Thymic CD1d tetramer+ NKT cells were isolated from two 22-week-old mixed breed pigs and subjected to scRNAseq analysis using the 10X Genomics platform. (A-B) Cell clustering of all analyzed cells depicting eight different NKT cell subtypes (A) and marker gene heatmap (B).

Mutant and control pig were compared for T cell, NK cell, monocyte, macrophage, dendritic cell, and granulocyte populations to determine how sub-clusters of these cell types are affected by different ITCs and virus strains. Each population cluster was analyzed using the FindMarkers function in Seurat to identify differentially expressed genes between mutant and control pigs. Such genes were analyzed using Ingenuity Pathway Analysis (Qiagen) software to identify ITC-controlled regulatory networks, including canonical pathways, upstream regulators, causal networks, and downstream biological effects. Flow cytometry was used to confirm molecules that are found to distinguish specific clusters or differentiation states.

Within each cell cluster, trajectories were compared between knockout and wildtype pigs to determine how CD1- and MR-1 restricted T cells affect cell fate decisions of pulmonary leukocytes, including innate and conventional T cells, NK cells, B cells, DCs, and myeloid-derived leukocytes, as well as the genes that regulate these decisions. It was discovered that the severity of IAV infections is different between various ITC-deficient pigs compared to their littermate controls. In this scenario, it was found that the CD1- and MR1-restricted T cells play a central role in modulating the activation of innate immune cells in the early phases of infection, such as regulating the expression of maturation or activation markers and pro-inflammatory cytokines and chemokines with broad-spectrum activity in the upper and lower respiratory tract. Furthermore, our scRNAseq analysis revealed that ITCs modulate the regulatory circuitry that governs the differentiation and recruitment of different pulmonary leukocytes during an IAV response. In the case of NKT cells, CD1D-deficient pigs were more resistant to IAV infection than CD1D-intact pigs. Thus, differences were found in the secreted and cellular proteins and cell types that constitute the antiviral innate defense systems of CD1D-deficient and -intact pigs. In particular, pro- and anti-inflammatory cytokines, type I and III interferons, and Interferon

Stimulated Genes (ISGs) that are rapidly and transiently induced after infection, and which establish an antiviral state in infected and neighboring host cells were compared. Using two diverse influenza viruses, the protection was further confirmed. Monocle orders cells according to progress through their differentiation program. An overview of the machine learning process used to construct the minimum spanning tree (MST) that is used to arrange cells in pseudotime. pathogenic roles of ITCs. CA04 strain was included because of the zoonotic importance of this strain. Creating CD1A and CD1B double knockout pigs is an efficient approach to establish if any group 1 CD1-restricted T cell subset contributes to IAV immunity. Finally, we may find that pig group I CD1- and MR1- restricted T cells have no effect on the early phases of an IAV infection.

Publications:

Publications are under preparation

iii) **Proposed utilization of the experience in India:** The experience provided by this training definitely improved my technical, scientific and analytical skills. The exposure of various protocols of isolation of live cells from different organs as spleen, lymph nodes, lungs etc. and handling and growing these for different assays in the lab has made me confident in carrying out the work at my lab. This experience will be utilized me in designing the projects to isolate the cells for different animal viruses for various immunological analyses. This will further be utilized for studying the pathogenesis of the associated disease and also it will help in development of the new diagnostics and identification of new cell receptors/cell types. The techniques learned will help in performing the experiments and generating the reliable and concrete data, which can prove the hypothesis of my projects.

In animal / veterinary research, the role of different innate immune cell types in the disease initiation and progression, and development of diagnostic has not been explored much. So, the exposure of the various cell type isolation and characterization will be utilized to develop project in which the role of the innate immune cells in the pathogenesis of the animal disease conditions will be explored. This will help in establishing the lab, where cellular and molecular immunological work can be done and collaborations with the researchers interested in this area of research can be made. The use of various gene edited animals in understanding the immune role in infectious diseases during the study will help me in exploring and understanding the pathways or mechanism of the different disease processes by utilizing the comparative analyses for different specific effector molecules or receptors.

Place: Mukteshwar
Date: 16/08/2024

Signature of ICMR-IF

ICMR Sanction No. INDO/FRC/452/(Y-62) 2022-23-IH&HRD