

S-H. Lee¹, H. Lillehoj¹, Y-H Hong^{1,2}, C. Baldwin³, D. Tompkins³, J. LaBresh⁴, Y. Sullivan⁴, B. Wagner⁵



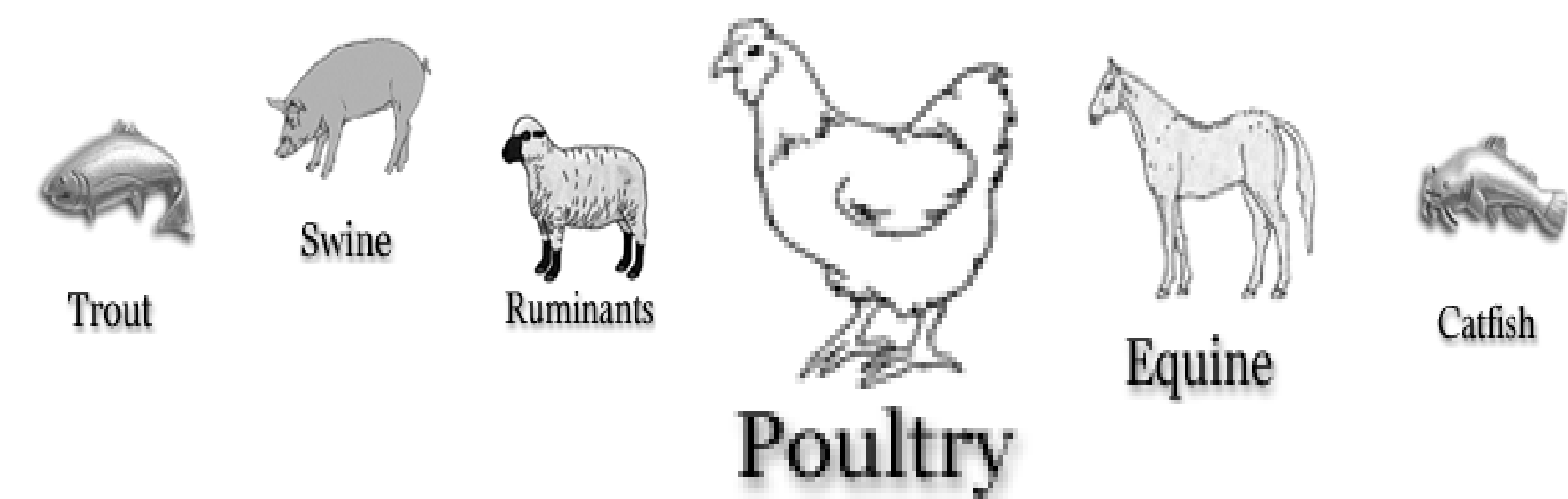
Agricultural Research Services, United States Department of Agriculture, Beltsville, MD, USA¹, Chung-Ang University, Anseong, Republic of Korea², University of Massachusetts, Amherst, MA, USA³, Kingfisher Biotech Inc., St. Paul, MN, USA⁴, Cornell University, Ithaca, NY, USA⁵



INTRODUCTION AND OBJECTIVES

- A major obstacle to advances in veterinary immunology and disease research is the lack of sufficient immunological reagents specific for veterinary animal species. In 2006, U. S. Veterinary Immune Reagent Network (VIRN) Consortium (www.vetimm.org) was developed to develop immune reagents against major veterinary and aquatic animal species.
- The initial priority list for poultry immune reagent development was focused on developing immune reagents against chicken cytokines and chemokines.
- This poster will report our progress with the poultry immune reagent development efforts of the U. S. VIRN from August 2006 to July 2011.
- During this period, 28 cytokine and chemokine genes have been cloned and recombinant cytokines become commercially available through the Kingfisher Biotechnology laboratory (www.kingfisherbiotech.com).
- In addition, transformed cell lines with a transient expression of cell surface molecules of CD25, CD80, CD83 and CD86 have been developed and these cells were used to immunize mice for mouse monoclonal antibodies (mAb) production.
- Because there are very few established chicken cell lines which can be used in *in vitro* cytokine/chemokine bioassays for poultry, validation of biological activity of recombinant chicken cytokines was carried out using primary lymphocytes and macrophages.
- Several stable mouse hybridomas secreting mAb against major chicken cytokines have been developed for immunoassay development.

These immune reagents will serve as valuable tools for basic and applied research in poultry immunology.



METHODS

Cytokines and chemokines

- 28 genes of chicken cytokine and chemokines (IL-1 β , IL-2, IL-4, IL-6, IL-7, IL-10, IL-12p35, IL-12p40, IL-15, IL-16, IL-17F, IL-17F, IL-18, IL-21, IL-22, GM-CSF, IFN- γ , TNFSF15 (TL1A), LITAF, LT, CCL4, CCL20, CXCL14, MIF, IL1R, IL-7R, IL-10R- β , IL-17R, IL-21R) have been cloned.
- The full-length genes were used for production of recombinant proteins in *E. coli* expression system.
- The full-length genes also have been sent to Kingfisher Biotechnology Laboratory (KF) for production of recombinant proteins in *Pichia pastoris* expression system.
- Some of their functional activities were measured in bioassays using chicken primary macrophages and lymphocytes or chicken cell lines.

Monoclonal antibody (mAb)

- BALB/c mice (6-week-old, Taconic Laboratories, Germantown, NY) were immunized intraperitoneally with 50 μ g of purified antigen protein (Ag) combined with Gerbu adjuvant (Accurate Chemical, Westbury, NY).
- Animals were boosted intraperitoneally with 25 μ g of the antigen/adjuvant emulsion at days 14 and 21, followed by injection of 25 μ g of Ag alone on days 28, 29, and 30.
- Mice were euthanized and spleen lymphocytes were fused with non-secreting mouse myeloma X63-Ag8.653 cells 3 days after the final boost.
- Hybridomas were selected in medium supplemented with HAT (Sigma, St. Louis, MO) and supernatants were screened for binding to CHO cells expressing *chAg*/IgG4 using a FACSCalibur flow cytometer (BD, Franklin Lakes, NJ).
- Each mAb was used for determination of the molecular size, immunolocalization, and functional activity assay.

Polyclonal antibody (pAb)

- New Zealand white Rabbits (3 months of age) were used.
- Subcutaneous injection was made in the loose skin behind the neck and shoulders.
- Polyclonal Abs have been used to develop chicken cytokine assay kit using capturing method.

Table 1. Cytokine, chemokine, and cell surface marker

Gene	Vector	Accession #	Size (bp)	mAb / pAb	Bioactivity
IL-2	pMAL	AF017645	432	mAb, pAb	✓
IL-6	pET32a(+), pMAL	NM_204628	726	mAb, pAb	
IL-15	pET32a	NM_204571	564	mAb, pAb	
IL-16	pcDNA3	AJ508678	1824	mAb	✓
IL-17F	pMAL	AJ493595	510	mAb, pAb	
IFN γ	pMAL	AH009942	481	mAb, pAb	✓
TNFSF15	pET32a	NM_001024578	720		✓
NK-lysin	pET32a	DQ186291	423		✓
LITAF	pET32a	AY765397	447		✓
IL-4	pET32a	NM_001007079	411	mAb, pAb	✓
IL-10	pET32a	NM_001004414	528	mAb	✓
CD80	pCR2.1	NM_001079739	951	mAb	✓
CD83	pCR.2.1	XM_418929	648	mAb	
CD86	pCR2.1	NM_001037839	852	mAb	
IL-1 beta	pcDNA1	Y15006	804		
IL-2 receptor γ	pcDNA3	NM_204596	636		
IL-18	pET32a	AJ277865	597	mAb	✓
Lymphotoctin	pcDNA3	AF006742	294		✓
CCL4 (MIP-1 β)	pBluescript-SK	NM_001030360	273		✓
CCL20 (MIP-3 α)	pET32a	NM_204438	303		✓
CXCL14	pcDNA3	NM_204617	1077		
IL-12p35	pET32a	NM_213588	618	mAb	
IL-12p40	pET32a	AY262752	948		
IL-17D	pET32a	EF570583	351	mAb, pAb	
GM-CSF	pET32a	NM_001007078	435		✓
MIF	pcDNA3.1	M95776	348		✓
IL-22	pET32a	NM_001199614	594		✓
IL7	pET32a	AM931037	557		
IL21	pET32a	NM_001024835	438	pAb	
IL1R	pET32a	NM_205485.1	1668		
IL7R	pET32a	NM_001080106	1383		
IL10R	pET32a	NM_204857.1	1026		
IL17R	pET32a	XM_416389	2229		
IL21R	pET32a	NM_001030640	1791	pAb	

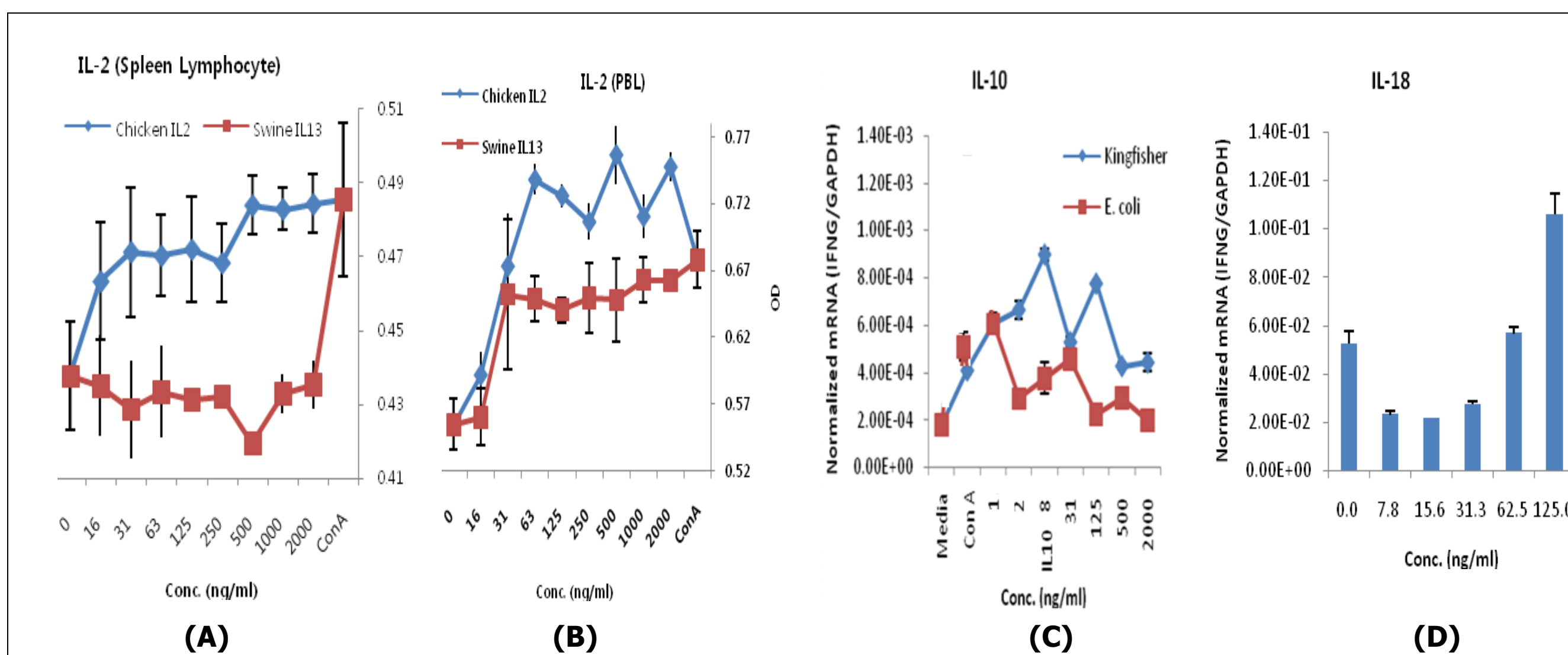


Fig. 1. Bioassays of recombinant cIL-2 (A, B), cIL-10 (C), and cIL-18 (D). Bioassays of recombinant cIL-2 (A, B) by cell proliferation assay. Chicken spleen and PBL blast cells were cultured with the serial dilutions of recombinant IL-2. Swine IL-13 was used as a negative control. IFN- γ transcript expression was measured following stimulation of chicken spleen lymphocytes (SPL) with the serial dilutions of recombinant cytokines. *E. coli* and/or yeast-expressed (Kingfisher) proteins were used for cIL-10 and cIL-18 assay.

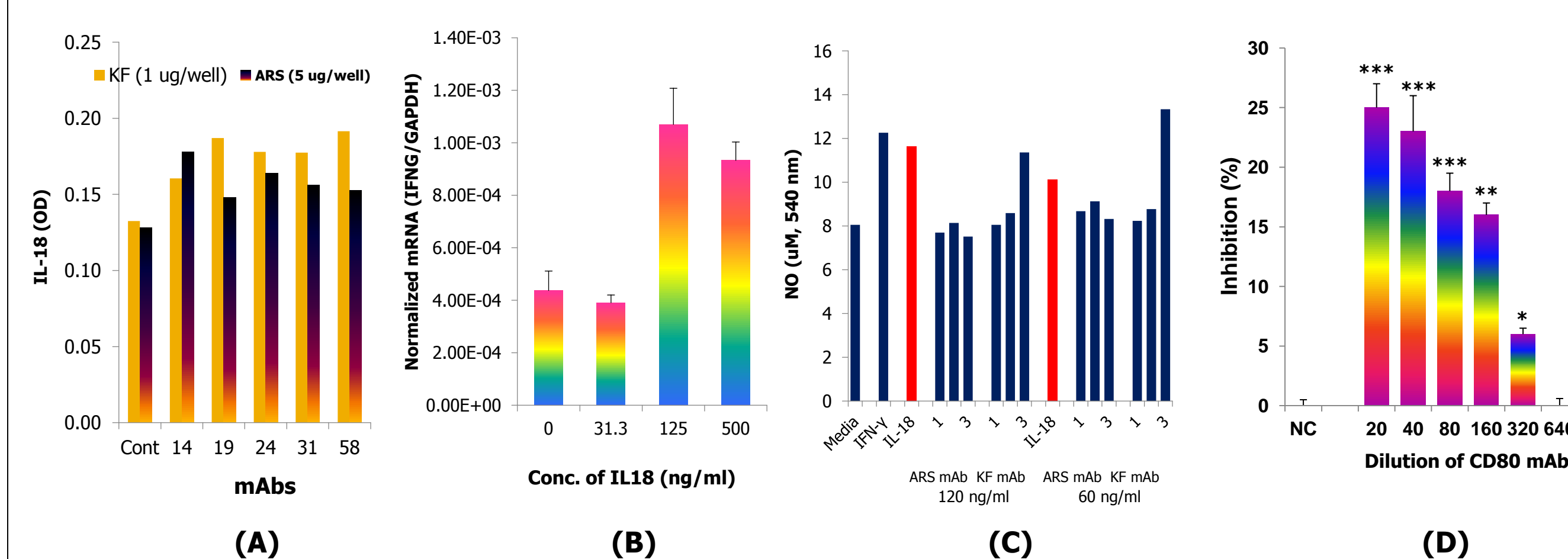


Fig. 2. Mouse mAb response against *E. coli* (ARS) and yeast-expressed (KF) rIL-18 (A). Expression of IFN-gamma in splenocytes cultured with IL-18 for 24 hours (B). NO produced in HD11 cells after 48 hours of incubation with supernatants of SPL cultured with IL-18 and mAb for 24 hours (C). Effect of *cCD80* mAb on IL-2 driven lymphoblast cell proliferation (D). Spleen lymphoblast cells (1.0×10^6 cells/ml) were cultured for 48 hr with medium alone or chicken IL-2 plus the indicated dilutions of *cCD80* mAb #112 or an undiluted isotype-matched negative control (NC) mAb. The percent inhibition of cell proliferation was calculated as described in the Materials and Methods. Each bar represents the mean \pm SD value ($n = 4$). * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

PROGRESSIVE RESULTS

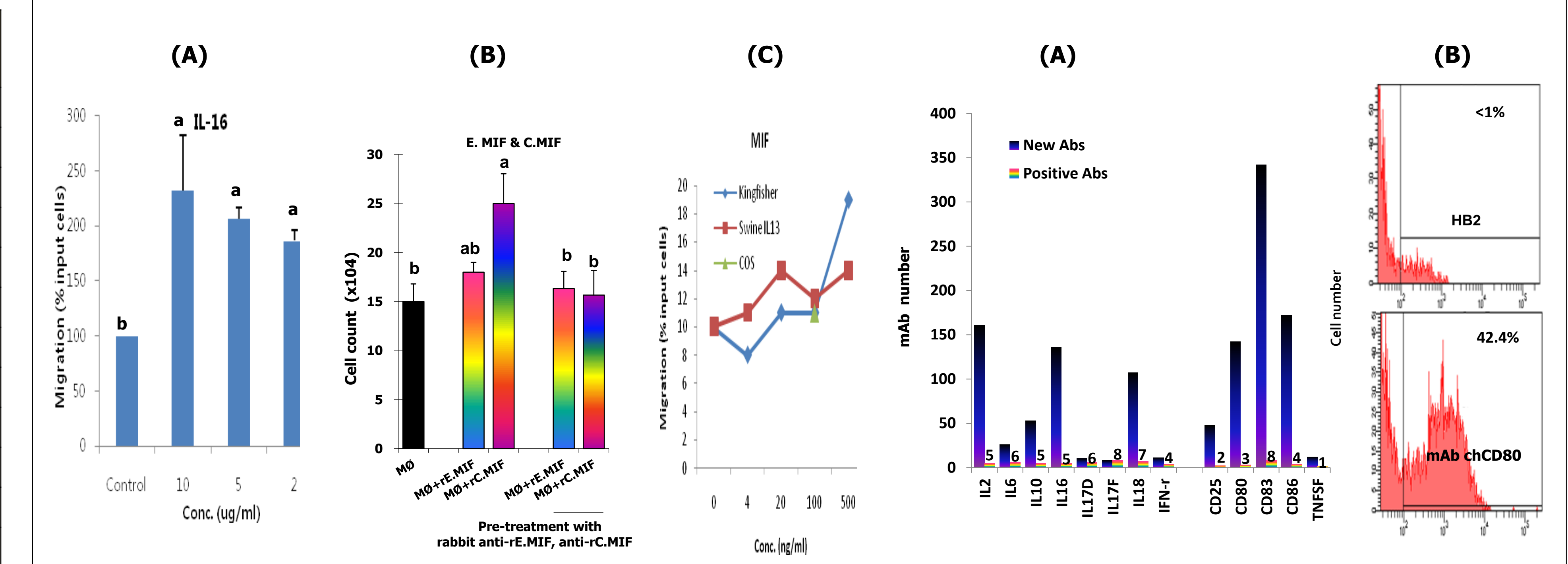


Fig. 3. Chemotaxis bioassays for recombinant cIL-16 (A) and MIF (B, C). Chicken macrophages were cultured with recombinant proteins and/or its pAb (B). Each bar represents the mean \pm SD ($n = 4$). Bars not sharing the same letter are significantly different ($P < 0.05$) according to the Duncan's multiple range test.

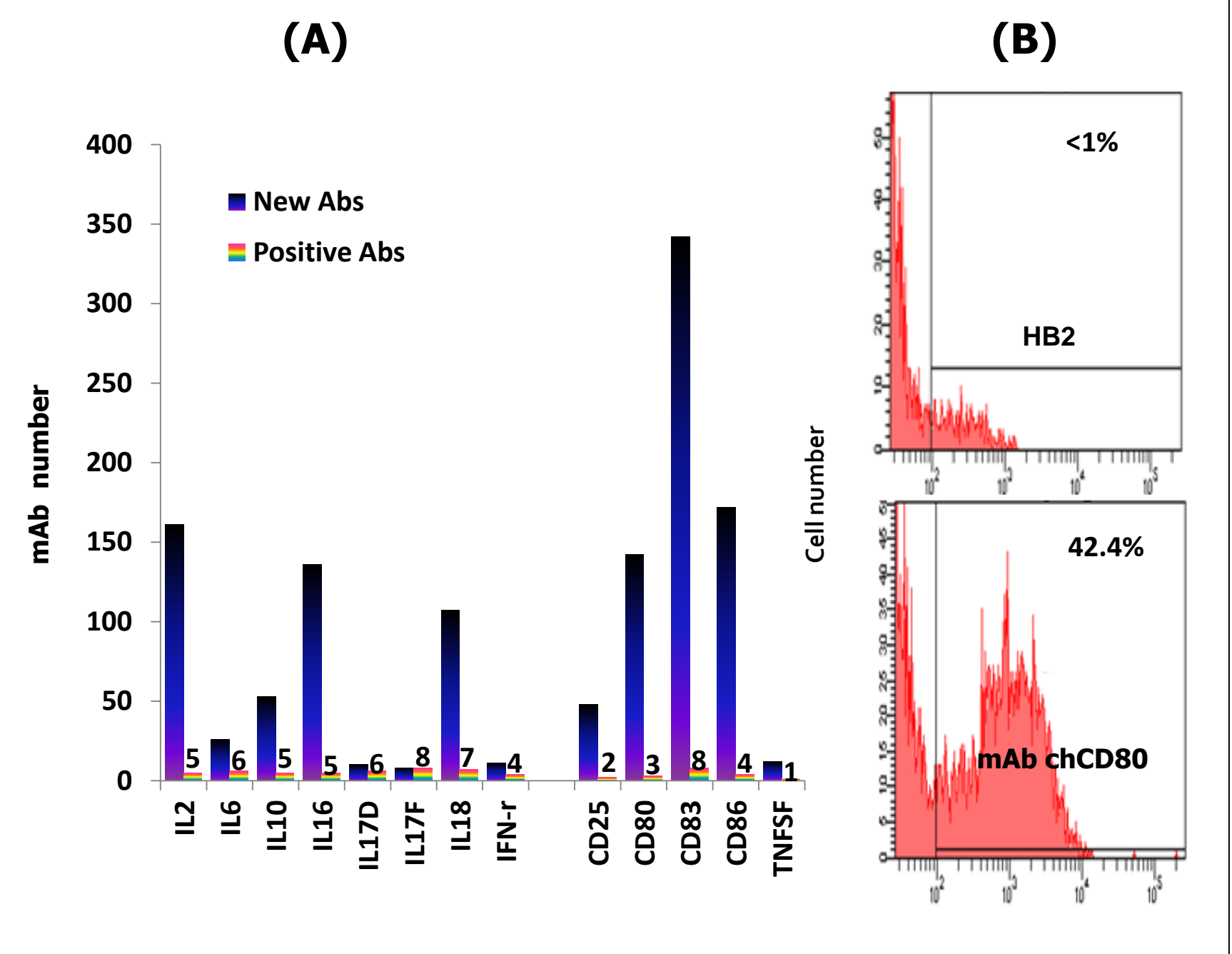


Fig. 4. mAbs against chicken cytokines (A). Flow cytometric analysis of *cCD80*-expressing cells (B). *CD80*/IgG4-CHO cells were stained with the HB2, an anti-human T cell mAb and *cCD80* mAb #112.

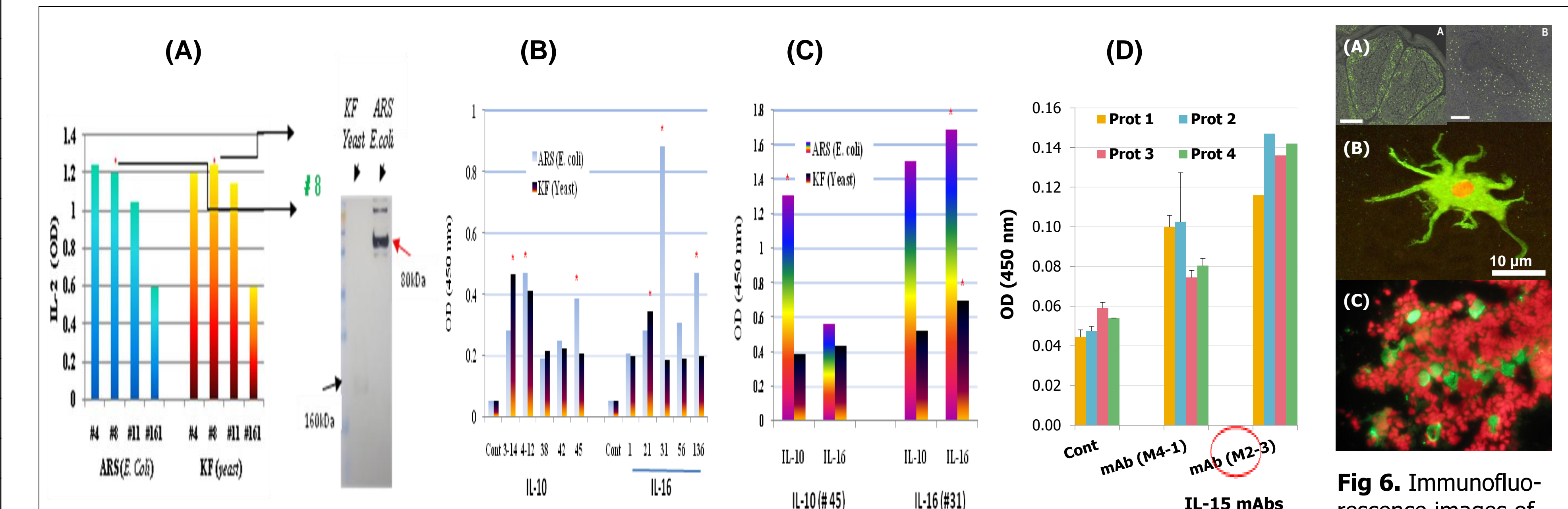


Fig. 5. Mouse mAb production against chicken IL-2 (A), IL-10, IL-16 (B, C), or IL-15 (D) was carried out and several stable hybridomas were selected.

Fig. 6. Immunofluorescence images of chCD25 (A), CD80 (B), and IL2 (C) in immune tissues.

Chicken Immune Reagent Planning

Molecule	Full length cDNA	Protein produced	Mice Immunized	Polyclonal sera reactivity	mAb Fusion	mAb reactivity	Assay development
IL-18	✓	✓	✓	✓	✓	✓	✓
IL-2	✓	✓	✓	✓	✓	✓	✓
IL-6	✓	✓	✓	✓	✓	✓	✓
IL-7	✓	✓	✓	✓	✓	✓	✓
IL-10	✓	✓	✓	✓	✓	✓	✓
IL-12p35	✓	✓	✓	✓	✓	✓	✓
IL-12p40	✓	✓	✓	✓	✓	✓	✓
IL-15	✓	✓	✓	✓	✓	✓	✓
IL-16	✓	✓	✓	✓	✓	✓	✓
IL-17A	✓	✓	✓	✓	✓	✓	✓
IL-17D	✓	✓	✓	✓	✓	✓	✓
IL-18	✓	✓	✓	✓	✓	✓	✓
IL-21	✓	✓	✓	✓	✓	✓	✓
IL-22	✓	✓	✓	✓	✓	✓	✓
CD25	✓	✓	✓	✓	✓	✓	✓
CD80	✓	✓	✓	✓	✓	✓	✓
CD83	✓	✓	✓	✓	✓	✓	✓
CD86	✓	✓	✓	✓	✓	✓	✓
CCL4	✓	✓	✓	✓	✓	✓	✓
CCL20	✓	✓	✓	✓	✓	✓	✓
CXCL14	✓	✓	✓	✓	✓	✓	✓
GM-CSF	✓	✓	✓	✓	✓	✓	✓
IFN- γ	✓	✓	✓	✓	✓	✓	✓
LITAF	✓	✓	✓	✓	✓	✓	✓
LT	✓	✓	✓	✓	✓	✓	✓
MIF	✓	✓	✓	✓	✓	✓	✓
TL1A	✓	✓	✓	✓	✓	✓	✓
IL1R1	✓	✓	✓	✓	✓	✓	✓
IL7R	✓	✓	✓	✓	✓	✓	✓
IL18R- β	✓	✓	✓	✓	✓	✓	✓
IL17R	✓	✓	✓	✓	✓	✓	✓
IL21R	✓	✓	✓	✓	✓	✓	✓
B-defensin	✓	✓	✓	✓	✓	✓	✓
IL-13	✓	✓	✓	✓	✓	✓	✓
VEGF	✓	✓	✓	✓	✓	✓	✓

Table 2. Commercially available reagents and Abs for poultry (Dr. Lillehoj's Lab)

Protein or Ab *	Details
IL2, IL-6, IL-4, IL-10, IL-16, IL-18, CCL4, CCL20, MIF, GM-CSF, IFN- γ , IL12p40, IL22, MIF, TNFSF15	Recombinant protein in Yeast
IL-16 GAPDH, IFN- γ , IL-1 β , IL-2, IL-6, IL-8, IL-12p40, IL-18, TGF- β 1, VEGF	BetSet primer set
IL-16	polyclonal Ab

• Accessibility and related information:
- Commercially available (Kingfisher Biotech Inc.)

PUBLICATIONS

- Hong et al., 2006a. Molecular cloning and characterization of chicken polysaccharide-induced TNF- α factor (LITAF). *Dev Comp Immunol.* 30: 919-929.
- Hong et al., 2006b. Analysis of chicken cytokine and chemokine gene expression following *E. acervulina* and *E. tenella* infections. *Vet Immunol Immunopathol.* 114:209-223.
- Park et al., 2007. Functional characterization of tumor necrosis factor superfamily 15 (TNFSF15) induced by lipopolysaccharides and *Emeria* infection. *Dev Comp Immunol.* 31:934-944.
- Hong et al., 2008. Cloning and functional characterization of chicken proinflammatory cytokine IL-17D. *Veterinary Immunology Immunopathol.* 126:1-8.
- Yoo et al., 2008. Monoclonal antibodies reactive with chicken interleukin-17. *Vet Immunol Immunopathol.* 121:359-363.
- Yoo et al., 2009. Molecular characterization of duck interleukin-17. *Vet Immunol Immunopathol.* 132: 318-322.
- Hong et al., 2010. Development and characterization of mouse monoclonal antibodies specific for chicken IL18. *Vet Immunol Immunopathol.* 138:144-148.
- Li et al., 2010. Cloning, prokaryotic expression and biological analysis of recombinant chicken IFN- γ . *Hybridoma* 29:1-6.
- Lee et al., 2011. Development and characterization of mouse monoclonal antibodies reactive with chicken CD80. *Comp Immunol Microbiol.* 34:273-9.
- Jeong et al., 2011. Molecular identification of duck and quail common cytokine receptor γ chain genes. *Vet Immunol Immunopathol.* 140: 159-165.

ACKNOWLEDGMENTS

This project was supported by the National Research Initiative of the USDA, U.S. Veterinary Immune Reagent Network Grant (NIFA #2010-65121-20649, USDA-CSREES #2005-01812) and the Next-Generation BioGreen 21 Program (No. PJ008084), Rural Development Administration, Republic of Korea. Special thanks to Dr. Cyril Gay, ARS National Program Leader in Animal Health, for initiating veterinary immunology project and S-I Jang, K-W Lee, M-S Park, D-K Kim, M. Nichols, S. Torreyson, and W-H Song for their technical assistance.