รายงานวิจัยฉบับสมบูรณ์

โครงการ การประเมินประสิทธิภาพของอินเตอร์ลิวคิน-10 small interfering RNA ในการเหนี่ยวนำการตอบสนองทางภูมิคุ้มกันแบบใช้เซลล์ต่อเชื้อไวรัส porcine reproductive and respiratory syndrome

โดย นาย วศิน เจริญตัณธนกุล และคณะ

กรกฎาคม 2552

สัญญาเลขที่ MRG5080167

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ผู้วิจัย	สังกัด
1 นายวศิน เจริญตัณธนกุล	มหาวิทยาลัยแม่โจ้
2 นายวัชระ กสิณฤกษ์	มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

ไวรัสพีอาร์อาร์เอส (porcine reproductive and respiratory syndrome; PRRS) กระตุ้นการสร้าง interleukin-10 (IL-10) ของสุกร ทำให้สุกรมีการตอบสนองทางภูมิคุ้มกันต่อไวรัสลดลง งานวิจัยนี้ศึกษาการใช้ phosphorothioate-modified IL-10 antisense oligodeoxynucleotides (IL-10AS) ที่จำเพาะกับ IL-10 mRNA ของสุกรเพื่อควบคุมการสร้าง IL-10 mRNA ใน peripheral blood mononuclear cell (PBMC) ของสุกรซึ่งบ่มร่วมกับไวรัสพีอาร์อาร์เอส และกระตุ้นด้วยสารกระตุ้นต่างๆ ได้แก่ concanavalin A และ phorbol 12-myristate 13-acetate/ionomycin พร้อมกันนี้ได้ศึกษาประสิทธิภาพของ IL-10AS ในการกระตุ้นการสร้าง IL-2, IL-4, tumor-necrosis factor alpha (TNFα), interferon alpha (IFNα) และ IFNγ mRNA ใน PBMC ของสุกรและ IFNγ protein ใน T lymphocyte ผลการศึกษาพบว่า IL-10AS สามารถยับยั้งการสร้าง IL-10 mRNA ได้อย่างมีนัยสำคัญ และช่วยกระตุ้นการสร้าง IFNγ mRNA และ protein ใน CD8β+ T lymphocyte IL-10AS ยับยั้งการสร้าง IL-2 mRNA อย่างมีนัยสำคัญ แต่ไม่มีผลต่อการสร้าง IL-4, TNFα และ IFNα mRNA ผลการศึกษาบ่งชี้ว่า IL-10AS สามารถนำไปพัฒนาเพื่อใช้เป็นสารกระตุ้นภูมิคุ้มกันแบบพึ่งเซลล์ (cell-mediated adaptive immunity) สำหรับวัคซีนป้องกันโรคพีอาร์อาร์เอสในอนาคต

คำสำคัญ: อินเตอร์ลิวคิน-10, antisense oligodeoxynucleotide, porcine reproductive and respiratory syndrome virus

Abstract

Up-regulation of interleukin-10 (IL-10) expression has been suggested to be the mechanism by which the porcine reproductive and respiratory syndrome virus (PRRSV) suppresses the innate and adaptive immune response in infected pigs. This study evaluates the potential of phosphorothioate-modified IL-10 antisense oligodeoxynucleotide (IL-10AS) specific to the translation initiation region of porcine IL-10 mRNA in enhancing innate and adaptive cytokine responses to PRRSV. Naïve peripheral blood mononuclear cells (PBMC) from eight PRRSV-seronegative pigs were transfected with IL-10AS *in vitro* prior to PRRSV inoculation and concanavalin A or phorbol 12-myristate 13-acetate plus ionomycin stimulation. The effects of IL-10AS on mRNA expression of IL-10, tumor necrosis factor alpha (TNFα), interferon alpha (IFNα), IL-2, IL-4, and IFNγ were tested by real-time PCR. The percentages of IFNγ-producing T cell subsets were determined by flow cytometry. Compared to the controls, the levels of IL-10 and IL-2 mRNA were significantly reduced, while those of IFNγ mRNA were increased, and TNFα, IFNα, and IL-4 mRNA were unchanged. An increase in the percent IFNγ^+ population was also observed in lymphocytes and CD8β^+ T cells. Our results suggest that IL-10AS has the potential to enhance the adaptive immune response to PRRSV infection.

Keywords: interleukin-10, antisense oligodeoxynucleotide, porcine reproductive and respiratory syndrome virus

หน้าสรุปโครงการ (Executive Summary)

งานวิจัยนี้ศึกษาประสิทธิภาพของ phosphorothioate-modified interleukin-10 antisense oligodeoxynucleotides (IL-10AS) ที่จำเพาะกับ IL-10 mRNA ของสุกรในการควบคุมการสร้าง IL-10 mRNA ใน peripheral blood mononuclear cell (PBMC) ของสุกรซึ่งบ่มร่วมกับไวรัสพีอาร์อาร์เอส (porcine reproductive and respiratory syndrome; PRRS) และกระตุ้นด้วยสารกระตุ้นต่างๆ ได้แก่ concanavalin A และ phorbol 12-myristate 13-acetate/ionomycin พร้อมกันนี้ได้ศึกษาประสิทธิภาพของ IL-10AS ในการกระตุ้นการสร้าง IL-2, IL-4, tumor-necrosis factor alpha (TNFα), interferon alpha (IFNα) และ IFNγ mRNA ใน PBMC ของสุกรและ IFNγ protein ใน T lymphocyte ผลการศึกษาพบว่า IL-10AS สามารถยับยั้งการสร้าง IL-10 mRNA ได้อย่างมีนัยสำคัญ และช่วยกระตุ้นการสร้าง IFNγ mRNA และ protein ใน CD8β+ T lymphocyte IL-10AS ยับยั้งการสร้าง IL-2 mRNA อย่างมีนัยสำคัญ แต่ไม่มีผลต่อการสร้าง IL-4, TNFα และ IFNα mRNA ผลการศึกษาบ่งชี้ว่า IL-10AS สามารถนำไปพัฒนาเพื่อใช้เป็นสารกระตุ้นภูมิคุ้มกันแบบพึ่งเซลล์ (cell-mediated adaptive immunity) สำหรับวัคซีนป้องกันโรคพีอาร์อาร์เอสในอนาคต

วัตถุประสงค์

1. เพื่อสังเคราะห์ IL-10AS ที่มีความจำเพาะต่อ IL-10 mRNA ของสุกร
2. เพื่อศึกษาปัจจัยที่มีผลต่อการเข้าสู่เซลล์ (transfection) PBMC ของ IL-10AS
3. เพื่อศึกษาความสามารถของ IL-10AS ในการยับยั้งการสร้าง IL-10 mRNA ใน PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและสารกระตุ้น
4. เพื่อศึกษาความสามารถของ IL-10AS ในการกระตุ้นการสร้าง IL-2, IL-4, TNFα, IFNα และ IFNγ mRNA ใน PBMC และ IFNγ protein ใน T lymphocyte ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและสารกระตุ้น

วิธีทดลอง

การสังเคราะห์ IL-10AS ที่มีความจำเพาะต่อ IL-10 mRNA ของสุกร

ทำการสังเคราะห์ IL-10AS ที่มีลำดับนิวคลีโอไทด์ (nucleotide) ที่จำเพาะกับ IL-10 mRNA ของสุกร ดังรายงานของ Sidahmed and Wilkie (2007) และ scramble control oligodeoxynucleotide จากบริษัท 1st BASE PTE จำกัด (Singapore) (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

การศึกษาปัจจัยที่มีผลต่อการเข้าสู่เซลล์ PBMC ของ IL-10AS

ทำการศึกษาปริมาณของ IL-10AS ที่เหมาะสมที่สามารถยับยั้งการสร้าง IL-10 mRNA ของ PBMC ที่บ่มร่วมกับ concanavalin A (conA) หรือ phorbol 12-myristate 13-acetate/ionomycin (PMA/I) ตรวจการมีชีวิตของเซลล์ที่ได้รับ IL-10AS ด้วยการย้อมสี trypan blue (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

การศึกษาความสามารถของ IL-10AS ในการยับยั้งการสร้าง IL-10 mRNA

ทำการบ่ม PBMC ที่ได้รับ IL-10AS กับไวรัสพีอาร์อาร์เอสและสารกระตุ้น conA หรือ PMA/I เปรียบเทียบความแตกต่างในการสร้าง IL-10 mRNA ของ PBMC ที่ได้รับ IL-10AS กับ PBMC ที่ไม่ได้รับ IL-10AS ด้วยวิธี real-time polymerase-chain reaction (PCR) (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

การศึกษาความสามารถของ IL-10AS ในการกระตุ้นการสร้าง IL-2, IL-4, TNFα, IFNα และ IFNγ mRNA และ IFNγ protein

ทำการบ่ม PBMC ที่ได้รับ IL-10AS กับไวรัสพีอาร์อาร์เอสและสารกระตุ้น conA หรือ PMA/I เปรียบเทียบความแตกต่างในการสร้าง IL-2, IL-4, TNFα, IFNα และ IFNγ mRNA ของ PBMC ที่ได้รับ

IL-10AS กับ PBMC ที่ไม่ได้รับ IL-10AS ด้วยวิธี real-time PCR วิเคราะห์จำนวน T lymphocyte และประชากรย่อยต่างๆ ได้แก่ CD4+, CD8β+, และ MAC320+ ที่สร้าง IFNγ ด้วยวิธี intracellular flow cytometry ทำการเปรียบเทียบค่าที่ได้กับค่าที่ได้จาก T lymphocyte ที่ไม่ได้รับ IL-10AS (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

ผลการทดลอง

ปัจจัยที่มีผลต่อการเข้าสู่เซลล์ PBMC ของ IL-10AS

IL-10AS ที่ปริมาณ 1 และ 2 μM สามารถยับยั้งการสร้าง IL-10 mRNA ได้อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มควบคุม IL-10AS ที่ปริมาณ 0.5 μM สามารถลดการสร้าง IL-10 mRNA ได้ แต่ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุม

การยับยั้งการสร้าง IL-10 mRNA ที่ตรวจพบนั้นไม่ได้เกิดจากการตายของ PBMC ที่ได้รับ IL-10AS เพราะสัดส่วนการมีชีวิตของเซลล์ภายหลังการ transfect ด้วย IL-10AS ไม่แตกต่างทางสถิติกับสัดส่วนการมีชีวิตของเซลล์ในกลุ่มควบคุม

ความสามารถของ IL-10AS ในการยับยั้งการสร้าง IL-10 mRNA

PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและ conA หรือ PMA/I มีการสร้าง IL-10 mRNA เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับ PBMC ที่บ่มร่วมกับสารกระตุ้นเพียงอย่างเดียว PBMC ที่ได้รับการ transfect ด้วย IL-10AS (2 μM) ก่อนบ่มร่วมกับไวรัสพีอาร์อาร์เอสและสารกระตุ้นมีระดับการสร้าง IL-10 mRNA ที่ลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับ PBMC ที่ไม่ได้รับ IL-10AS

เช่นเดียวกับผลการทดลองข้างต้น การสร้าง IL-10 mRNA ที่ลดลงไม่ได้เกิดจากการตายของ PBMC ที่ได้รับ IL-10AS เพราะสัดส่วนการมีชีวิตของเซลล์ภายหลังการ transfect ด้วย IL-10AS และบ่มด้วยไวรัสพีอาร์อาร์เอสและสารกระตุ้นไม่แตกต่างทางสถิติกับสัดส่วนการมีชีวิตของเซลล์ในกลุ่มที่ไม่ได้รับ IL-10AS

ความสามารถของ IL-10AS ในการกระตุ้นการสร้าง IL-2, IL-4, TNFα, IFNα และ IFNγ mRNA และ IFNγ protein

PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและ conA มีการสร้าง IL-2 mRNA เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับ PBMC ที่บ่มร่วมกับสารกระตุ้นเพียงอย่างเดียว PBMC ดังกล่าวไม่มีการสร้าง IL-4 mRNA แตกต่างจาก PBMC ของกลุ่มควบคุม

PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและ PMA/I มีการสร้าง IFNγ mRNA ลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับ PBMC ที่บ่มร่วมกับสารกระตุ้นเพียงอย่างเดียว PBMC ดังกล่าวไม่มีการสร้าง TNFα และ IFNα mRNA แตกต่างจาก PBMC ของกลุ่มควบคุม

PBMC ที่ได้รับการ transfect ด้วย IL-10AS (2 μM) ก่อนบ่มร่วมกับไวรัสพีอาร์อาร์เอสและสารกระตุ้นมีระดับการสร้าง IL-2 mRNA ที่ลดลงอย่างมีนัยสำคัญทางสถิติและมีระดับการสร้าง IFNγ mRNA เพิ่มขึ้น ($p>0.05$) เมื่อเทียบกับ PBMC ที่ไม่ได้รับ IL-10AS PBMC ดังกล่าวไม่มีการสร้าง IL-4, TNFα และ IFNα mRNA แตกต่างจาก PBMC ที่ไม่ได้รับ IL-10AS

Lymphocytes และ CD8β+ T lymphocytes จาก PBMC ที่ได้รับการ transfect ด้วย IL-10AS (2 μM) ก่อนบ่มร่วมกับไวรัสพีอาร์อาร์เอสและสารกระตุ้นมีระดับการสร้าง IFNγ protein เพิ่มขึ้น ($p>0.05$) เมื่อเทียบกับ lymphocytes และ CD8β+ T lymphocytes จาก PBMC ที่ไม่ได้รับ IL-10AS ไม่พบความแตกต่างของระดับการสร้าง IFNγ protein ใน CD4+ และ MAC320+ T lymphocyte ที่ได้รับและไม่ได้รับ IL-10AS

สรุปและวิจารณ์ผลการทดลอง

ไวรัสพีอาร์อาร์เอสกระตุ้นการสร้าง IL-10 mRNA และ IL-2 mRNA แต่ยับยั้งการสร้าง IFNγ mRNA ใน PBMC อย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับ PBMC ของกลุ่มควบคุม ไวรัสไม่มีผลต่อการสร้าง IL-4, TNFα และ IFNα mRNA

IL-10AS สามารถยับยั้งการสร้าง IL-10 mRNA ใน PBMC ของสุกรซึ่งบ่มร่วมกับไวรัสพีอาร์อาร์เอสและสารกระตุ้นต่างๆ IL-10AS สามารถกระตุ้นการสร้าง IFNγ mRNA และ protein ใน PBMC และ CD8β+ T lymphocyte ตามลำดับ IL-10AS ยับยั้งการสร้าง IL-2 mRNA อย่างมีนัยสำคัญ แต่ไม่มีผลต่อการสร้าง IL-4, TNFα และ IFNα mRNA

การที่ IL-10AS สามารถยับยั้งการสร้าง IL-10 mRNA และกระตุ้นการสร้าง IFNγ mRNA บ่งชี้ถึงประสิทธิภาพของ IL-10AS ที่สามารถกระตุ้นภูมิคุ้มกันแบบพึ่งเซลล์ (cell-mediated adaptive immunity) ต่อไวรัสพีอาร์อาร์เอสได้ อย่างไรก็ตาม การที่ IL-10AS ยับยั้งการสร้าง IL-2 mRNA อาจทำให้ภูมิคุ้มกันที่เกิดขึ้นไม่มีประสิทธิภาพเพียงพอสำหรับการป้องกันโรค

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

งานวิจัยในอนาคตควรศึกษาหาลำดับนิวคลีโอไทด์ของ IL-10AS ที่ไม่มี off-target effect ต่อ IL-2 mRNA

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 **Charerntantanakul W**. Adjuvants for porcine reproductive and respiratory syndrome virus vaccines. *Vet immuno immunopathol*. 2009; 129, 1-13.

1.2 **Charerntantanakul W**., Kasinrerk W. Interleukin-10 antisense oligodeoxynucleotide suppresses IL-10 expression and effects on innate and adaptive cytokine responses to porcine reproductive and respiratory syndrome virus. *Viral immunol*. Submitted for publication.

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงวิชาการ

สร้างเครือข่ายความร่วมมือทางวิชาการระหว่างหน่วยงานของผู้วิจัย (ภาควิชาชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยแม่โจ้) และอาจารย์ที่ปรึกษางานวิจัย (ภาควิชาเทคนิคการแพทย์ คณะเทคนิคการแพทย์ มหาวิทยาลัยเชียงใหม่) ผู้วิจัยได้รับคำแนะนำทางวิชาการในระหว่างทำการทดลองและสามารถใช้เครื่อง flow cytometry สำหรับตรวจวัดการแสดงออกของ IFNγ ภายในเซลล์ของลิมโฟซัยต์ subset ต่างๆ จากห้องปฏิบัติการของอาจารย์ที่ปรึกษางานวิจัย รวมถึงได้รับคำแนะนำในระหว่างการจัดเตรียมต้นฉบับ (manuscript) เพื่อการตีพิมพ์ในวารสารทางวิชาการระดับนานาชาติ

3. การเสนอผลงานในที่ประชุมวิชาการ

3.1 **Charerntantanakul W**., Kasinrerk W. Evaluation of interleukin-10 antisense oligodeoxynucleotides in induction of cell-mediated immune response to porcine reproductive and respiratory syndrome virus. The Second International Conference on Science and Technology for Sustainable Development of the Greater Mekong sub-region, Hanoi University of Agriculture, Gialam, Hanoi, **Vietnam**. October 2-3, 2008. *Oral presentation given* (20 minutes).

3.2 **Charerntantanakul W**., Kasinrerk W. Evaluation of interleukin-10 antisense oligodeoxynucleotides in induction of cell-mediated immune response to porcine reproductive and respiratory syndrome virus. The Thailand Research fund 8th annual meeting, Holiday inn Resort Regent Beach, Cha-am, Petchburi, Thailand. October 16-18, 2008. *Oral presentation given* (15 minutes).

ภาคผนวก

Veterinary Immunology and Immunopathology 129 (2009) 1–13

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Review paper

Adjuvants for porcine reproductive and respiratory syndrome virus vaccines

Wasin Charerntantanakul *

Department of Biology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

ARTICLE INFO

Article history:
Received 23 February 2008
Received in revised form 3 December 2008
Accepted 8 December 2008

Keywords:
Vaccine adjuvant
Porcine reproductive and respiratory syndrome virus
Cell-mediated immune response
Antibody

ABSTRACT

This review deals with present and past efforts in utilization of vaccine adjuvants for porcine reproductive and respiratory syndrome virus (PRRSV) vaccines. PRRSV vaccines elicit delayed and weak cell-mediated immune (CMI) and antibody responses after vaccination. Several kinds of vaccine adjuvants have been utilized to accelerate and magnify immune responses to PRRSV vaccines. These adjuvants include cytokines, chemical reagents, and bacterial products. Of 11 vaccine adjuvants tested, five (i.e. interleukin-2 (IL-2), IL-12, interferon α (IFNα), polyinosinic and polycytidylic acid, and cytidine–phosphate–guanosine oligodeoxynucleotides (CpG ODN)) significantly enhance CMI response to PRRSV vaccines. The response is characterized by proliferation, cytotoxicity, and IFNγ secretion of peripheral blood mononuclear cells or T cells in response to recall PRRSV antigens in vitro. Two (i.e. CpG ODN and cholera toxin) significantly enhance PRRSV-specific antibody response after vaccination. Two (i.e. IL-2 and CpG ODN) significantly enhance protective efficacy of PRRSV vaccines in challenge models. Improvement of immune responses to PRRSV vaccines should focus in future studies on assessing more vaccine adjuvants for their efficiency in enhancing both CMI and antibody responses and on identifying PRRSV components and strategies that down-modulate pig immune responses in order to devise vaccine adjuvants that can regulate such strategies of the virus.

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* Tel.: +66 53 873535x121; fax: +66 53 878225.
E-mail address: wasin@mju.ac.th.

Abbreviations: PRRSV, porcine reproductive and respiratory syndrome virus; CMI, cell-mediated immunity; MLV, modified-live virus; KV, killed virus; ORF, open reading frame; Poly IC, polyinosinic-polycytidylic acid; Poly ICLC, polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose; LMS, levamisole; CpG ODN, cytidine–phosphate–guanosine oligodeoxynucleotides; CT, cholera toxin; LPS, lipopolysaccharide; MPL, monophosphoryl lipid A; APC, antigen-presenting cell; DC, dendritic cell; MDC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; PBL, peripheral blood lymphocyte; PAM, pulmonary alveolar macrophage; NK, natural killer; Th, T-helper; Rp, recombinant porcine; Rh, recombinant human; Rb, recombinant bovine; IFN, interferon; TNFα, tumor-necrosis factor alpha; TLR, Toll-like receptor; Ig, immunoglobulin; MHC, major histocompatibility complex; PRV, pseudorabies virus; FMDV, foot and mouth disease virus; SSSK, swine streptococcic septicemia killed; MLN, mesenteric lymph node.

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doi:10.1016/j.vetimm.2008.12.018

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1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-sense single-stranded enveloped RNA virus of the family *Arteriviridae* (Meulenberg et al., 1993). The viral genome is approximately 15 kb in size and composed of nine open-reading frames (ORF), designated ORF1a, 1b, 2a, 2b, and 3-7 (Meulenberg et al., 1993). PRRSV causes respiratory disease in growing pigs and reproductive failure in breeding age swine (Christianson et al., 1992; Rossow et al., 1994). The virus causes approximately $560.32 millions in losses each year in the US swine industry (Neumann et al., 2005).

PRRSV infects myeloid antigen-presenting cells (APC) of the pigs, which include monocytes, macrophages, and dendritic cells (DC) (Charerntantanakul et al., 2006a; Loving et al., 2007; Thacker et al., 1998; Voicu et al., 1994). The virus elicits poor innate and adaptive immune responses after infection. Innate immune response determined by phagocytic ability, microbial killing activity, production of reactive oxygen species, e.g. superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), and production of pro-inflammatory cytokines, e.g. interleukin-1 (IL-1), tumor necrosis factor alpha (TNFα), and type I interferon (IFN) was significantly reduced in PRRSV-infected pulmonary alveolar macrophages (PAM), pulmonary intravascular macrophages, and peripheral blood mononuclear cells (PBMC) (Albina et al., 1998; Chiou et al., 2000; Lopez-Fuertes et al., 2000; Van Reeth et al., 1999). The levels of IL-1, TNFα, and type I IFN were barely detected in serum and bronchoalveolar lavage fluid of PRRSV-infected pigs (Albina et al., 1998; Van Reeth et al., 1999). Humoral immune response to PRRSV was somewhat delayed. PRRSV-specific antibodies were detected after one to two weeks of infection and majority of them were against non-neutralizing epitopes of the virus (Mulupuri et al., 2008; Yoon et al., 1995). Neutralizing antibodies to PRRSV appeared approximately four weeks after infection and had relatively low titers throughout the course of infection (Wills et al., 1997; Yoon et al., 1995). Cell-mediated immune (CMI) response to PRRSV determined by lymphocyte blastogenesis and adaptive cytokine production was delayed, primarily detectable in the in vitro recall response of PBMC around 4–8 weeks after infection (Bassaganya-Riera et al., 2004; Bautista and Molitor, 1997; Charerntantanakul et al., 2006b; Lopez Fuertes et al., 1999; Meier et al., 2003; Royaee et al., 2004).

Innate and adaptive immune responses are required for effective PRRS protection (Murtaugh et al., 2002). Innate cytokines, i.e. TNFα and type I IFN inhibit PRRSV replication and activate porcine APC to produce pro-inflammatory cytokines and stimulate lymphocyte proliferation (Albina et al., 1998; Buddaert et al., 1998; Johansson et al., 2003; Lopez-Fuertes et al., 2000; Murtaugh and Foss, 2002). Neutralizing antibodies suppress PRRSV infection and protect pigs from viremia and clinical diseases following homologous PRRSV challenge infection (Lopez and Osorio, 2004). Adaptive cytokine, i.e. IFNγ effectively inhibits PRRSV replication and enhances porcine APC activation and pro-inflammatory cytokine production (Bautista and Molitor, 1999; Rowland et al., 2001).

Vaccination is a strategy currently used to control clinical diseases caused by PRRSV. There are at least two types of PRRSV vaccine commercially available; a modified-live virus (MLV) vaccine and a killed virus (KV) vaccine. In most vaccine producers, PRRSV MLV vaccines are recommended for use in sows and gilts for the reduction of viremia or reproductive failure and in piglets for the reduction of viremia or respiratory disease. The vaccines are efficacious but induce delayed antibody and CMI responses (Charerntantanakul et al., 2006b; Foss et al., 2002; Meier et al., 2004). PRRSV KV vaccines are recommended for use in sows and gilts for the reduction of reproductive failure, or in sows, gilts, boars, and piglets for the reduction of reproductive and respiratory disorders. The efficacy of PRRSV KV vaccines is less than ideal. The vaccines induce poor CMI response and do not induce an antibody response (measured by IDEXX ELISA) (Bassaganya-Riera et al., 2004; Piras et al., 2005; Zuckermann et al., 2007).

Several experimental PRRSV vaccines have been developed for more potent, safe, and efficacious vaccines. These are, for examples, DNA, recombinant peptide, and synthetic peptide vaccines. To date, DNA vaccines induce antibody and CMI responses and show some efficacy in pig protection from viremia and respiratory diseases (Pirzadeh and Dea, 1998; Rompato et al., 2006; Xue et al., 2004). Recombinant and synthetic peptide vaccines are not as potent and efficacious as MLV and DNA vaccines. They require numerous injections, yet do not confer protection (Charerntantanakul et al., 2006b; Pirzadeh and Dea, 1998).

PRRSV vaccines (i.e. MLV and DNA) generally provide full protection against PRRSV that are antigenically

homologous to the vaccine virus or vaccine antigen. The vaccines, however, provide only partial protection against heterologous PRRSV infection and clinical diseases (Mateu and Diaz, 2008). Poor immunogenicity and poor cross protective efficacy of PRRSV vaccines are current important issues in PRRSV immunology.

Recent interest in improving immune response to PRRSV vaccines is the utilization of vaccine adjuvants. Several kinds of vaccine adjuvants have been studied for their ability to potentiate immune response to PRRSV vaccines. These adjuvants include cytokines, chemical reagents, and bacterial products. These vaccine adjuvants possess either T helper1 (Th1)- or Th2-inducing properties. Some of them also possess innate immune stimulatory property (i.e. APC activation and pro-inflammatory cytokine production). To date, commercial PRRSV MLV vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim, St. Joseph, MO), in-house KV vaccine, DNA vaccine, and recombinant and synthetic peptide vaccine have tried these adjuvants. Only some of them, however, received appreciative results of enhanced immune response or increased vaccine efficacy. Future studies are, therefore, required to seek new vaccine adjuvants that can potentiate immunogenicity and protective efficacy of PRRSV vaccines.

The objective of this work is to review current knowledge of vaccine adjuvants for PRRSV vaccines. The review covers in vitro and in vivo effects of vaccine adjuvants in pigs and other veterinary models. The first part of the review covers immunostimulatory effects of cytokines, either in the form of recombinant protein or cytokine-encoded plasmid. The second part describes immunostimulatory effects of various chemical reagents. The third part reviews immunostimulatory effects of bacterial products. The last part provides summary and suggestions for future study on PRRSV vaccine adjuvants.

2. Cytokines

2.1. Combination of IL-1 and IL-6

A combination of recombinant porcine (rp) IL-1 and rpIL-6 was used to enhance Th1 and Th2 responses to PRRSV MLV vaccine. In pigs, IL-1 and IL-6 effectively promote lymphocyte trafficking to the draining lymph nodes, lymphocyte proliferation, immunoglobulin (Ig) production, hematopoiesis, and natural killer (NK) activity of peripheral blood lymphocytes (PBL) (Binns et al., 1992; Emery et al., 1996; Knoblock and Canning, 1992; Murtaugh, 1994; Murtaugh et al., 1996). IL-1 and IL-6 act synergistically in enhancing proliferation of thymocytes and peripheral blood T cells (Dinarello, 1994). The adjuvanticity of IL-1 and IL-6 has been reported in pigs immunized with *Streptococcus suis* vaccine (Blecha et al., 1995) and pseudorabies virus (PRV) MLV vaccine, respectively (Ling-Hua et al., 2006) (Table 1).

A combination of rpIL-1 and rpIL-6 was administered to pigs by conditions described in Table 2. The cytokines did not enhance PRRSV-specific antibody (determined by IDEXX and anti-ORF5 ELISA) and PRRSV-specific CMI responses (determined by ELISPOT assay for IFNγ production by PBMC and delayed-type hypersensitivity (DTH) test), compared to pigs vaccinated with PRRSV MLV vaccine alone (Foss et al., 2002). Pigs receiving MLV vaccine either with or without cytokine adjuvant were completely protected from viremia after VR-2332 (a parental strain of MLV) challenge. The absence of adjuvant effect of rpIL-1 and rpIL-6 was proposed to be due to insufficient dose and/or inappropriate timing of cytokine administration, or ineffectiveness of the cytokines in potentiating anti-PRRSV immune responses. In addition, it might be due to the existence of substantial immunogenicity of PRRSV MLV vaccine which minimizes the opportunity for rpIL-1 and rpIL-6 adjuvanticity to be detected. PRRSV MLV vaccine can generate robust antibody (determined by IDEXX ELISA) and some CMI responses (determined by ELISPOT assay for IFNγ production by PBMC). The level of immune responses elicited by the vaccine is high enough for clinical protection from homologous virus challenge and for partial protection from heterologous virus challenge. The vaccine adjuvant is utilized in order to improve immune responses and thereby increase cross protective efficacy of the vaccine. It might be possible that rpIL-1 and rpIL-6 have little opportunity to show significantly their contribution to anti-PRRSV immune enhancement in the presence of substantial immunogenicity of PRRSV MLV vaccine. More effective vaccine model for elucidation of rpIL-1 and rpIL-6 adjuvant property may be PRRSV KV and peptide vaccines, since these vaccines by themselves barely generate anti-PRRSV immunity. The immunoenhancing property of rpIL-1 and rpIL-6 should be detected more clearly in these vaccine models than in PRRSV MLV vaccine. In addition, future experiments may optimize dose and administration schedule of rpIL-1 and rpIL-6 for more effective condition. Also, future studies may be done using heterologous PRRSV for challenge infection which will allow an evaluation of enhanced immune responses, if any, in cross protection.

2.2. IL-2

IL-2 was used in the form of genetic adjuvant to enhance Th1 response to PRRSV DNA vaccine. In pigs, the cytokine promotes proliferation and NK activity of PBL (Hennessy et al., 1990; Knoblock and Canning, 1992). Its adjuvanticity has been reported in foot and mouth disease virus (FMDV) DNA (Wong et al., 2002), inactivated or subunit PRV (Kawashima and Platt, 1989; Lin et al., 2005), *S. suis* (Blecha et al., 1995), and *Escherichia coli* J5 vaccines (Hennessy et al., 1990) (Table 1). The IL-2 DNA was cloned into plasmids expressing PRRSV ORF5 (ORF5/IL-2) and ORF7 (ORF7/IL-2) (Xue et al., 2004), or into separate expression plasmids (Rompato et al., 2006) (Table 2). By either administration strategy, pigs receiving porcine IL-2 showed significantly reduced virus loads in their serum, PAM, and lymphoid tissues after homologous virus challenge (i.e. PRRSV strain used for generation of DNA vaccine), when compared to pigs receiving PRRSV DNA vaccine alone. Pigs immunized with PRRSV ORF7 DNA vaccine and separate IL-2 expression plasmids also demonstrated significantly increased T cell proliferation

Table 1
Experimental vaccine adjuvants utilized in other swine vaccines.

Adjuvant					Vaccine[a]	Day of challenge	Adjuvant effects	References
Type	Form	Route	Dose/ administration	Day[b]				
IL-1β	rb Protein[c]	i.m.	10 μg/kg	0, 1, 2	*S. suis*	21	↑%$CD8^+$ T cells; Ab; ↓mortality	Blecha et al. (1995)
IL-2	Plasmid DNA	i.m.	200 μg	0, 28	FMDV DNA (d 0, 28)	38	↑T cell proliferation	Wong et al. (2002)
	rp Protein[d]	i.m.	2×10^5 units	0, 7, 14	PRV inactivated	56	↑Neutralizing Ab; cytotoxicity and IFNγ production of PBMC; ↓nasal virus excretion	Lin et al. (2005)
	rh Protein[c]	s.c.	10^5 unit/kg	0, 1, 2, 3, 4 21, 22, 23, 24, 25	PRV subunit (d 0, 21)	42	↑Neutralizing Ab	Kawashima and Platt (1989)
	rb Protein[c]	i.m.	25 μg/kg	0, 1, 2	*S. suis*	21	↑%$CD8^+$ T cells; NK activity of PBMC; Ab	Blecha et al. (1995)
	rh Protein[c]	i.m.	10^5 unit/kg	0, 1, 2, 3, 4	*E. coli* J5	–	↑NK activity of PBMC	Hennessy et al. (1990)
	Plasmid DNA	i.m.	200 μg	0	PRV DNA	21	No	Somasundaram et al. (1999)
IL-6	Plasmid DNA	s.c.	10 μg	0	PRV MLV	–	↑Ab; lymphocyte proliferation	Ling-Hua et al. (2006)
	rp Protein[d]	i.m.	2×10^5 units	0, 7, 14	PRV inactivated	56	No	Lin et al. (2005)
	Plasmid DNA	i.d.	7.5 μg	0, 27	Swine influenza virus HA–DNA (d 0, 27)	48	No	Larsen and Olsen (2002)
IL-12	rh Protein[e]	i.m.	2 μg	0, 14 (2 h before and after each vaccination)	PRV inactivated (d 0, 14)	35	↑#IFNγ-secreting PBMC	Zuckermann et al. (1998)
	IL-12p35-and IL-12p40-Encoded plasmid DNA	i.m.	500 μg each	0, 21, 42	*S. japonicum* DNA (d 0, 21, 42)	51, 82	↓#Hepatic *S. japonicum*	Zhu et al. (2004)
IFNγ	Plasmid DNA	i.m.	200 μg	0	PRV DNA	21	No	Somasundaram et al. (1999)
IFNα	Plasmid DNA	i.m.	200 μg	0	PRV DNA	28	No	Dufour et al. (2000)
Poly IC	–	i.p.	1 mg/kg	0	FMDV	28	↑Neutralizing Ab	Cunliffe et al. (1977)
Poly ICLC	–	Orally	0.25 μg/kg	0	TGEV MLV (d 0,10)	–	↑Neutralizing Ab	Derbyshire and Lesnick (1990b)
LMS	–	i.m.	2.5 mg/kg	−3, −2, −1	*E. coli*	7	↑T cell and macrophage activation and migration to MLN and ileal Peyer's patches	Bozic et al. (2003); Bozic et al. (2006)
CpG ODN	ssDNA	s.c.	10 μg	0	PRV MLV	–	↑Ab; lymphocyte proliferation	Ling-Hua et al. (2006); Linghua et al. (2006a)
	Plasmid DNA	i.m.	200 μg	0	PRV DNA	21	↑Ab; IFNγ expression of PBMC; ↓nasal virus excretion and mortality	Dory et al. (2005)
	ssDNA	i.m.	500 μg/kg	0, 14	SSSK (d 0, 14)	–	↑Ab; proliferation, IFNγ, IL-6, MHC II, and CD14 expression by PBMC	Linghua et al. (2006c, 2007b); Linghua et al. (2006b)
	ssDNA	i.m.	250 μg/kg	0	*P. multocida*	–	↑Ab; proliferation, IFNγ, and IL-6 of PBMC	Zhang et al. (2007)
CpG ODN (cont)	ssDNA	s.c.	1 mg	0, 21	*A. pleuro–pneumoniae* OmlA (d 0,21)	35	↑Ab; ↓pneumonia	Alcon et al. (2003)
	ssDNA	i.m.	500 μg	0, 17	*E. coli* F4 fimbriae (d 0,17)	25	↑Proliferation of PBMC; ↓fecal *E. coli* shedding	Van der Stede et al. (2005)
CT	Commercial (Sigma)	i.n.	25 μg	0, 21, 35	*A. suum* antigens (d 0, 21, 35)	42	↑Ab; IL-4 and IL-10 expression of PBMC	Tsuji et al. (2004)
	Source not available	Orally	50 μg	0, 1, 2, 16, 35	F4/HSA	–	Ab	Verdonck et al. (2005a)
	Source not available	Orally	25 μg	0, 1, 2, 16	RecombinantFaeG	24	↑Ab; proliferation of PBMC; ↓fecal *E. coli* shedding	Verdonck et al. (2005b)

[a] In case of repeated vaccinations, the days of vaccination are placed in the parenthesis.
[b] Day of vaccine adjuvant administration after vaccination. Vaccination day refers to day 0 of the experiment.
[c] Recombinant protein expressed in *E. coli* (rb, recombinant bovine; rh, recombinant human).
[d] Recombinant protein expressed in *Pichia pastoris* (rp, recombinant porcine).
[e] Recombinant protein expressed in Sf21 insect cells.

Table 2
Adjuvants in PRRSV vaccine.

Adjuvant					Vaccine[a]	# Pigs/ group	Day of challenge	Adjuvant effects	References
Type	Form	Route	Dose/ administration	Day[b]					
IL-1+ IL-6	rp Protein[c]	i.m.	20 μg	0, 2, 4, 7	Ingelvac®	3	42	No	Foss et al. (2002)
IL-2	Plasmid DNA	i.m.	500 μg	0, 14, 28, 42	ORF5 DNA, ORF7 DNA	3	63	↓Virus load in tissues and lung lesion	Xue et al. (2004)
	Plasmid DNA	i.d.	60 μg	−1, 0, 1, 20, 21, 22	ORF7 DNA (d 0, 21)	4	49	↑T cell proliferation, ↓virus load in serum and PAM	Rompato et al. (2006)
IL-4	Plasmid DNA	i.d.	60 μg	−1, 0, 1, 20, 21, 22	ORF7 DNA (d 0, 21)	4	49	No	Rompato et al. (2006)
IL-12	rp Protein[d]	i.m.	4 μg	1	Ingelvac®	10	55	↑%$CD4^+CD8^+$ and $CD8^+\gamma\delta^+$ T cells expressing IFNγ	Charerntantanakul et al. (2006b)
	rp Protein[c]	i.m.	20 μg	0, 2, 4, 7	Ingelvac®	3	42	↑#IFNγ-secreting PBMC	Foss et al. (2002)
	rp Protein[c]	i.m.	40 μg	0, 1	Ingelvac®	5	–	No	Meier et al. (2004)
	Plasmid DNA	i.m.	200 μg	0	Ingelvac®	5	56	No	Meier et al. (2004)
	Plasmid DNA	i.d.	5 μg	0	Ingelvac®	5	–	↑#IFNγ-secreting PBMC	Meier et al. (2004)
IFNγ	Plasmid DNA	i.m.	500 μg	0, 14, 28, 42	ORF5 DNA, ORF7 DNA	3	63	↓virus load in tissues and lung lesion	Xue et al. (2004)
IFNα	Plasmid DNA	i.m.	200 μg	0	Ingelvac®	5	56	↑#IFNγ-secreting PBMC	Meier et al. (2004)
	Plasmid DNA	i.m.	200 μg	0	Ingelvac®	5	–	↑#IFNγ-secreting PBMC	Royaee et al. (2004)
	Plasmid DNA+ rp protein[e]	i.m.	400 μg (DNA) 10^5 U (protein)	−1, 0, 1	Ingelvac®	10	55	No	Charerntantanakul et al. (2006b)
Poly IC	–	i.m.	0.25 mg/kg	0	Ingelvac®	6	–	↑#IFNγ-secreting PBMC	Meier et al. (2004)
Poly ICLC	–	i.m.	0.4 mg	1	Ingelvac®	10	55	No	Charerntantanakul et al. (2006b)
CpG ODN	ssDNA	s.c.	50 μg	0	In-house KV	10	–	↑%$CD4^+$ and $CD8^+$ T cells, and Ab titer	Linghua et al. (2007c); Linghua et al. (2006d)
CpG ODN (cont)	ssDNA	s.c.	200 μg/kg	0	In-house KV	5	28	↑Ab; proliferation, IFNγ, and MHC class II expression of PBMC. ↓mortality	Linghua et al. (2007c)
	ssDNA	i.n.	1 mg/kg	0, 21	In-house KV	10	–	↑Ab; proliferation, IFNγ, and IL-6 production of PBMC	Zhang et al. (2007)
LMS[f]	–	i.m.	0.25–2% v/v	0, 14	China commercial KV (d 0,14)	7	–	At 0.5% v/v; ↑Ab; IL-2 and IFNγ production and proliferation of PBMC. At 2% v/v;↑ IL-4 and IL-10 production.	Kang et al. (2005)
CT	Commercial (Sigma)	i.m.	20 μg	0, 2, 4, 7	Ingelvac®	3	42	↑GP5-specific Ab	Foss et al. (2002)
	CT-A1-deleted peptides	Orally	1 mg	0	Recombinant PRRSV N peptides	4	–	↑Anti-N Ab in intestinal tract	Hyland et al. (2004)
	Commercial (Sigma)	i.m.	60 μg	0, 14, 28	Synthetic ORF5 ectodomain peptides (d 0,14,28)	10	55	No	Charerntantanakul et al. (2006b)
LPS	–	i.m.	Proprietary	0	Ingelvac®	10	55	No	Charerntantanakul et al. (2006b)

[a] In case of repeated vaccinations, the days of vaccination are placed in the parenthesis.
[b] Day of vaccine adjuvant administration after vaccination. Vaccination day refers to day 0 of the experiment.
[c] Recombinant protein expressed in *Pichia pastoris* (rp, recombinant porcine).
[d] Recombinant protein expressed in Chinese hamster ovary cells.
[e] Recombinant protein expressed in *E. coli*.
[f] Study in murine model.

in the recall stimulation in vitro after the second immunization of the vaccine (Rompato et al., 2006). Future IL-2 studies may evaluate cytokine contribution in cross protection against heterologous PRRSV challenge.

2.3. IL-4

IL-4 was used in the form of genetic adjuvant to enhance Th2 response to PRRSV DNA vaccine. In pigs, IL-4

induces proliferation of mitogen-stimulated $CD4^+$ T cells; generation of monocyte-derived DC (MDC) in combination with granulocyte macrophage-colony stimulating factor; and suppression of pro-inflammatory cytokines favoring Th1 differentiation, e.g. IL-1, IL-18, and TNFα (Foss et al., 2003; Nuntaprasert et al., 2005; Zhou et al., 1994). The cytokine has been utilized as adjuvant only in experimental human vaccines but not in any veterinary vaccine. It significantly enhanced antigen-specific antibody production in rhesus macaques vaccinated with human immunodeficiency virus-1 (HIV-1) env/rev and simian immunodeficiency virus gag/pol-encoded DNA (Kim et al., 2000). The cytokine did not demonstrate any adjuvant effect when administered in mice immunized with herpes simplex virus type 2 DNA vaccine (Sin et al., 1999) and influenza virus DNA vaccine (Lee et al., 1999).

When utilized as adjuvant for PRRSV DNA vaccine, the IL-4-encoded plasmids were injected several times in pigs immunized with PRRSV ORF7 DNA vaccine (Rompato et al., 2006). Plasmid expression of IL-4 and PRRSV nucleocapsid (N) protein was confirmed by indirect ELISA following plasmid injection in BALB/c mice. Pigs that received ORF7 DNA vaccine plus IL-4-encoded plasmids did not develop any improved antibody (determined by IDEXX ELISA) and CMI responses (determined by lymphocyte proliferation in response to recall PRRSV in vitro) or clinical protection after homologous PRRSV challenge (determined by virus load in serum), compared to pigs that received ORF7 DNA vaccine alone (Rompato et al., 2006) (Table 2). The lack of IL-4 adjuvanticity to PRRSV DNA vaccine was proposed to be attributed to insufficient levels of cytokine expression in vivo, insufficient dose and/or inappropriate timing of cytokine administration, or inefficiency of IL-4 in potentiating anti-PRRSV immunity. In addition, it might be attributed to lack of expression or insufficient levels of N protein production in pigs in vivo by DNA vaccine. In the absence of vaccine antigens or presence of inadequate amount of antigens, the cytokine cannot work efficiently as adjuvant. Future studies on PRRSV DNA vaccine should determine DNA expression of PRRSV N protein in pigs in vivo. Also, more studies on IL-4 adjuvanticity are required in pigs as well as in other veterinary species to evaluate its adjuvant property.

2.4. IL-12

IL-12 was used to enhance Th1 response to PRRSV MLV vaccine. In pigs, rpIL-12 promotes proliferation and IFNγ production of T and NK cells; IgG and IgA production of B cells; and IFNγ production of PAMs (Carter and Curiel, 2005; Domeika et al., 2002; Foss et al., 1999b). Recombinant human (rh) IL-12 (approximately 85% amino acid sequence homology to porcine IL-12 (Foss and Murtaugh, 1997)) promotes cytotoxicity and TNFα production of porcine NK cells (Cho et al., 1996). Its adjuvanticity has been reported in numerous experimental human and some veterinary vaccines (Toka et al., 2004; Uzonna et al., 2003). In pigs, IL-12 has been utilized as vaccine adjuvant for inactivated PRV (Zuckermann et al., 1998) and *Schistosoma japonicum* Chinese strain 23-kDa membrane protein-encoded DNA vaccines (Zhu et al., 2004) (Table 1).

IL-12 is efficient in suppressing PRRSV replication in vitro and in vivo (Carter and Curiel, 2005). In in vitro, it significantly reduced PRRSV replication in IL-12-pretreated PAMs. In in vivo, pigs pretreated with rpIL-12 showed significantly reduced viremia after subsequent PRRSV infection. PAMs collected from these pigs had reduced PRRSV genome levels and produced greater amount of IFNγ and lesser amount of IL-10 in response to recall PRRSV than PAMs of PRRSV-infected control pigs (Carter and Curiel, 2005). The mechanism of IL-12 in PRRSV suppression is currently not known.

IL-12, when used as adjuvant for PRRSV MLV vaccine, was administered to pigs in the form of recombinant protein or cytokine-encoded plasmids. In either form, the cytokine significantly augmented populations of PRRSV-specific IFNγ-secreting PBMC (Table 2). The cytokine expression by plasmid DNA was confirmed by functional assay in porcine T cell stimulation for IFNγ production in response to recall antigens in vitro. The adjuvanticity of IL-12-encoded plasmids was seen only after intradermal (i.d.) administration, but not after intramuscular (i.m.) administration. This was possibly due to differential ability of APC of epidermis and muscular tissues in uptaking plasmid DNA (Donnelly et al., 1998). IL-12, in either form, did not enhance humoral immune response (determined by IDEXX ELISA, anti-ORF5 ELISA, and serum virus neutralization (SVN) test), and protective efficacy of PRRSV MLV vaccine (determined by serum virus titers, virus loads in tissues, and lung lesions after virulent heterologous PRRSV challenge) (Charerntantanakul et al., 2006b; Foss et al., 2002; Meier et al., 2004) (Table 2). The absence of cross protection in the presence of enhanced CMI response suggests that only increased CMI response alone is not sufficient for cross protection. Protective immune response to heterologous PRRSV may require both enhanced CMI and antibody production (Mateu and Diaz, 2008).

2.5. IFNγ

IFNγ was used to enhance Th1 response to PRRSV DNA vaccine. In pigs, the cytokine promotes co-stimulatory molecule and pro-inflammatory cytokine production, i.e. IL-1 and IL-12, and reduces anti-inflammatory cytokine production, i.e. IL-13 in activated myeloid APC (Charley et al., 1990; Foss et al., 1999a; Raymond et al., 2006). It also promotes E-selectin and vascular cell adhesion molecule (VCAM) expressions in endothelial cells and enhances lymphocyte proliferation in immunosuppressed pigs (Batten et al., 1996; Saulnier et al., 1991). IFNγ inhibits replication of PRRSV (Bautista and Molitor, 1999; Buddaert et al., 1998), influenza virus (Horisberger, 1992), transmissible gastroenteritis virus (TGEV) (Charley et al., 1988), vesicular stomatitis virus (VSV) (Xia et al., 2005), PRV (Yao et al., 2007), and FMDV (Moraes et al., 2007). The cytokine has been utilized as vaccine adjuvant for enhancing Th1 response in pigs in the form of expression plasmids for PRV DNA vaccine (Somasundaram et al., 1999). It did not augment antibody (determined by IgG1- and IgG2-specific anti-PRV ELISA) nor improve vaccine efficacy in protecting pigs from nasal PRV excretion after virulent PRV challenge (Table 1).

When used as adjuvant for PRRSV DNA vaccine, the IFNγ gene was cloned into plasmids expressing PRRSV ORF5 (ORF5/IFNγ) or ORF7 (ORF7/IFNγ) (Xue et al., 2004). Plasmid expression of IFNγ and PRRSV glycoprotein 5 (GP5) and N proteins was tested by indirect immunofluorescence assay in MARC-145 cells. Pigs immunized i.m. with ORF5/IFNγ and ORF7/IFNγ showed no increased PRRSV-specific antibody (determined by ELISA and Western blotting using PRRSV GP5 and N proteins as antigens) and CMI responses (determined by frequencies of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells in fresh PBMC) (Table 2). ORF5/IFNγ-immunized pigs, however, tended to be protected from lung lesions and virus replication in lymphoid and non-lymphoid tissues, e.g. lung and kidney after virulent homologous PRRSV challenge, when compared to pigs immunized with ORF5 DNA vaccine alone (Xue et al., 2004). No statistical analysis was done for this report. Failure of IFNγ in enhancing CMI response to PRRSV DNA vaccine was probably due to lack of expression or insufficient level of virus antigen and cytokine expression in pigs in vivo and/or insufficient dose of antigen and cytokine administration. This may be improved in future study by determining PRRSV protein expression in pigs in vivo, increasing dose and frequency of plasmid administration, and using i.d. as a route of administration (Donnelly et al., 1998).

2.6. IFNα

IFNα is an antiviral cytokine reportedly produced by various cell types in response to virus infection. Its production was, however, very low in pigs acutely infected with PRRSV (Albina et al., 1998; Van Reeth et al., 1999). IFNα has been reported for its ability to activate APC, increase antibody and cytotoxic T cell response, and direct Th1 differentiation (Bracci et al., 2008). It was thought that administration of IFNα in PRRSV-vaccinated pigs might help increase IFNγ response to PRRSV vaccine. In pigs, IFNα promotes IL-1 production of monocytes and cytotoxicity of lymphocytes (Cepica and Derbyshire, 1986). The cytokine also inhibits replication of influenza virus (Horisberger, 1992), classical swine fever virus (Xia et al., 2005), PRV (Pol et al., 1991; Yao et al., 2007), VSV (Horisberger, 1992), TGEV (Jordan and Derbyshire, 1994), FMDV (Chinsangaram et al., 2001), and PRRSV (Albina et al., 1998).

IFNα has been utilized in the form of expression plasmids (Meier et al., 2004; Royaee et al., 2004) or expression plasmids plus rpIFNα (Charerntantanakul et al., 2006b) for PRRSV MLV vaccine. Vaccinated pigs receiving plasmids expressing porcine IFNα (200 μg) demonstrated significantly increased numbers of IFNγ-secreting PBMC in response to recall PRRSV in vitro (Meier et al., 2004; Royaee et al., 2004). On the other hand, vaccinated pigs receiving higher dose of plasmids expressing porcine IFNα (400 μg) and *E. coli*-expressed rpIFNα (10^5 units) did not show significantly increased $\%IFN\gamma^+$ T cells in response to recall PRRSV in vitro when compared to pigs vaccinated with PRRSV MLV vaccine alone (Charerntantanakul et al., 2006b). The expression of IFNα by plasmid DNA was tested by functional assay in inhibiting VSV replication in Madin Derby bovine kidney cells. Neither administration strategy enhanced PRRSV-specific antibody response (determined by IDEXX ELISA and SVN test) nor vaccine efficacy (determined by viremia and lung lesions after virulent heterologous PRRSV challenge) (Charerntantanakul et al., 2006b; Meier et al., 2004). Like IL-12 studies, the results of IFNα studies suggest that only CMI response alone is not sufficient for cross protection. Protective immunity to heterologous PRRSV may need both enhanced CMI and antibody production.

3. Chemical reagents

3.1. Poly IC and Poly ICLC

Poly IC (polyinosinic and poly cytidylic acid) and poly ICLC (poly IC complexed with poly-L-lysine and carboxymethylcellulose) were used to enhance Th1 response to PRRSV MLV vaccine. They are potent type I IFN inducer in pigs (Derbyshire and Lesnick, 1990a,b; Jordan and Derbyshire, 1994; Lesnick and Derbyshire, 1988; Loewen and Derbyshire, 1988a,b; Weingartl and Derbyshire, 1990). Poly IC induces surface expression of MHC class II and CD80/CD86, and IFNγ gene expression in porcine monocytes and MDC; and also induces Toll-like receptor-5 (TLR-5), TLR-9, and IL-12p35 gene expression in porcine MDC (Raymond and Wilkie, 2005). Poly ICLC induces cytolytic activity of porcine NK cells (Derbyshire and Lesnick, 1990a; Lesnick and Derbyshire, 1988).

The adjuvanticity of poly IC and poly ICLC has been reported in some veterinary vaccines; for examples, inactivated Venezuelan equine encephalomyelitis virus (Houston et al., 1976) and inactivated Newcastle disease virus vaccines (Knight et al., 1977). In pigs, their adjuvanticity has been reported in FMDV (Cunliffe et al., 1977; Maes et al., 1977) and TGEV vaccines (Derbyshire and Lesnick, 1990b) (Table 1). Poly IC, when used as adjuvant for PRRSV MLV vaccine, induces significantly increased number of antigen-specific IFNγ-secreting PBMC (determined by ELISPOT assay) (Meier et al., 2004). The increased response was detected as early as one week and sustained until three weeks after vaccination (Table 2). Poly ICLC, on the other hand, did not induce increased PRRSV-specific $\%IFN\gamma^+$ T cells in pigs vaccinated with PRRSV MLV vaccine (Charerntantanakul et al., 2006b) (Table 2). Neither poly IC nor poly ICLC induced increased PRRSV-specific antibody response (determined by IDEXX ELISA) and vaccine efficacy (determined by viremia and lung lesion after virulent heterologous PRRSV challenge) (Charerntantanakul et al., 2006b; Meier et al., 2004). Like the results of IL-12 and IFNα studies, the results of poly IC studies suggest that both enhanced CMI and antibody responses may be required for cross protection.

3.2. Levamisole (LMS)

LMS was studied for its adjuvanticity to PRRSV KV vaccine in murine model (Kang et al., 2005). Its adjuvant property is not well reported in pigs. It has been utilized as adjuvant for some bovine vaccines, e.g. bovine herpesvirus-1 and *Pasteurella multocida* vaccines to which it significantly enhanced antibody response (Babiuk and

Misra, 1982; Sharma et al., 1990). In pigs, LMS augmented lymphocyte blastogenesis in vitro in response to lectins (Hennessy et al., 1987). The drug induced migration of porcine T cells to mesenteric lymph nodes (MLN) and ileal Peyer's patches, and enhanced T cell and macrophage activation in those tissues in response to oral immunization with attenuated *E. coli* vaccine (Bozic et al., 2003; Bozic et al., 2006) (Table 1).

LMS was administered i.m. in BALB/c and C57BL/6 mice at varying doses. At low dose (0.25-0.5% v/v), LMS significantly induced both types of mice to develop increased PRRSV-specific T cell proliferation and Th1 cytokine gene expression, i.e. IL-2 and IFNγ, as compared to mice immunized with oil-adjuvanted PRRSV vaccine only. At high dose (1-2% v/v), LMS significantly induced both types of mice to develop increased PRRSV-specific antibody response and Th2 cytokine gene expression, i.e. IL-4 and IL-10. This study indicated that LMS has a potential effect in directing Th1 and Th2 response to PRRSV KV vaccine, depending on its dose. This effect needs to be proven in pigs.

3.3. Cytidine–phosphate–guanosine (CpG) oligodeoxynucleotides (ODN)

CpG ODN containing palindromic hexamer 'ATCGAT' has been reported for its immunostimulatory effects in pigs (Kamstrup et al., 2001). It induces gene expression of MHC class II, B7, TLR-5, and TLR-9 in monocytes, MDC, and immature plasmacytoid DC; gene expression and protein production of IL-6, IL-12, TNFα, type I IFN, and IFNγ in monocytes, MDC, and PBMC; and proliferation of PBMC and B cells (Guzylack-Piriou et al., 2004; Kamstrup et al., 2001; Raymond and Wilkie, 2005; Van der Stede et al., 2005). CpG ODN, when administered in pigs, enhanced IFNγ level in serum and MHC class II expression in PBMC (Linghua et al., 2007a; Zhang et al., 2006).

The adjuvant effects of CpG ODN have been reported in numerous vaccine studies in mice and non-human primates, and some in pigs (Davis et al., 2000; Wu et al., 2004). In pigs, these effects were reported in, for examples, PRV MLV (Ling-Hua et al., 2006; Linghua et al., 2006a), PRV DNA (Dory et al., 2005), and swine streptococcic septicemia killed (SSSK) antigen (Linghua et al., 2006c, 2007b; Linghua et al., 2006b) (Table 1). CpG ODN were used to enhance both Th1 and Th2 responses to in-house PRRSV KV vaccine. It significantly enhanced PRRSV-specific antibody response (determined by IgA- and IgG-specific ELISA), lymphocyte proliferation, MHC class II expression, and IFNγ and IL-6 production of PBMC after in vitro PRRSV restimulation (Linghua et al., 2007c, 2006d; Zhang et al., 2007) (Table 2). The CpG ODN significantly increased in-house PRRSV KV vaccine efficacy in protecting pigs from respiratory difficulty and death after virulent heterologous PRRSV challenge (Linghua et al., 2007c). To date, CpG ODN is the only experimental vaccine adjuvant that successfully enhances both humoral and CMI responses to PRRSV KV vaccine, and enhances efficacy of PRRSV KV vaccine in protecting pigs from heterologous virus challenge. Its adjuvanticity should be tested in future studies with other strains of PRRSV as well as other PRRSV vaccine models in order to evaluate its potential as PRRSV vaccine adjuvant.

4. Bacterial products

4.1. Cholera toxin (CT)

CT was used to enhance Th2 response to PRRSV MLV, recombinant and synthetic peptide vaccines. The idea of using CT, and other bacterial products, e.g. lipopolysaccharide (LPS), to enhance anti-PRRSV immune response comes from the evidence that these bacterial components activate APC's antigen presentation and T cell stimulation (Dougan and Hormaeche, 2006). APC activation is essentially required for immunogens like PRRSV which elicit poor innate and adaptive immune responses. CT provides danger signals to APC through its interaction with GM1 gangliosides on APC surface (Lavelle et al., 2004). The toxin enhances cAMP production, resulting in activation of protein kinases and downstream transcription factors (Lavelle et al., 2004). In pigs, stimulation of PAM with CT increased MHC class II, CD80-CD86, and IL-1 mRNA expression (Foss et al., 1999a). In mice, CT induces DC's antigen presentation, T cell activation, and cytokine production that favor Th2 differentiation, e.g. IL-6 and IL-10 (Lavelle et al., 2004). CT, when used as adjuvants for swine vaccines, i.e. *Ascaris suum* antigens, purified F4 fimbrae of enterotoxigenic *E. coli* conjugated with human serum albumin, and recombinant F4 fimbrial adhesin FaeG, reportedly induced strong mucosal and serum IgA and serum IgG production (Tsuji et al., 2004; Verdonck et al., 2005a,b) (Table 2).

In PRRSV vaccine studies, CT reportedly induced humoral immune response to PRRSV vaccines with varying results. It significantly enhanced kinetics and magnitude of serum anti-PRRSV GP5 antibody response induced by PRRSV MLV vaccine (Foss et al., 2002). The response was detected three weeks after vaccination whereas the maximum response induced by MLV vaccine alone was at four weeks after vaccination. When administered with synthetic PRRSV ORF5 ectodomain peptides, CT did not enhance PRRSV-specific antibody response (determined by IDEXX ELISA and SVN test) and clinical protection (determined by serum virus titers and lung lesions after virulent heterologous PRRSV challenge) (Charerntantanakul et al., 2006b). CT, when applied orally as recombinant CT-A1-deleted peptides fused with PRRSV N peptides and Myc, slightly enhanced mucosal IgA and more robust serum IgG specific for PRRSV N peptides (Hyland et al., 2004). These vaccine studies showed no efficacy of CT in enhancing CMI response (determined by ELISPOT and flow cytometric assays for IFNγ production by PBMC and T cells, and DTH test) to PRRSV MLV vaccine and antigens. CT may be utilized with Th1-inducing adjuvants to enhance both humoral and CMI response to PRRSV MLV vaccine in future experiments. Its application as adjuvant for synthetic and recombinant PRRSV peptides may be optimized by increasing frequency of CT and antigen peptide co-administrations.

4.2. LPS

LPS was used to enhance Th1 response to PRRSV MLV vaccine. The toxin complex (LPS:LPS-binding protein:CD14) binds to TLR-4 of myeloid APC and stimulates production of pro-inflammatory cytokines, e.g. IL-1, IL-12, and TNFα

(Kaisho and Akira, 2002). The toxin itself can bind to radioprotective 105 of B cells and activate nuclear factor kappa B (Cox et al., 2006). In pigs, LPS induces expression of MHC class II and B7 molecules on peripheral blood monocytes and MDC (Raymond and Wilkie, 2005). LPS-stimulated monocytes showed increased gene expression of IFNγ, TLR-5, IL-10, and IL-13, while LPS-stimulated MDC showed increased gene expression of IFNγ, IL-12p35, TLR-4, TLR-9, IL-10, and IL-13 (Raymond and Wilkie, 2005). LPS potently stimulates PAMs and monocyte-derived macrophages to express IL-1, IL-6, IL-8, and TNFα mRNA (Foss et al., 1999a; Lin et al., 1994; Sacco et al., 1996; Scamurra et al., 1996). It also induces expression of VCAM-1 in porcine endothelial cells (Tsang et al., 1994).

LPS has limited use in animals as a vaccine adjuvant and is not permitted for use in humans due to its high toxicity. With careful dosing, LPS was demonstrated in mice to induce a Th1 response which was characterized by increased IgG2a production, antigen-specific $CD4^+$ T cell proliferation, and IL-2 production after immunization with vaccine antigens (Costalonga and Zell, 2007). Its use as adjuvant for swine vaccine other than PRRSV MLV has not been reported.

In PRRSV MLV vaccine study, LPS at proprietary dose did not enhance CMI (determined by IFNγ production of T cells in recall reaction in vitro) nor antibody responses (determined by IDEXX ELISA and SVN test) to the vaccine (Charerntantanakul et al., 2006b). The toxin also did not enhance vaccine efficacy in protecting pigs from viremia and lung lesions after virulent heterologous PRRSV challenge (Table 2) (Charerntantanakul et al., 2006b). Failure of LPS to stimulate immune response to PRRSV MLV vaccine was probably due to its inappropriate dose and schedule of administration. Additional studies are needed to optimize the condition of LPS administration.

Extensive data on adjuvant properties of LPS were obtained from a LPS derivative, monophosphoryl lipid A (MPL). MPL is a detoxified derivative of LPS of *Salmonella enterica* serovar Minnesota R595 that lacks many of endotoxic properties of LPS, yet retains its immunostimulatory activities (Persing et al., 2002). MPL has been utilized as vaccine adjuvant for some experimental veterinary vaccines; most of which were conducted in guinea pigs and a few in domestive species. These vaccines are, for examples, parainfluenza virus type 3 (PI3) subunit, *Bacillus anthracis* subunit, *Mycobacterium tuberculosis* subunit, FMDV, and recombinant leishmaniae antigens. MPL significantly enhanced antibody response of guinea pigs vaccinated with PI3 subunit, *B. anthracis* subunit, and *M. tuberculosis* subunit vaccines (Doherty et al., 2002; Ewasyshyn et al., 1992; McBride et al., 1998). It significantly enhanced antibody response of cattle vaccinated with FMDV vaccine (Solyom and Bertok, 1985), and of dogs vaccinated with recombinant leishmanial antigens (Fujiwara et al., 2005). MPL has not been used as a vaccine adjuvant in any swine vaccines and could be tested for its adjuvant property in PRRSV vaccine.

5. Conclusions and future studies

Efforts to improve immune responses to PRRSV vaccines have included utilization of vaccine adjuvants. Numerous vaccine adjuvants have been tested for their efficiency in enhancing CMI and antibody responses to the vaccine. These vaccine adjuvants include cytokines, chemical reagents, and bacterial products. Of 11 vaccine adjuvants tested, five (IL-2, IL-12, IFNα, poly IC, and CpG ODN) significantly enhance CMI response to PRRSV vaccines (Table 2). The increased response is generally transient for a few weeks after adjuvant administration. Two vaccine adjuvants, i.e. CpG ODN and CT significantly enhance antibody response after PRRSV vaccination (Table 2). The increased response is also transient for a couple weeks after adjuvant administration. Only two vaccine adjuvants, i.e. IL-2 and CpG ODN, significantly enhanced protective efficacy of PRRSV vaccines in reducing viremia, lung lesions, and mortality of pigs after PRRSV challenge. To date, CpG ODN is the only vaccine adjuvant that can significantly enhance CMI and antibody responses to PRRSV vaccine and increase PRRSV vaccine efficacy after virulent PRRSV challenge. However, only one study has been reported (Linghua et al., 2007c). Additional post-vaccination challenge studies are required to fully evaluate the efficacy of CpG ODN as a PRRSV vaccine adjuvant. For these studies, various strains of PRRSV should be used in order to evaluate cross protection. In addition, other models of PRRSV vaccines, e.g. MLV, DNA, and peptide vaccines should be tested with CpG ODN in order to evaluate its adjuvanticity in different types of vaccine. In addition to CpG ODN, there is a need to seek other vaccine adjuvants that can enhance CMI and antibody responses to PRRSV vaccine. To be effective, the vaccine adjuvant candidates should stimulate robust CMI and antibody responses since both types of immune responses may be required for optimal PRRSV immunity (Charerntantanakul et al., 2006b; Lopez et al., 2007; Lopez and Osorio, 2004; Mateu and Diaz, 2008; Zuckermann et al., 2007). These vaccine adjuvants may be simply a combination of Th1-inducing adjuvants and Th2-inducing adjuvants, e.g. a combination of IL-12 and CT, or other vaccine adjuvants reportedly used in other veterinary species, e.g. immune-stimulating complex, LMS, and MPL (Spickler and Roth, 2003).

A recent finding reported that poor CMI response to PRRSV might result from increased IL-10 production (Suradhat et al., 2003). PRRSV upregulates IL-10 expression in monocytes after infection (Charerntantanakul et al., 2006a). The increased IL-10 production can be detected both in vivo and in vitro (in a recall antigen stimulation of PBMC) (Chung and Chae, 2003; Feng et al., 2003; Johnsen et al., 2002; Labarque et al., 2003; Royaee et al., 2004; Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003). The mechanism of PRRSV upregulation of IL-10 is not known. In pigs, IL-10 reportedly inhibits IFNγ production of T cells (Charerntantanakul et al., 2006a; Waters et al., 1999). Inhibition of IL-10 gene expression might be a strategy to improve CMI response to PRRSV vaccine. The inhibition may be accomplished by the use of RNA interference technology, i.e. small interfering RNA that is specific for IL-10 mRNA of pigs. This technology may also be used with other mRNA targets as well as PRRSV ORFs that might be responsible for the reduced CMI response. To date, PRRSV ORFs involving in reduced CMI response have not been identified. The

mechanisms of PRRSV reduction of CMI responses also remain to be studied.

Neutralizing antibody is a major component of humoral immunity that provides protection to PRRS (Lopez et al., 2007; Lopez and Osorio, 2004). In many PRRSV vaccine adjuvant studies (including CpG ODN and CT), appearance of neutralizing antibody was not determined. The antibody parameters measured were total antibody (determined by IDEXX ELISA), total Ig (determined by IgG- and IgA-specific ELISA), and anti-GP5 antibody. These antibody parameters contain predominantly anti-N and some anti-decoy neutralizing epitopes of PRRSV which are not protective to PRRSV infection (Lopez and Osorio, 2004; Yoon et al., 1995). Future experiments on PRRSV vaccine adjuvants should determine the production of neutralizing antibody in order to assess the efficiency of vaccine adjuvants in enhancing protective humoral immunity.

Various forms of vaccine adjuvants have been studied with PRRSV vaccine. To date, vaccine adjuvants in the form of plasmid DNA and recombinant protein have been studied with PRRSV MLV vaccine; CpG ODN with PRRSV KV vaccine; plasmid DNA with PRRSV DNA vaccine; and recombinant protein with PRRSV recombinant and synthetic peptide vaccine. In any form, the adjuvants can significantly enhance immune response to PRRSV vaccine (except to synthetic peptide vaccine) (Table 2). The magnitude of immune response enhanced by vaccine adjuvant can be different, however, when the adjuvants were administered by different route. This was seen clearly when adjuvants in the form of plasmid DNA, i.e. IL-12 were administered i.d. and i.m. Intradermal administration of IL-12-encoded DNA yielded significantly enhanced CMI response to PRRSV MLV vaccine, while i.m. administration of the DNA did not. Improper route of administration can result in failure of immune enhancement and lead to misinterpretation of adjuvant effectiveness. The same concept applies to dose and schedule of adjuvant administration.

The vaccine model used for PRRSV vaccine adjuvant studies is also an important factor for evaluation of adjuvant effectiveness. Considering the fact that PRRSV MLV vaccine by itself can enhance CMI and antibody responses and provides full protection against homologous virus challenge and partial protection against heterologous virus challenge, it is quite difficult for any vaccine adjuvant to potentiate further immunogenicity and protective efficacy of PRRSV MLV vaccine. This difficulty of immune potentiation is demonstrated by the fact that no adjuvant studied to date significantly enhances the protective efficacy of PRRSV MLV vaccine, either after homologous or heterologous virus challenge. Little opportunity for vaccine adjuvants to improve immunogenicity and protective efficacy of PRRSV MLV vaccine may lead to misinterpretation of vaccine adjuvant effectiveness. The vaccine model that might demonstrate the effectiveness of vaccine adjuvant better is the PRRSV KV, recombinant peptide, and synthetic peptide vaccines, since these vaccines by themselves barely induce CMI and antibody responses and poorly confer PRRS protection. The contribution of vaccine adjuvants to improvement of immunogenicity and protective efficacy of these vaccines should be detectable more clearly than their contribution to PRRSV MLV vaccine. This should therefore minimize the opportunity for misinterpretation of the effectiveness of vaccine adjuvants.

Results from several PRRSV vaccine adjuvant studies suggest that only data of enhanced immune response alone is not predictive for PRRS protection (Table 2). Pigs can show significantly enhanced CMI response, but not disease reduction. It is therefore essential that future PRRSV vaccine adjuvant studies have a challenge infection. The challenge virus should be antigenically heterologous to the vaccine virus or vaccine antigens for determination of cross protection.

It is noteworthy that all challenge infection models in PRRSV vaccine adjuvants studied to date are only respiratory model. No reproductive model of infection has been conducted. In reproductive model of challenge infection, enhanced immune response should not only protect sow from viremia, but should also protect fetuses from transplacental infection and late term abortion (Christianson et al., 1992). Like respiratory model of challenge infection, PRRSV that are antigenically heterologous to the vaccine virus should be used for virus challenge in order to determine cross protection.

Acknowledgement

The author thanks Dr. James A. Roth for critical review of the manuscript.

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Interleukin-10 antisense oligodeoxynucleotide suppresses IL-10 expression and effects on innate and adaptive cytokine responses to porcine reproductive and respiratory syndrome virus

Wasin Charerntantanakul[1*], Watchara Kasinrerk[2, 3]

[1] Department of Biology, Faculty of Science, Maejo University, Chiang Mai, Thailand

[2] Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand

[3] Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand

* Corresponding author: Wasin Charerntantanakul, DVM., Ph.D.
Department of Biology, Faculty of Science,
Maejo University, Chiang Mai, 50290, Thailand.
Tel: (+66 53) 873535 ext 121
Fax: (+66 53) 878225
E-mail: wasin@mju.ac.th

ABSTRACT

Up-regulation of interleukin-10 (IL-10) expression has been suggested to be the mechanism by which the porcine reproductive and respiratory syndrome virus (PRRSV) suppresses the innate and adaptive immune response in infected pigs. This study evaluates the potential of phosphorothioate-modified IL-10 antisense oligodeoxynucleotide (IL-10AS) specific to the translation initiation region of porcine IL-10 mRNA in enhancing innate and adaptive cytokine responses to PRRSV. Naïve peripheral blood mononuclear cells (PBMC) from eight PRRSV-seronegative pigs were transfected with IL-10AS *in vitro* prior to PRRSV inoculation and concanavalin A or phorbol 12-myristate 13-acetate plus ionomycin stimulation. The effects of IL-10AS on mRNA expression of IL-10, tumor necrosis factor alpha (TNFα), interferon alpha (IFNα), IL-2, IL-4, and IFNγ were tested by real-time PCR. The percentages of IFNγ-producing T cell subsets were determined by flow cytometry. Compared to the controls, the levels of IL-10 and IL-2 mRNA were significantly reduced, while those of IFNγ mRNA were increased, and TNFα, IFNα, and IL-4 mRNA were unchanged. An increase in the percent $IFN\gamma^+$ population was also observed in lymphocytes and $CD8\beta^+$ T cells. Our results suggest that IL-10AS has the potential to enhance the adaptive immune response to PRRSV infection.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is an enveloped positive-sense single-stranded RNA virus of the family *Arteriviridae* (39). The virus causes reproductive failure in breeding age swine and respiratory disease and poor growth performance in growing pigs (13,47). After infection, PRRSV elicits poor innate and adaptive immune responses. The virus minimally induces, or even reduces, innate cytokine production, i.e. interleukin-1 (IL-1), IL-12, type I interferon (IFN), and tumor necrosis factor alpha (TNFα) in pulmonary alveolar macrophages (PAM), monocyte-derived dendritic cells (MDC), bone marrow-derived DC (BDC), and peripheral blood mononuclear cells (PBMCs) (1,5,35,36,60,62). The virus suppresses phagocytic and microbicidal activities, and production of reactive oxygen species in mononuclear phagocytes (12,45,59). The virus also suppresses surface expression of MHC class I and II (5,23,42,62), CD11b/c, CD14, and CD80/CD86 (23,62), Toll-like receptor 3 (TLR3) and TLR7 (10), and reduces the capacity of MDC in inducing allogeneic T cell proliferation (23,42,62). PRRSV robustly elicits non-neutralizing antibody production, which is detectable at about two weeks of infection (41,67). However, it elicits relatively delayed and low neutralizing antibody production, primarily detectable at around four weeks after infection, and has a mean titer of approximately 2^3 to 2^5 throughout the course of infection (64,67). Cell-mediated immune (CMI) response to PRRSV is much delayed, primarily detectable around four to eight weeks after infection (2,3,9,34,38,48). The CMI response to PRRSV is extremely delayed compared to CMI response to other swine viral pathogens, appearing within three days to one week after infection (58).

Numerous studies have suggested that the poor innate and adaptive immune responses to PRRSV might be attributed to increased IL-10 production (54,55). PRRSV has been demonstrated to up-regulate IL-10 production in PAM, MDC, BDC and PBMCs (5,8,10,14,20-24,26,29,42,48,54,55). PRRSV-induced increased IL-10 expression contributed significantly to the reduction of IFNγ production in T cells (8). In humans and mice, IL-10 suppresses antigen processing and presentation, and production of IL-1, IL-12, IL-18, TNFα, and type I IFN by monocytes, macrophages, and DC; and IL-2, IL-4, and IFNγ by T helper 1 (Th1), Th2, and natural killer cells (16-19,27,40,44,56,57,65).

Suppression of PRRSV-induced IL-10 production may be a strategy to enhance innate and adaptive immune responses to the virus. Our previous study reported the utilization of monoclonal antibodies (mAbs) specific for porcine IL-10 in neutralizing IL-10 activity and enhancing the percentage of $IFN\gamma^+$ T cells to PRRSV (8). Recently, Sidahmed and Wilkie (50) utilized phosphorothioate-modified IL-10 antisense oligodeoxynucleotides (IL-10AS) specific to the translation initiation region of porcine IL-10 mRNA in decreasing IL-10 mRNA expression in concanavalin A (ConA)-stimulated porcine PBMC. In the present study, we further investigate the potential of using IL-10AS in decreasing IL-10 mRNA expression and enhancing innate and adaptive cytokine expression in naïve PBMC inoculated with PRRSV. We report here that IL-10AS is efficient in decreasing IL-10 mRNA expression and in increasing IFNγ mRNA expression. An increase in the percentage of $IFN\gamma^+$ cells was also observed.

MATERIALS AND METHODS

IL-10AS

The IL-10AS sequence used was 5'TGAGCTGGGCATGGTAGA3' as described by Sidahmed and Wilkie (50). Scramble control oligodeoxynucleotide (ODN) sequences were 5'TGATTGTATACTTCTTGAT3' (Scr1), 5'CCAGTGACGGTGCAGGCA3' (Scr2), and 5'CAAGTGCACACAAGGCTA3' (Scr3). All phosphorothioate-modified ODN were synthesized by 1st BASE PTE Ltd (Singapore).

Antibodies

All primary and secondary mAbs were purchased from BD Pharmingen (San Diego, CA). Isotype control mAbs were from Southern Biotechnology Associates, Inc. (Birmingham, AL). Primary mAbs included fluorescein isothiocyanate (FITC)-conjugated mouse anti-porcine CD4α (clone 74-12-4, isotype IgG2b), unconjugated mouse anti-porcine CD8β (clone 295/33-25, isotype IgG2a), and unconjugated rat anti-porcine MAC320 (clone MAC320, isotype IgG2a). Secondary mAbs were FITC-conjugated rat anti-mouse IgG2a (clone R19-15) and FITC-conjugated mouse anti-rat IgG1/2a (clone G28-5). Intracellular staining mAbs were R-phycoerythrin (R-PE)-conjugated mouse anti-porcine IFNγ (clone P2G10, isotype IgG1).

Virus and cell line

PRRSV isolate 01NP1 and MARC-145 cells were kindly provided by Dr.Roongroje Thanawongnuwech at the Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University. The virus was propagated in MARC-145 cells grown in $DMEM^{++}$ (DMEM, 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml) (all from Gibco, Grand Island, NY)) to the titer of 10^6 tissue culture infectious dose 50%/ml ($TCID_{50}$/ml). Virus titer was determined from

cytopathic effects of the virus in MARC-145 cells after three days of PRRSV inoculation. PRRSV used in this study was at its seventh passage. Mock antigens were prepared in the same fashion as virus antigens, except that no viruses were inoculated.

Isolation of PBMCs

Blood was collected from eight 24-week old PRRSV-seronegative pigs from a commercial producer using ethylene diamine tetra-acetic acid (EDTA) (J.T.Baker, Phillipsburg, NJ) as an anti-coagulant. PBMCs were isolated from the blood sample by Ficoll-Hypaque gradient centrifugation using Histopaque®-1077 (Sigma, St. Louis, MO). Contaminating red blood cells in the isolated PBMC were lysed with cold red blood cell lysis buffer (0.156M ammonium chloride, 10 mM sodium bicarbonate, and 1 mM EDTA). PBMCs were resuspended in reduced serum medium (Optimem® I, Gibco) to the concentration of 10^6 cells/ml.

Transfection of PBMC with IL-10AS

Optimization of IL-10AS concentration

IL-10AS mixtures containing 0.5, 1, or 2 µM IL-10AS in transfection media (Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA) in Optimem® I at 1.5% v/v) were prepared and incubated at room temperature for 30 minutes. Twenty µl of IL-10AS mixture were added to each well of a 96-well flat-bottom plate, followed by 100 µl of PBMC (10^6 cells/ml). The cultures were mixed gently by rocking the plates back and forth for 5 minutes, then incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 hours. The media were removed and replaced with 200 µl of RPMI^{++} (RPMI-1640 with L-glutamine, 10% heat-inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (250

ng/ml) (all from Gibco)) and 50 µl of either ConA (5 µg/ml final concentration) (Sigma, St. Louis, MO) or phorbol 12-myristate 13-acetate plus ionomycin (PMA/I; 7 and 430 ng/ml final concentrations, respectively) (Calbiochem, Germany). Plates were incubated at 37°C in a CO_2 incubator for 16 and 12 hours after ConA and PMA/I stimulation, respectively. Cells were then harvested and evaluated for IL-10 mRNA expression. PBMCs cultured without IL-10AS transfection in the presence or absence of cytokine inducers were used as positive and negative controls, respectively. PBMCs treated with scramble ODN or transfection media prior to ConA or PMA/I stimulation served as scramble and transfection media controls, respectively.

Evaluation of IL-10AS specificity

PBMCs were transfected with IL-10AS (2 µM) and stimulated with either ConA or PMA/I as described above. ConA-stimulated cells were determined for IL-10, IL-2 and IL-4 mRNA expressions (61), and PMA/I-stimulated cells were evaluated for IL-10, TNFα, IFNα, and IFNγ mRNA expressions (8).

Evaluation of the effect of IL-10AS on cytokine mRNA expressions in PRRSV-inoculated PBMC

PBMCs were transfected with IL-10AS as described above. After transfection, media were removed and replaced with 100 µl $RPMI^{++}$ and 100 µl PRRSV (10^6 $TCID_{50}$/ml). Plates were incubated at 37°C in a 5% CO_2 incubator for 48 hours. For determination of IL-10, IL-2, and IL-4 mRNA expressions, 50 µl of ConA (5 µg/ml final concentration) were added to the culture and the plates were incubated for an additional 16 hours (61). For evaluation of IL-10, TNFα, IFNα, and IFNγ mRNA expressions, 50 µl of PMA/I (7 and 430 ng/ml final concentrations, respectively) were added and the plates were incubated for an additional 12 hours (8). For evaluation of intracellular IFNγ production, 50 µl of PMA/I/G (PMA/I plus GolgiStop®

solution (BD Pharmingen, San Diego, CA) at the manufacturer-recommended dilution) were added and the plates were incubated for an additional 12 hours (46). PBMCs treated with transfection media alone (without IL-10AS) served as the PRRSV-inoculated transfection media control, and those left untransfected served as the PRRSV-inoculated control. Other controls included PBMCs receiving culture media alone (negative control); culture media plus ConA, PMA/I, or PMA/I/G (positive control); and mock antigens plus ConA, PMA/I, or PMA/I/G (mock control). All PBMCs were determined for cell viability at the end of the transfection period, PRRSV inoculation, and ConA, PMA/I, or PMA/I/G stimulation using trypan blue (Gibco).

Reverse transcriptase-PCR

RNA extraction and reverse transcription

Total RNA was extracted from PBMCs using the RNeasy® kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Contaminating DNA was eliminated by Dnase I (Fermentas, Glen Burnie, MD). Total RNA was eluted in 40 µl of RNase-free water. Ten µl of which were assigned to reverse transcription, using Omniscript RT kit (Qiagen, Valencia, CA), 0.5 µg random hexamers (Qiagen, Valencia, CA), and 40 U ribonuclease inhibitor (RiboLock™, Fermentas, Glen Burnie, MD).

Real-time PCR

Complementary DNA (cDNA) was used as a template for real-time PCR of porcine IL-10, IL-2, IL-4, TNFα, IFNα, IFNγ, and ribosomal protein L32 (RPL32). The PCR was performed on the MJ Research PTC-200 thermal cycler in a total reaction volume of 50 µl, consisting of 2 µl cDNA template, 0.3 µM each of forward and reverse primers, and 25 µl PCR

buffer (QuantiTect SYBR Green PCR master mix, Qiagen, Valencia, CA). All primer sequences were from Royaee et al. (48). The PCR conditions were 'initial activation' at 95^{o}C for 15 minutes; and 40 cycles of 'denaturation' at 94^{o}C for 15s, 'annealing' at 55^{o}C for 30s, and 'extension' at 72^{o}C for 30s. The threshold cycles (C_T) of all genes were collected and used for calculation of cytokine mRNA expression by the $\Delta\Delta C_T$ method. A melting curve analysis was performed after the completion of the PCR cycles. PCR products were determined for size correction by agarose gel electrophoresis and visualized under ultraviolet light with Quantity One software (version 4.5.0, Bio-Rad, Hercules, CA).

Immunofluorescent staining

At the end of the PMA/I/G stimulation period, PBMC culture plates were chilled on ice for 15 minutes. The cells were harvested and washed twice with PBS^{++} (PBS, 0.5% heat-inactivated FBS, and 0.1% sodium azide (Fisher Scientific, Pittsburgh, PA)). The washed cells were then incubated with 50 µl of primary mAbs (anti-CD4α, anti-CD8β, or anti-MAC320) at an optimum dilution in the dark at 4^{o}C for 30 minutes, followed by three washes with PBS^{++}. For anti-CD8β and anti-MAC320 mAb staining, cells were subsequently incubated with 50 µl of appropriate secondary antibody at 4^{o}C in the dark, followed by three washes with PBS^{++}. All cells were then fixed with 100 µl of BD Cytofix/Cytoperm solution (BD Pharmingen) for 30 minutes at 4^{o}C in the dark, washed twice with 500 µl of BD Perm/Wash (BD Pharmingen), and 50 µl of R-PE-conjugated anti-IFNγ mAb were added. Cells were incubated in the dark for 30 minutes at 4^{o}C, washed twice, and fixed with 300 µl of 1% formaldehyde (Fisher Scientific) in PBS. Membrane fluorescence of the stained cells was analyzed by a FACScan cytometer (BD Pharmingen). Flow cytometry data were analyzed using CellQuest analysis software (version

3.3, BD Biosciences, San Jose, CA). A dot plot of linear side scatter versus linear forward scatter was generated to gate lymphocyte populations from total PBMCs. A histogram based on FITC-conjugated CD4α, CD8β, or MAC320 was applied to lymphocyte populations to differentiate T cell subsets. A histogram based on R-PE-conjugated IFNγ was applied to each T cell subset to obtain the percentage of $IFN\gamma^+$ cells.

Statistical analysis

All statistical analyses were performed using the JMP6 software (SAS Institute Inc., Cary, NC). Mean differences of IL-10, IL-2, IL-4, TNFα, IFNα, and IFNγ mRNA expressions were tested by one-way analysis of variance, followed by Dunnett's test using the mean of the positive control group as a control, and, where indicated, using the mean of the transfection media control group or PRRSV-inoculated group as a control. The same statistical methods were applied for analysis of mean differences of percent viable cells and percent $IFN\gamma^+$ in lymphocytes and T cell subsets. $P<0.05$ was set as a statistically significant level throughout this study.

RESULTS

IL-10AS suppressed IL-10 mRNA expression in PRRSV-inoculated PBMC

IL-10AS has been demonstrated to down-regulate IL-10 mRNA expression in ConA-stimulated porcine PBMCs (50). In this study, we further investigated the potential of using IL-10AS to down-regulate IL-10 mRNA expression in porcine PBMCs inoculated with PRRSV in the presence of a cytokine inducer, i.e. ConA or PMA/I. To determine the optimal concentration

of IL-10AS, PBMCs were transfected with IL-10AS at various concentrations and subsequently stimulated with ConA or PMA/I and the IL-10 mRNA expression was analyzed by real-time PCR. The IL-10AS at 2 μM showed significant suppression of IL-10 mRNA expression in ConA- and PMA/I-stimulated PBMCs (Fig. 1); thus this concentration was selected for further investigation. The IL-10AS used was demonstrated to specifically suppress IL-10 mRNA expression, as it had no effect on IL-2, IL-4, TNFα, IFNα, and IFNγ mRNA expression in the stimulated PBMCs (data not shown). No reduction in IL-10 mRNA expression was observed in the scramble controls (Fig. 1).

PRRSV-inoculated PBMCs demonstrated significantly increased IL-10 mRNA expression in response to ConA or PMA/I stimulation, as compared to the absence of PRRSV (positive control) (Fig. 2). Transfection of such PBMCs with IL-10AS significantly reduced IL-10 mRNA expression (Fig. 2). This observation was not due to different numbers of viable PBMCs after the treatments. The percent viability of PBMCs in the absence and presence of IL-10AS in ConA or PMA/I stimulation were 47.4±3.4% and 47.1±3.4%, and 49.3±4.1% and 50.2±3.8% , respectively; these values were not significantly different from those of the positive control and the PRRSV-inoculated group**.**

IL-10AS effects on innate and adaptive cytokine mRNA expressions in PRRSV-inoculated PBMC

PRRSV-inoculated PBMCs showed significantly increased IL-2 mRNA but unchanged IL-4 mRNA expression in response to ConA stimulation (Fig. 3). However, the PBMCs had significantly reduced levels of IFNα and IFNγ mRNA, but levels of TNFα mRNA expression were unchanged in response to PMA/I stimulation (Fig. 3). No significant changes in cytokine mRNA expression were detected in the mock control (Fig. 3).

Compared to PRRSV-inoculated PBMCs, transfection of PBMCs with IL-10AS prior to PRRSV inoculation and ConA stimulation resulted in significantly reduced IL-2 mRNA expression, while IL-4 mRNA expression was unchanged (Fig. 3). On the other hand, transfection of PBMCs with IL-10AS prior to PRRSV inoculation and PMA/I stimulation resulted in increased IFNγ mRNA expression, but TNFα and IFNα mRNA expression was unchanged (Fig. 3). No significant modulation in any cytokine mRNA expression was observed in PRRSV-inoculated transfection media control (Fig. 3).

IL-10AS effects on %IFNγ$^+$ cells in lymphocytes and T cell subsets

The lymphocyte population from PRRSV-inoculated PBMCs had significantly reduced percentage of IFNγ$^+$ cells in response to PMA/I/G stimulation in comparison to the absence of PRRSV (positive control) (Fig. 4). Lymphocytes from mock control PBMCs did not show such a significant reduction (Fig. 4). The significant reduction of the percentage of IFNγ$^+$ cells was restricted to CD8β$^+$, and not observed for the CD4$^+$ and MAC320$^+$ T cell subsets (Fig. 5). Significant reduction of %IFNγ$^+$ cells was not due to virus-induced cell death. The percentage of viable PBMCs after PRRSV inoculation and subsequent PMA/I/G stimulation was 45.7±3.1%, which was not significantly different from that in the absence of virus (positive control) (48.1±5.4%).

Compared to lymphocyte population and CD8β$^+$ T cells from PRRSV-inoculated PBMCs, transfection of PBMCs with IL-10AS prior to PRRSV inoculation and PMA/I/G stimulation resulted in an increased percentage of IFNγ$^+$ cells in lymphocytes ($p=0.11$) and CD8β$^+$ T cells ($p=0.068$) (Fig. 4 and 5). No increase in the percentage IFNγ$^+$ cells was detected

in lymphocytes and T cell subsets of PRRSV-inoculated transfection media control (Fig. 4 and 5).

DISCUSSION

In this study we investigated the potential of IL-10AS in down-regulating IL-10 mRNA expression and in inducing innate (TNFα and IFNα) and adaptive (IL-2, IL-4, and IFNγ) cytokine mRNA expression in naïve PBMCs inoculated with PRRSV in the presence of cytokine inducers, ConA or PMA/I. The effect of IL-10AS on an induction of %IFNγ^+ cells was also investigated.

The antisense activity of IL-10AS was observed in PBMCs stimulated with ConA or PMA/I via significant reduction of IL-10 mRNA expression (Fig. 1). No significant reduction of IL-2, IL-4, TNFα, IFNα, and IFNγ mRNA expression was observed. In addition, no significant reduction of IL-10 mRNA expression was observed in scramble ODN controls (Fig. 1). These findings demonstrated the specificity of IL-10AS to IL-10 mRNA. Phosphorothioate-modified AS ODN reportedly regulates mRNA expression through RNaseH-mediated degradation of target mRNA (49,53). The phosphorothioate-modified AS ODN binds to target mRNA in a sequence-specific manner, and forms an mRNA/DNA duplex which triggers RNaseH to cleave hybridized target mRNA, resulting in reduced amounts or absence of intact mRNA template for translation, and thereby reduced target protein production (49,53).

PRRSV has been demonstrated to up-regulate IL-10 mRNA expression in various immune cell types, including PAM, MDC, BDC, and PBMCs (5,8,10,14,20-24,26,29,42,48,54,55). In this study, PRRSV-up-regulated IL-10 mRNA expression in PBMCs

in response to ConA and PMA/I stimulation (Fig. 2). Transfection of PBMCs with IL-10AS prior to PRRSV inoculation and cell activation significantly down-regulated IL-10 mRNA expression (Fig. 2). The reduced IL-10 mRNA expression was neither due to IL-10AS- and transfection media-induced cell death nor transfection media-mediated reduced cytokine mRNA expression. These observations suggest that IL-10AS was efficient in suppressing IL-10 mRNA expression in PBMCs in response to PRRSV.

PRRSV modulating innate and adaptive cytokine mRNA expression was observed in PBMCs in response to cytokine inducers (Fig. 3). The virus significantly up-regulated IL-2 mRNA expression in response to ConA stimulation, and significantly down-regulated IFNα and IFNγ mRNA expression in response to PMA/I stimulation (Fig. 3). PRRSV up-regulation of IL-2 mRNA expression has been reported in naïve PBMCs inoculated with the virus and stimulated with ConA, and in primed PBMCs stimulated *in vitro* with recall virus (8,34). PRRSV suppression of IFNα mRNA expression and IFNα and IFNγ productions has been reported *in vitro* and in PRRSV-infected pigs (1,4,8,15,24,31,36,48,60). No significant modulation of IL-4 and TNFα mRNA expression was observed in PRRSV-inoculated PBMCs in response to ConA and PMA/I stimulation, respectively (Fig. 3). This was in accordance with previous reports that PRRSV barely induces, or even reduces, IL-4 and TNFα mRNA expression (5,20,21,24,26,34,52,55).

Compared to PRRSV-inoculated PBMCs, transfection with IL-10AS prior to PRRSV inoculation resulted in significantly reduced IL-10 and IL-2 mRNA expression. Increased IFNγ and unchanged IFNα mRNA expression were also observed in response to IL-10AS treatment (Fig. 3). IL-10 has been reported to suppress IL-2, IFNγ, and IFNα productions in human and murine PBMCs and T cells (17,19,27,43,44,56,57). The cytokine reportedly suppresses IFNγ

production in porcine T cells (8,63). Its effect on porcine IL-2 and IFNα transcription and translation, however, has not been investigated. In humans and mice, neutralization of IL-10 by anti-IL-10 mAbs significantly increased IL-2, IFNα, and IFNγ production (27,43,57). In pigs, neutralization of IL-10 by anti-IL-10 mAbs significantly increased IFNγ production by T cells (8). Considering the evidence in humans and mice, PRRSV-inoculated PBMCs should have shown increased IL-2 and IFNα mRNA expression in response to IL-10AS transfection, rather than reduced or unchanged cytokine mRNA expression. Our present results were thus unexpected. A possible reason for the reduced IL-2 mRNA expression may be that IL-10AS interfered with the PRRSV mechanism of IL-2 induction. This may be through IL-10AS binding to and thereby facilitating degradation of PRRSV-up-regulated mRNA molecules signaling for IL-2 induction. Such mRNA molecules should not be up-regulated by ConA alone, since no reduction of IL-2 mRNA expression was observed in IL-10AS-transfected PBMCs stimulated with ConA. To date, the PRRSV mechanism for IL-2 induction in ConA-stimulated naïve PBMCs is unknown. Further studies are required to investigate such mechanisms of the virus, as well as the cause of IL-10AS-mediated IL-2 mRNA suppression in PRRSV-inoculated PBMCs. In addition, modification of IL-10AS may be needed to minimize its suppressive effect on IL-2 mRNA expression, as this may affect adaptive immune response to PRRSV.

In contrast to the possible reasons for reduced IL-2 mRNA expression, the explanation for the lack of IFNα mRNA up-regulation in IL-10AS-transfected PBMCs inoculated with PRRSV may be that PRRSV does not utilize IL-10 to down-regulate this cytokine's mRNA expression. The virus may exploit other mechanisms to suppress IFNα mRNA expression. Recently, Luo et al (37) reported that PRRSV inhibited the retinoic acid-inducible gene I (RIG I)-mediated pathway, resulting in inhibition of IFNβ translation, a cytokine required for IFNα

mRNA expression (30,32). It is not known whether PRRSV suppression of the RIG I-mediated pathway is the sole mechanism of PRRSV used to suppress IFNα mRNA expression. It is also unclear what triggers viral suppression of the RIG I-mediated pathway. Further studies are needed to investigate PRRSV mechanism of IFNα mRNA suppression, as well as to elucidate the effects of IL-10 on innate and adaptive cytokine mRNA expression in porcine PBMCs.

PRRSV-inoculated PBMCs had significantly reduced percentages of $IFN\gamma^+$ cells in response to PMA/I/G stimulation (Fig. 4). The reduction was detected in the $CD8\beta^+$ T cell subset, but not in $CD4^+$ and $MAC320^+$ T cell subsets (Fig. 5). In pigs, T cells expressing the CD8β subunit are cytotoxic T cells, and T cells expressing CD4 and MAC320 molecules are T helper cells and $CD2^-\gamma\delta^+$ T cells, respectively (7,66). All of these porcine T cell subsets ($CD8^+$, $CD4^+$, $\gamma\delta^+$) were reportedly susceptible to PMA/I/G stimulation for IFNγ production (46). The observation that PRRSV significantly reduced $\%IFN\gamma^+$ in $CD8\beta^+$ T cells in response to PMA/I/G stimulation was evidence for the presence of a suppressive effect of PRRSV on CMI response (6).

Transfection of PBMCs with IL-10AS prior to PRRSV inoculation and PMA/I/G stimulation contributed to increased percentage of $IFN\gamma^+$ cells (Fig. 4 and Fig. 5). Increased IFNγ production in response to IL-10 knockdown has been reported in numerous human and murine studies in which $CD4^+$ and $CD8^+$ T cells demonstrated significantly increased IFNγ production after cultivation with antigens and IL-10AS- or IL-10 small interfering RNA-transfected antigen-presenting cells (11,25,28,33,51). The observation that IL-10AS enhanced the percentage of $IFN\gamma^+$ in lymphocytes and $CD8\beta^+$ T cells suggests that IL-10AS might be useful for enhancing CMI response to PRRSV.

In conclusion, the present study reports that IL-10AS is efficient in down-regulating IL-10 mRNA expression and in up-regulating IFNγ mRNA as well as in enhancing the percentage of $IFN\gamma^+$ cells. IL-10AS, however, significantly down-regulates IL-2 mRNA expression, and has no effect on IL-4, TNFα, and IFNα mRNA expression. The capacity of IL-10AS in reducing IL-10 mRNA expression and inducing IFNγ response suggests its potential to enhance CMI response to PRRSV. However, modification of IL-10AS to minimize its suppressive effect on IL-2 mRNA response may be needed for ideal CMI enhancement.

ACKNOWLEDGEMENTS

The authors thank Dr.Ratree Platt at Iowa State University for critical review of the manuscript and appreciate excellent technical support of Ms.Reunkaew Praphrute. This work is supported by the Thailand Research Fund (grant# MRG5080167), the Commission on Higher Education, and National Science and Technology Development Agency. We thank Dr. Dale E. Taneyhill for proofreading the manuscript.

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FIGURE CAPTIONS

FIGURE 1. IL-10 mRNA expression in PBMCs transfected with various concentrations of IL-10AS and stimulated with ConA and PMA/I. PBMCs were transfected with indicated concentrations of IL-10AS for 4 hours and subsequently stimulated with ConA or PMA/I for 16 or 12 hours, respectively, prior to real-time PCR analysis. PBMCs treated with culture media alone prior to stimulation with either cytokine inducer served as positive controls. PBMCs transfected with scramble ODN (Scr1, Scr2, Scr3) at 2 μM or treated with transfection media prior to stimulation with ConA or PMA/I served as scramble controls and transfection media control, respectively. Data were normalized with RPL32 mRNA expression of the same animal. Error bar indicates the standard error of the mean (SEM). * represents significant mean difference of IL-10 mRNA expression between treatment and positive control groups ($p<0.05$).

FIGURE 2. IL-10AS suppresses IL-10 mRNA expression induced by PRRSV. PBMCs were transfected with IL-10AS at 2 μM for 4 hours, then cultivated with PRRSV for 48 hours, and stimulated with ConA or PMA/I for 16 or 12 hours, respectively. The IL-10 mRNA was determined by real-time PCR analysis. PBMCs stimulated with ConA and PMA/I, in the absence of IL-10AS and PRRSV, served as the positive control. PBMCs treated with culture media plus mock antigens or PRRSV and stimulated with either cytokine inducer served as the mock control and PRRSV-inoculated control (represented by PRRSV), respectively. PBMCs treated with transfection media (no IL-10AS) plus PRRSV and stimulated with either cytokine inducer served as PRRSV-inoculated transfection media control. Data were normalized with RPL32 mRNA expression from the same animal. Error bars indicates the SEM. * represents a

significant mean difference in IL-10 mRNA expression between treatment and positive control groups ($p<0.05$). Different letters (a, b) indicate significant mean differences of IL-10 mRNA expression between transfected and PRRSV-inoculated control groups ($p<0.05$).

FIGURE 3. Effect of IL-10AS on IL-2, IL-4, TNFα, IFNα, and IFNγ mRNA expression. Mean increase in IL-2, IL-4, TNFα, IFNα, and IFNγ mRNA expression in PBMCs transfected with IL-10AS at 2 μM prior to PRRSV inoculation, and ConA (for IL-2 and IL-4) or PMA/I (for TNFα, IFNα, and IFNγ) stimulation. PBMCs were transfected with IL-10AS at 2 μM for 4 hours, then cultivated with PRRSV for 48 hours, and stimulated with ConA or PMA/I for 16 or 12 hours, respectively, prior to real-time PCR analysis. PBMCs treated with culture media alone and stimulated with cytokine inducer served as the positive control. PBMCs treated with culture media plus mock antigens or PRRSV and stimulated with cytokine inducer served as the mock control and PRRSV-inoculated control (represented by PRRSV), respectively. PBMCs treated with transfection media (no IL-10AS) plus PRRSV and stimulated with cytokine inducer served as PRRSV-inoculated transfection media control. Data were normalized with RPL32 mRNA expression from the same animal. Error bars indicates the SEM. * represents significant mean difference of cytokine mRNA expression between treatment and positive control groups ($p<0.05$). Different letters (a, b) indicate significant mean differences in cytokine mRNA expression between transfected and PRRSV-inoculated control groups ($p<0.05$).

FIGURE 4. Effect of IL-10AS on %IFNγ$^+$ cells in lymphocytes after PRRSV inoculation. PBMCs were transfected with IL-10AS at 2 μM for 4 hours, then cultivated with PRRSV for 48 hours, and stimulated with PMA/I/G for another 12 hours prior to flow cytometric analysis.

PBMCs treated with culture media alone and stimulated with PMA/I/G served as the positive control. PBMCs treated with culture media plus mock antigens or PRRSV and stimulated with PMA/I/G served as a mock control and PRRSV-inoculated control (represented by PRRSV), respectively. PBMCs treated with transfection media (no IL-10AS) plus PRRSV and stimulated with PMA/I/G served as a PRRSV-inoculated transfection media control. Data were normalized with RPL32 mRNA expression from the same animal. Error bars indicates the SEM. * represents a significant mean difference in the percentage of $IFN\gamma^+$ between treatment and positive control groups ($p<0.05$). Same letters (a) indicate no significant mean difference in the percentage of $IFN\gamma^+$ cells between transfected and PRRSV-inoculated control groups ($p>0.05$).

FIGURE 5. Induction of $IFN\gamma^+$ cells in T cell subsets by IL-10AS. PBMCs were transfected with IL-10AS for 4 hours, then cultivated with PRRSV for 48 hours, and stimulated with PMA/I/G for another 12 hours prior to flow cytometric analysis. T cell subsets from PBMCs treated with culture media alone and stimulated with PMA/I/G served as a positive control. T cell subsets from PBMCs treated with culture media plus mock antigens or PRRSV and stimulated with PMA/I/G served as a mock control and PRRSV-inoculated control (represented by PRRSV), respectively. T cell subsets from PBMCs treated with transfection media (no IL-10AS) plus PRRSV and stimulated with PMA/I/G served as PRRSV-inoculated transfection media control. Error bars indicates the SEM. * represents a significant mean difference in the percentage of $IFN\gamma^+$ of $CD4^+$, $CD8\beta^+$, and $MAC320^+$ T cells between treatment and positive control groups ($p<0.05$). Same letters (a) indicate no significant mean difference in the percentage of $IFN\gamma^+$ cells between T cell subsets of transfected and PRRSV-inoculated control groups ($p>0.05$).

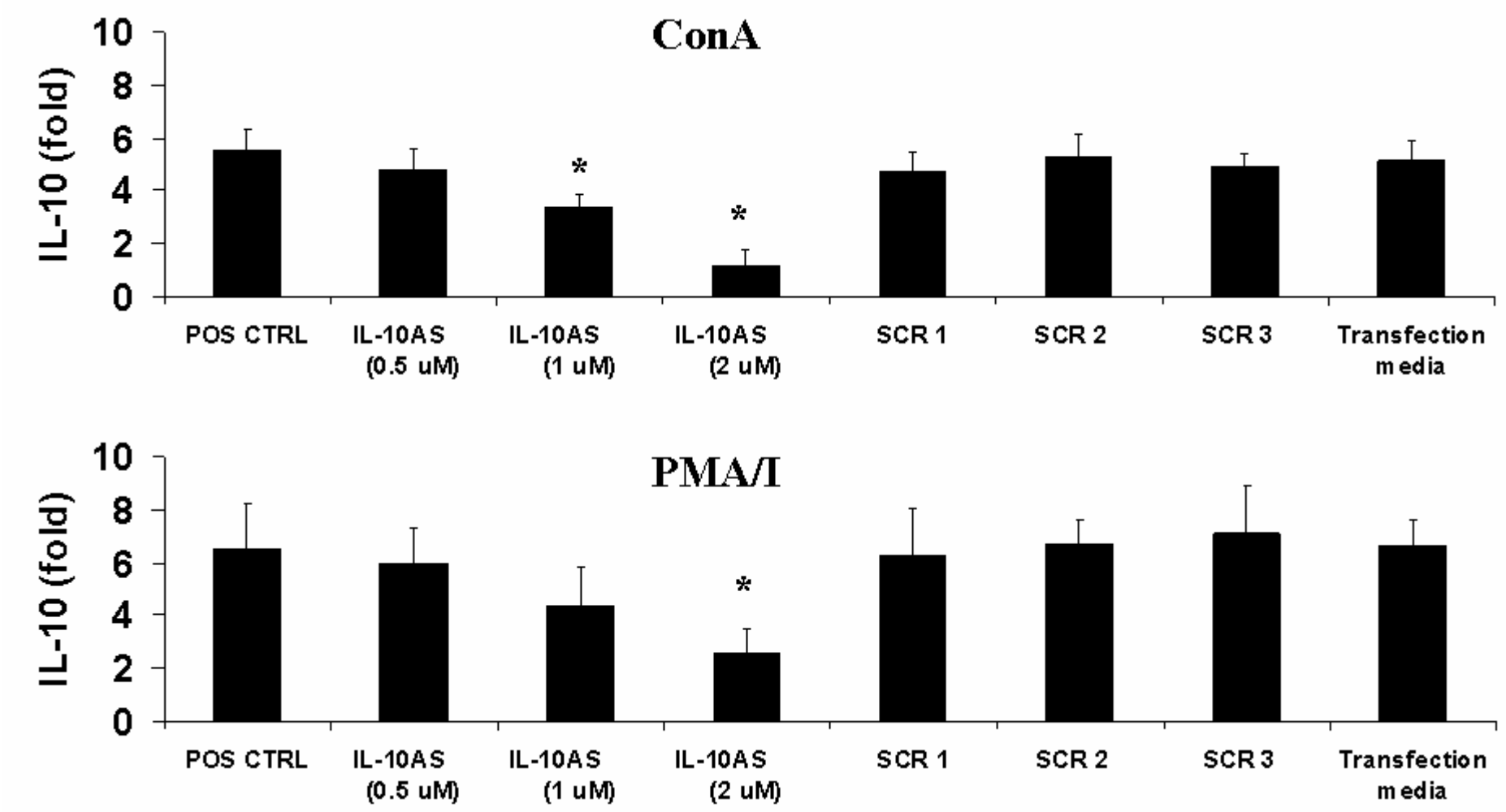
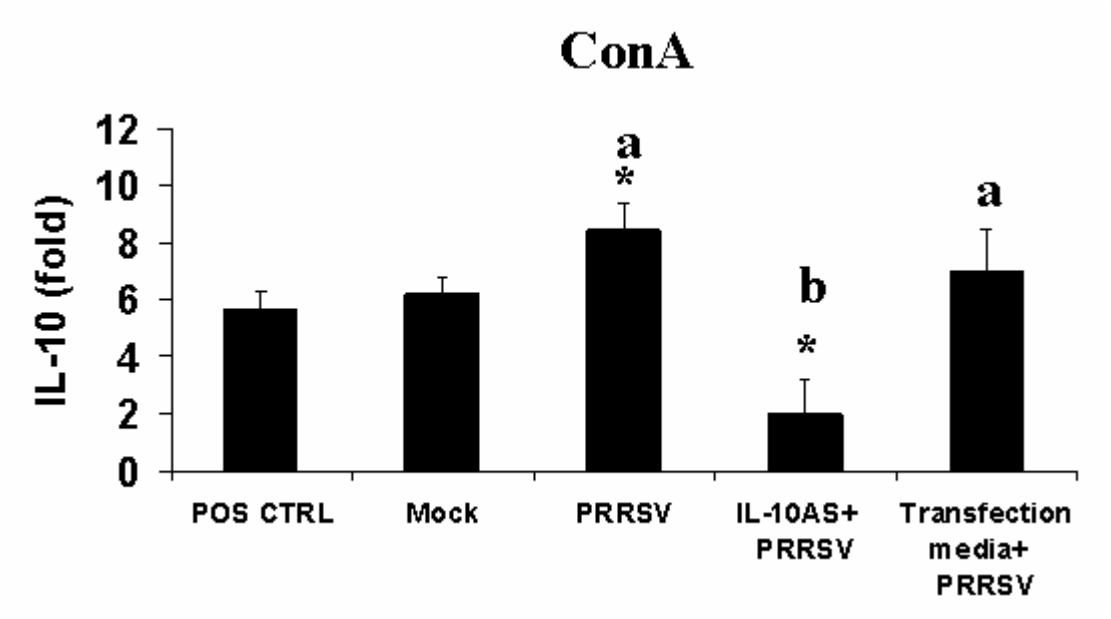


FIGURE 1. (Charerntantanakul and Kasinrerk)



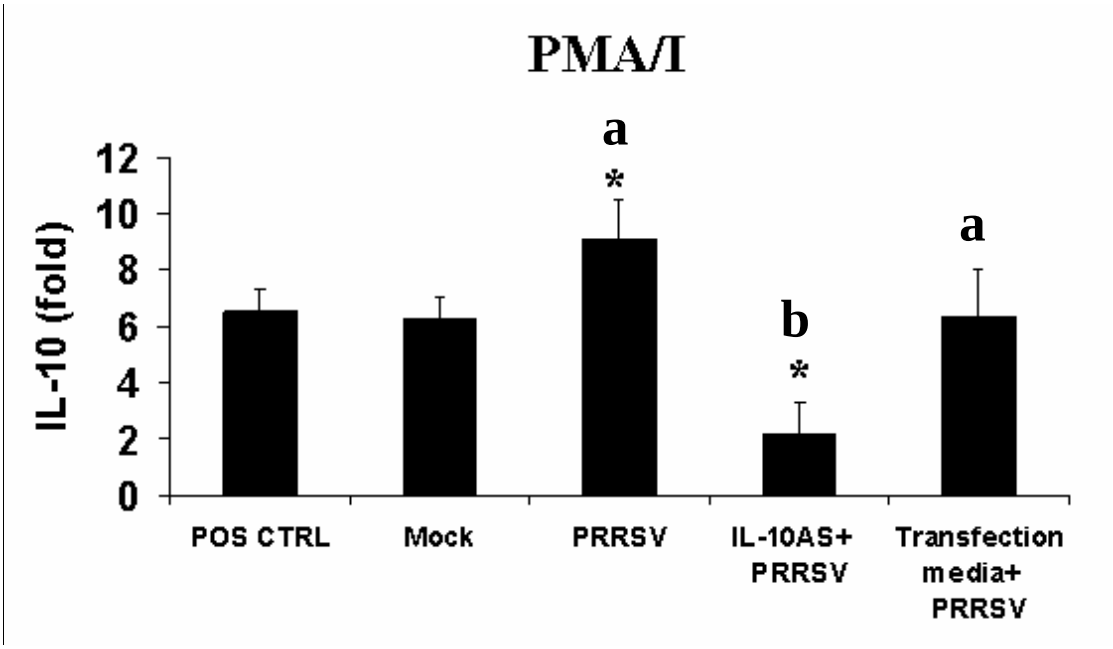


FIGURE 2. (Charerntantanakul and Kasinrerk)

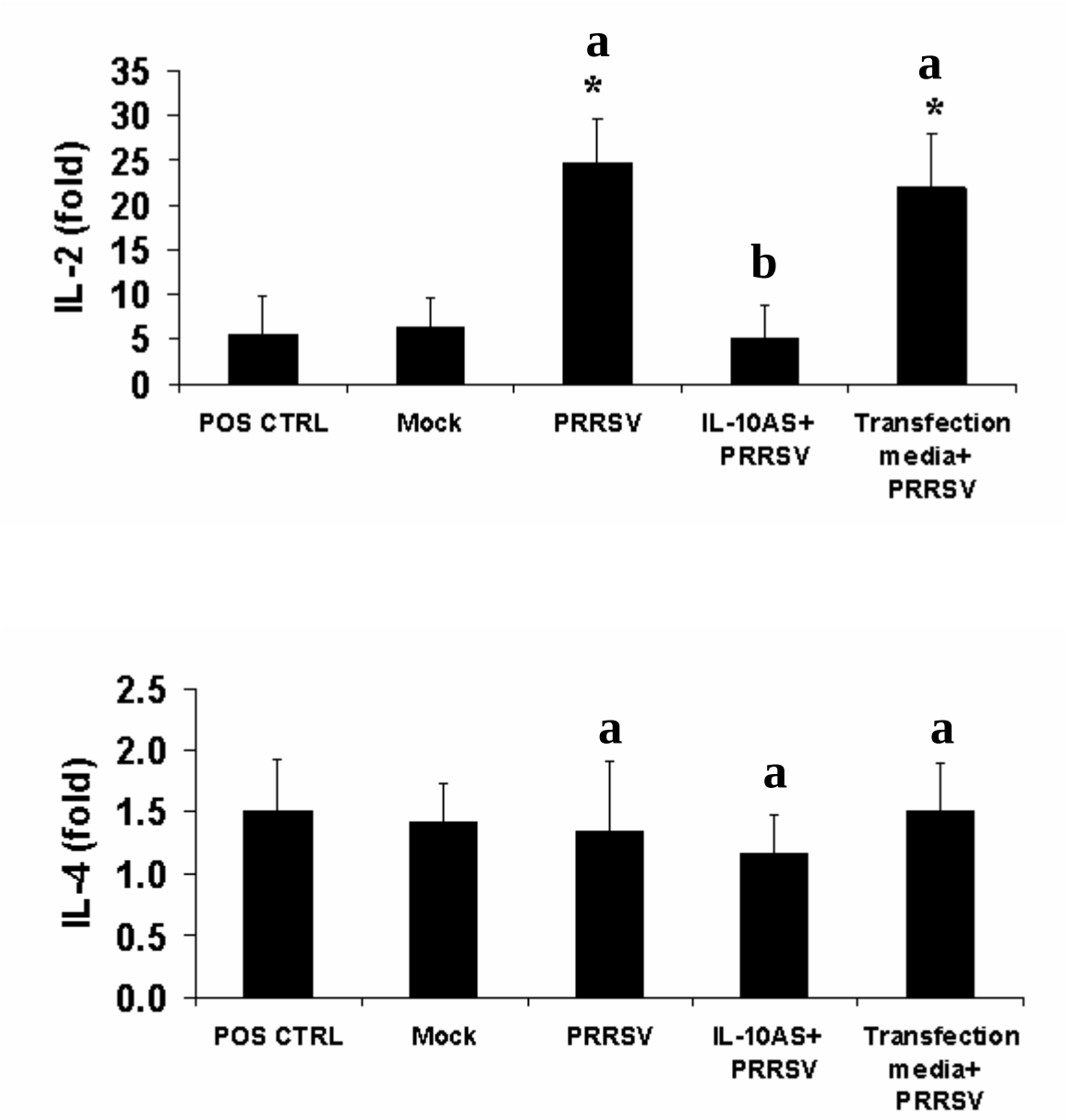


FIGURE 3. (Charerntantanakul and Kasinrerk)

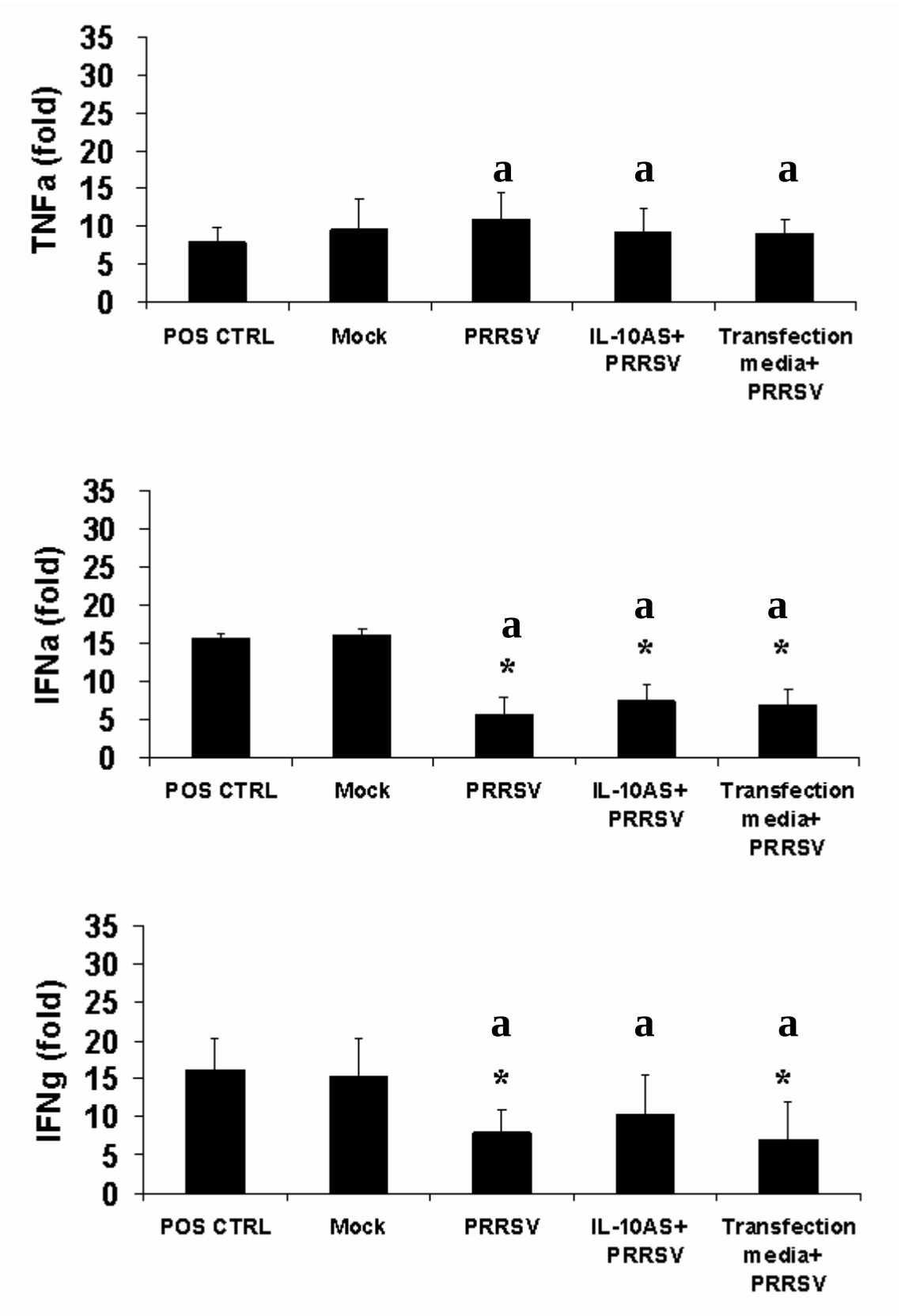


FIGURE 3 (cont). (Charerntantanakul and Kasinrerk)

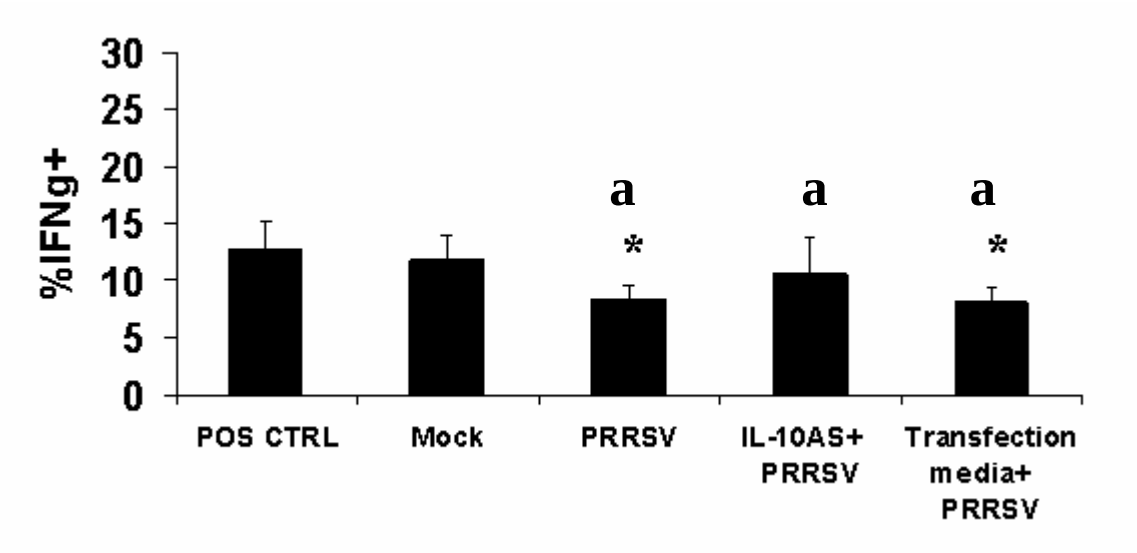


FIGURE 4. (Charerntantanakul and Kasinrerk)

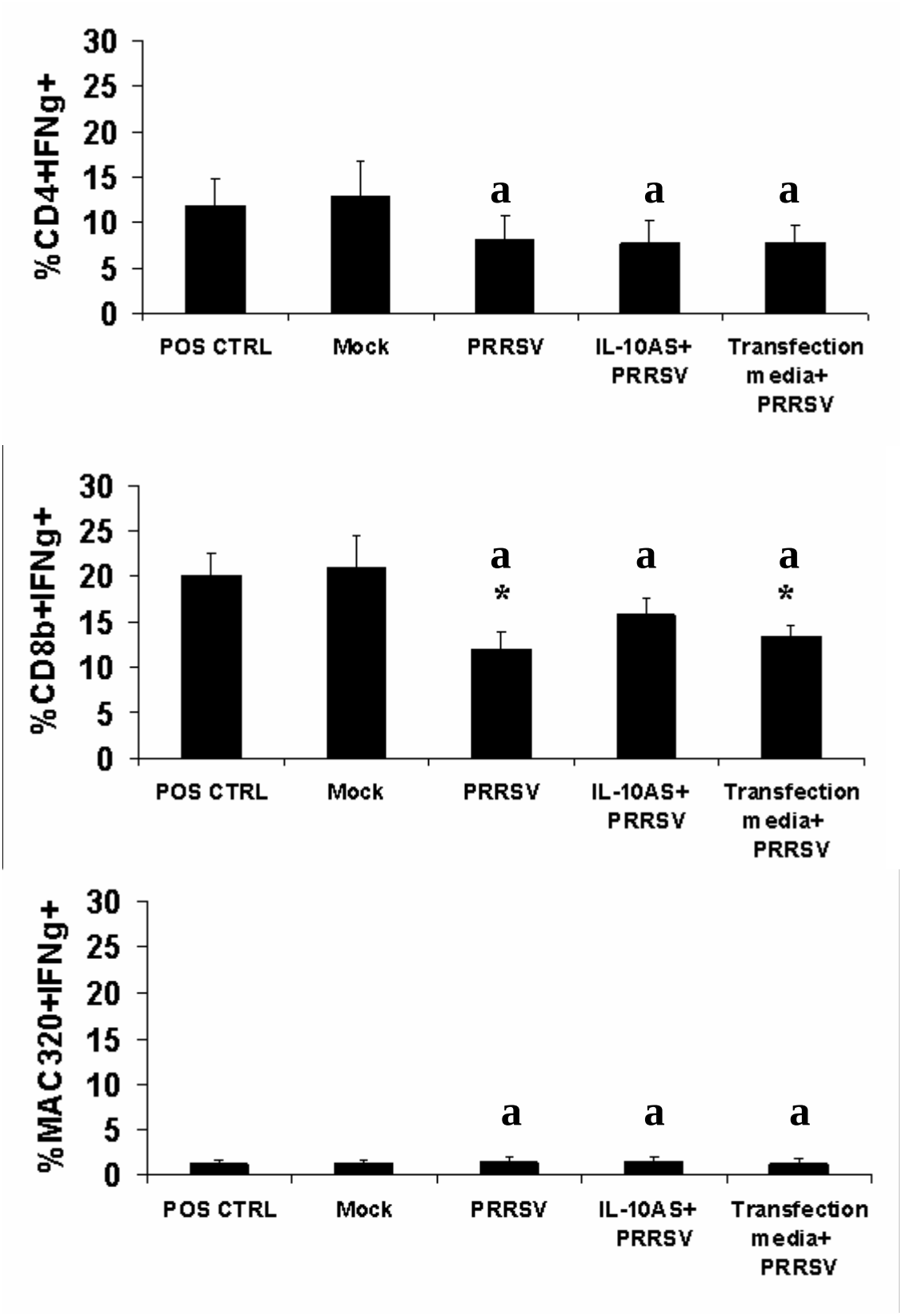


FIGURE 5. (Charerntantanakul and Kasinrerk)