รายงานวิจัยฉบับสมบูรณ์

โครงการ การประเมินฤทธิ์แอดจูแวนท์ของ Matrix สำหรับวัคซีนพีอาร์อาร์เอสและฤทธิ์ยับยั้งไวรัสพีอาร์อาร์เอส

โดย นาย วศิน เจริญตัณธนกุล

กรกฎาคม 2561

สัญญาเลขที่ RSA5880053

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และมหาวิทยาลัยแม่โจ้

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยแม่โจ้ที่ให้ทุนสนับสนุนงานวิจัยนี้จนสำเร็จลุล่วงไปด้วยดี และขอขอบคุณสาขาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ และคณะสัตวศาสตร์และเทคโนโลยี มหาวิทยาลัยแม่โจ้ที่ให้ความอนุเคราะห์ครุภัณฑ์ วัสดุและอุปกรณ์ต่างๆที่จำเป็นตลอดงานวิจัย

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ชื่อโครงการ : การประเมินฤทธิ์แอดจูแวนท์ของ Matrix สำหรับวัคซีนพีอาร์อาร์เอสและฤทธิ์ยับยั้งไวรัสพีอาร์อาร์เอส

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ไวรัสพีอาร์อาร์เอส (porcine reproductive and respiratory syndrome virus) สร้างความสูญเสียให้กับอุตสาหกรรมการผลิตสุกรของประเทศไทย ไวรัสมีสองสายพันธุ์ คือ สายพันธุ์ยุโรปและสายพันธุ์อเมริกาเหนือ ซึ่งทั้งสองสายพันธุ์ก่อปัญหาในประเทศไทย วัคซีนที่มีใช้อยู่ในปัจจุบันกระตุ้นภูมิคุ้มกันได้ไม่สูงและคุ้มโรคที่เกิดจากการติดเชื้อข้ามสายพันธุ์ของไวรัสได้ไม่ดี Saponin quil A เป็นสารในธรรมชาติที่มีรายงานว่าสามารถกระตุ้นภูมิคุ้มกันทั้งแบบแอนติบอดีและแบบพึ่งเซลล์ในหนูทดลองได้ดี งานวิจัยนี้ประเมินฤทธิ์การกระตุ้นภูมิคุ้มกันของ quil A ในเม็ดเลือดขาวชนิดโมโนนิวเคลียร์เซลล์ (mononuclear cell) ของสุกรที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสในระดับห้องปฏิบัติการ ซึ่งพบว่า quil A สามารถกระตุ้นการแสดงออกของยีน Mx1, IRF3, IRF7, OAS1, STING, IFNb และ IFNg ได้เมื่อเทียบกับเม็ดเลือดขาวที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสเพียงอย่างเดียว โดย quil A ไม่มีผลต่อการติดเข้าสู่เซลล์และการยับยั้งการแบ่งตัวของไวรัสพีอาร์อาร์เอสในเซลล์เพาะเลี้ยง MARC-145 และเมื่อฉีด quil A ให้กับสุกรร่วมกับวัคซีนพีอาร์อาร์เอสสายพันธุ์ยุโรปพบว่าสุกรที่ได้รับ quil A ร่วมด้วยมีการแสดงออกของยีน IRF3, OAS1, osteopontin, IFNa, IFNb, IFNg, IL-2, IL-13 และ TNFa เพิ่มขึ้นเมื่อเทียบกับสุกรที่ได้รับวัคซีนเชื้อเป็นเพียงอย่างเดียว เมื่อสุกรได้รับการฉีดเชื้อพิษทับด้วยไวรัสพีอาร์อาร์เอสสายพันธุ์อเมริกาเหนือ พบว่าสุกรที่ได้รับ quil A ร่วมกับวัคซีนพีอาร์อาร์เอสเชื้อเป็นมีไตเตอร์ของไวรัสพีอาร์อาร์เอสลดลงเมื่อเทียบกับสุกรที่ได้รับวัคซีนเชื้อเป็นเพียงอย่างเดียว การศึกษานี้บ่งชี้ว่า quil A กระตุ้นภูมิคุ้มกันแบบพึ่งเซลล์ต่อวัคซีนพีอาร์อาร์เอสเชื้อเป็นและเพิ่มความคุ้มโรคที่เกิดจากการติดไวรัสพีอาร์อาร์เอสต่างสายพันธุ์

คำหลัก : ไวรัสพีอาร์อาร์เอส Quil A ภูมิคุ้มกันปฐมภูมิ อินเตอร์เฟอรอน ซัยโตคายน์ส่งเสริมการอักเสบ

Abstract

Project Code : RSA5880053

Project Title :

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Porcine reproductive and respiratory syndrome virus (PRRSV) devastates pig industry in Thailand. The virus comprises two genotypes, i.e. European and North American. Both genotypes cause problems in Thailand. Currently available vaccine elicits poor immune response and confers limited cross protection. Saponin quil A is a natural product that has been reported to induce balanced immune response in murine models. This study evaluated the immunogenicity of quil A in porcine peripheral blood mononuclear cells (PBMC) incubated with PRRSV. Quil A was found to up-regulate Mx1, IRF3, IRF7, OAS1, STING, IFNb and IFNg gene expressions, compared with PRRSV-inoculated PBMC control. Quil A did not affect PRRSV infectivity and replication in MARC-145 cells. When quil A was injected with European PRRSV modified-live virus (MLV) vaccine, pigs showed significantly increased IRF3, OAS1, osteopontin, IFNa, IFNb, IFNg, IL-2, IL-13 and TNFa gene expressions, compared with pigs vaccinated with PRRSV MLV vaccine alone. When quil A+vaccinated pigs were challenged with North American PRRSV, the animals had significantly reduced PRRSV titer in serum as compared to pigs vaccinated with PRRSV MLV vaccine alone. Our findings indicate that quil A stimulates cell-mediated immune response to and enhances cross-protective efficacy of PRRSV MLV vaccine.

Keywords : Porcine reproductive and respiratory syndrome virus; Quil A; Innate immunity, Interferon, Inflammatory cytokines

บทนำ

งานวิจัยนี้ศึกษาประสิทธิภาพของ saponin quil A ต่อการกระตุ้นการแสดงออกของซัยโตคายน์ที่ส่งเสริมกระบวนการอักเสบ (pro-inflammatory cytokine) ในเม็ดเลือดขาวชนิดโมโนนิวเคลียร์เซลล์ (peripheral blood mononuclear cell) ของสุกรที่บ่มร่วมกับไวรัสพีอาร์อาร์เอส (porcine reproductive and respiratory syndrome virus) และศึกษาประสิทธิภาพของ quil A ในการยับยั้งการติดเข้าสู่เซลล์และการแบ่งตัวของไวรัสพีอาร์อาร์เอสในเซลล์เพาะเลี้ยง MARC-145 รวมถึงศึกษาประสิทธิภาพของ quil A ในการกระตุ้นภูมิคุ้มกันและการคุ้มโรคในสุกรเมื่อฉีดร่วมกับวัคซีนพีอาร์อาร์เอสสายพันธุ์ยุโรปและสุกรได้รับการฉีดเชื้อพิษทับด้วยไวรัสพีอาร์อาร์เอสสายพันธุ์อเมริกาเหนือ

วิธีการทดลอง

การศึกษาความเข้มข้นที่ไม่เป็นพิษของ quil A ต่อ PBMC ของสุกร

เจาะเก็บเลือดและปั่นแยก PBMC จากสุกรที่ไม่มีแอนติบอดีต่อไวรัสพีอาร์อาร์เอส บ่ม PBMC ร่วมกับ saponin quil A ที่ความเข้มข้นต่างๆ ตรวจการมีชีวิตของเซลล์ที่ได้รับ quil A ด้วยการย้อมสี trypan blue และ MTT solution (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript) เลือกความเข้มข้นที่สูงที่สุดของ quil A ที่ไม่เป็นพิษต่อ PBMC เพื่อใช้ในการศึกษาต่อไป

การศึกษาผลของ quil A ต่อการแสดงออกของยีนที่เกี่ยวข้องกับภูมิคุ้มกันใน PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอส

บ่ม PBMC ร่วมกับ quil A และไวรัสพีอาร์อาร์เอส ศึกษาระดับการแสดงออกของยีน Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNa, IFNb, IL-10, TGFb, IFNg, IL-2, IL-13, and TNFa ด้วยวิธี real-time PCR โดยใช้ RPL32 และ YWHAZ เป็น housekeeping gene เปรียบเทียบระดับการแสดงออกของยีนดังกล่าวกับ PBMC ที่บ่มร่วมกับ quil A เพียงอย่างเดียว และ PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสเพียงอย่างเดียว (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

การศึกษาผลของ quil A ต่อการแสดงออกของยีนที่เกี่ยวข้องกับภูมิคุ้มกันใน PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและกระตุ้นด้วย Poly IC หรือ LPS

บ่ม PBMC ร่วมกับ quil A และไวรัสพีอาร์อาร์เอส จากนั้นกระตุ้นด้วย Poly IC หรือ LPS ศึกษาระดับการแสดงออกของยีน Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNa, IFNb, IL-10, TGFb, IFNg, IL-2, IL-13, and TNFa ด้วยวิธี real-time PCR โดยใช้ RPL32 และ YWHAZ เป็น housekeeping gene เปรียบเทียบระดับการแสดงออกของยีนดังกล่าวกับ PBMC ที่บ่มร่วมกับ quil A+poly IC/LPS และ PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอส+poly IC/LPS (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

การศึกษาผลของ quil A ต่อการติดเข้าสู่เซลล์และการแบ่งตัวของไวรัสพีอาร์อาร์เอส

บ่ม quil A ร่วมกับเซลล์ MARC-145 จากนั้นปิเปตไวรัสพีอาร์อาร์เอสใส่ หรือบ่มไวรัสพีอาร์อาร์เอสร่วมกับเซลล์ MARC-145 จากนั้นปิเปต quil A ใส่ บ่มทั้งหมดนาน 96 ชั่วโมง แล้วศึกษาไตเตอร์ของไวรัสด้วยวิธี immunoperoxidase monolayer assay (IPMA) (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

การศึกษาผลของ quil A เมื่อฉีดร่วมกับวัคซีนพีอาร์อาร์เอสเชื้อเป็นสายพันธุ์ยุโรปในสุกรและสุกรได้รับเชื้อพิษทับด้วยไวรัสพีอาร์อาร์เอสสายพันธุ์อเมริกาเหนือ

สุกรอายุ 4 สัปดาห์ที่ไม่มีแอนติบอดีต่อไวรัสพีอาร์อาร์เอสจำนวน 24 ตัว แบ่งออกเป็น 4 กลุ่ม กลุ่มละ 6 ตัว กลุ่มแรกได้รับวัคซีนพีอาร์อาร์เอสเชื้อเป็นสายพันธุ์ยุโรป (Amervac®; Hipra) ในวันที่ 0 ของงานทดลอง กลุ่มที่สองได้รับวัคซีนพีอาร์อาร์เอสเชื้อเป็นสายพันธุ์ยุโรป (Amervac®; Hipra) ในวันที่ 0 ของงานทดลองและได้รับ quil A (Invivogen) ในวันที่ -1, 0 และ 1 ของงานทดลอง กลุ่มที่สามได้รับตัวทำละลายวัคซีน (vaccine solvent) ในวันที่ 0 ของงานทดลอง และกลุ่มที่สี่ไม่ได้รับสิ่งใด ในวันที่ 28 ของการทดลอง สุกรในกลุ่มที่หนึ่งถึงสามได้รับเชื้อพิษทับด้วยไวรัสพีอาร์อาร์เอสสายพันธุ์อเมริกาเหนือ 01NP1

เจาะเลือดสุกรทุกตัวในวันที่ 0, 7, 14, 21, 28, 35, 42 และ 49 ของการทดลองเพื่อศึกษาระดับแอนติบอดีด้วยวิธี ELISA การแสดงออกของยีน Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNa, IFNb, IL-10, TGFb, IFNg, IL-2, IL-13, and TNFa ด้วยวิธี real-time PCR โดยใช้ RPL32 และ YWHAZ เป็น housekeeping gene และปริมาณสารพันธุกรรมของไวรัสในซีรัม (serum) ด้วยวิธี real-time PCR โดยใช้ ORF7-encoded recombinant plasmid เป็น standard (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

ตรวจวัดอุณหภูมิร่างกายทางทวารหนัก (rectal temperature) และอาการแสดงทางคลินิก ในสุกรทุกตัวในวันที่ 28 เป็นต้นไปจนถึงวันที่ 49 ของการทดลอง ชั่งน้ำหนักสุกรทุกตัวในวันที่ 28 และ 49 ของการทดลองเพื่อวิเคราะห์การเจริญเติบโตต่อวัน (รายละเอียดแสดงในภาคผนวก ในส่วน material and method ของ manuscript)

ผลการทดลอง

การศึกษาความเข้มข้นที่ไม่เป็นพิษของ quil A ต่อ PBMC ของสุกร

ความเข้มข้นที่สูงที่สุดของ quil A ที่ไม่เป็นพิษต่อ PBMC ของสุกรเท่ากับ 4 ไมโครกรัม/มล. (รายละเอียดแสดงในภาคผนวก ในส่วน results ของ manuscript)

การศึกษาผลของ quil A ต่อการแสดงออกของยีนที่เกี่ยวข้องกับภูมิคุ้มกันใน PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอส

PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและ quil A มีการแสดงออก Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNa, IFNb, IFNg และ IL-13 เพิ่มขึ้น และมีการแสดงออก IL-2 และ TGFb ลดลงเมื่อเทียบกับ PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอส

PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและ quil A มีการแสดงออก Mx1, IRF7, OAS1, STING, IFNa และ IL-10 เพิ่มขึ้นเมื่อเทียบกับ PBMC ที่บ่มร่วมกับ quil A (รายละเอียดแสดงในภาคผนวก ในส่วน results ของ manuscript)

การศึกษาผลของ quil A ต่อการแสดงออกของยีนที่เกี่ยวข้องกับภูมิคุ้มกันใน PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและกระตุ้นด้วย Poly IC หรือ LPS

PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและกระตุ้นด้วย Poly IC หรือ LPS มีการแสดงออก Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNa, IFNb, IFNg และ TNFa ลดลง และมีการแสดงออก IL-2, IL-10 และ IL-13 เพิ่มขึ้นเมื่อเทียบกับ PBMC ที่กระตุ้นด้วย Poly IC หรือ LPS

PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและ quil A และกระตุ้นด้วย Poly IC หรือ LPS มีการแสดงออก Mx1, IRF3, IRF7, OAS1, STING, IFNb และ IFNg เพิ่มขึ้น และมีการแสดงออก TGFb ลดลงเมื่อเทียบกับ PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและกระตุ้นด้วย Poly IC หรือ LPS (รายละเอียดแสดงในภาคผนวก ในส่วน results ของ manuscript)

การศึกษาผลของ quil A ต่อการติดเข้าสู่เซลล์และการแบ่งตัวของไวรัสพีอาร์อาร์เอส

เซลล์ MARC-145 ที่บ่มด้วยไวรัสพีอาร์อาร์เอสและ quil A มีไตเตอร์ของไวรัสพีอาร์อาร์เอสไม่แตกต่างจากเซลล์ MARC-145 ที่บ่มด้วยไวรัสพีอาร์อาร์เอสเพียงอย่างเดียวทั้งในระยะก่อนที่ไวรัสติดเข้าสู่เซลล์และระยะหลังจากที่ไวรัสติดเข้าสู่เซลล์แล้ว (รายละเอียดแสดงในภาคผนวก ในส่วน results ของ manuscript)

การศึกษาผลของ quil A เมื่อฉีดร่วมกับวัคซีนพีอาร์อาร์เอสเชื้อเป็นสายพันธุ์ยุโรปในสุกรและสุกรได้รับเชื้อพิษทับด้วยไวรัสพีอาร์อาร์เอสสายพันธุ์อเมริกาเหนือ

สุกรกลุ่มที่ 1 (ได้รับวัคซีนเพียงอย่างเดียว) และกลุ่มที่ 2 (ได้รับวัคซีน+Quil A) เริ่มสร้างแอนติบอดีหลังจากได้รับวัคซีนไปแล้ว 14 วัน และระดับแอนติบอดีค่อยๆเพิ่มสูงขึ้นจนถึงสูงที่สุดในวันที่ 28 หลังได้รับวัคซีน สุกรไม่สร้างแอนติบอดีเพิ่มขึ้น (anamnestic response) หลังได้รับการฉีดเชื้อพิษทับ สุกรทั้งสองกลุ่มมีระดับแอนติบอดีไม่แตกต่างกันตลอดการทดลอง

ก่อนการฉีดเชื้อพิษทับ สุกรกลุ่มที่ 2 มีการแสดงออกของ Mx1, IRF3, IRF7, IFNa, OPN, IL-2, IL-13, IFNb และ IFNg เพิ่มขึ้น และมีการแสดงออกของ IL-10 และ IL-13 ลดลง แต่มีการแสดงออกของ OAS1, STING, TNFa และ TGFb ไม่แตกต่างกันเมื่อเทียบกับสุกรกลุ่มที่ 1

หลังการฉีดเชื้อพิษทับ สุกรกลุ่มที่ 2 มีการแสดงออกของ IFNb, OAS1, OPN, IFNa, IFNg, IL-2, TNFa, IL-13 และ IRF3 เพิ่มขึ้น และมีการแสดงออกของ TGFb ลดลง แต่มีการแสดงออกของ Mx1, IRF7, sTING และ IL-10 ไม่แตกต่างกันเมื่อเทียบกับสุกรกลุ่มที่ 1

หลังการฉีดเชื้อพิษทับ สุกรกลุ่มที่ 2 มีปริมาณไวรัสพีอาร์อาร์เอสในกระแสเลือดลดลงประมาณ 1 $\log_{10}$ ในวันที่ 7 หลังการฉีดเชื้อ

หลังการฉีดเชื้อพิษทับ สุกรกลุ่มที่ 2 แสดงภาวะไข้ อาการทางคลินิก และการเจริญเติบโตไม่แตกต่างจากสุกรกลุ่มที่ 1 (รายละเอียดแสดงในภาคผนวก ในส่วน results ของ manuscript)

บทวิจารณ์

Quil A มีฤทธิ์ช่วยกระตุ้นการแสดงออกของ Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNa, IFNb, IFNg และ IL-13 ใน PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอส ซึ่งยีนเหล่านี้แสดงออกน้อยใน PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอส และมีฤทธิ์ช่วยลดการแสดงออกของ IL-2 และ TGFb ซึ่งยีนเหล่านี้ถูกเหนี่ยวนำให้แสดงออกโดยไวรัสพีอาร์อาร์เอส

เมื่อใช้ poly IC หรือ LPS กระตุ้นการแสดงออกของยีน พบว่า PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสมีการแสดงออกของ Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNa, IFNb, IFNg และ TNFa ลดลงเมื่อเทียบกับ PBMC ที่บ่มร่วมกับ poly IC หรือ LPS ผลการทดลองนี้แสดงให้เห็นว่าไวรัสพีอาร์อาร์เอสกดการแสดงออกของยีนเหล่านี้

เมื่อเติม quil A ให้กับ PBMC ที่บ่มร่วมกับไวรัสพีอาร์อาร์เอสและกระตุ้นด้วย poly IC หรือ LPS พบว่า PBMC มีการแสดงออกของ Mx1, IRF3, IRF7, OAS1, STING, IFNb และ IFNg เพิ่มขึ้นเมื่อเทียบกับ PBMC ที่ไม่ได้เติม quil A ผลการทดลองนี้บ่งชี้ว่า quil A สามารถกระตุ้นการแสดงออกของยีนเหล่านี้ได้

เมื่อนำ quil A มาใช้เป็นแอดจูแวนท์ให้กับวัคซีนพีอาร์อาร์เอสเชื้อเป็นสายพันธุ์ยุโรป พบว่า PBMC ของสุกรที่ได้รับวัคซีนร่วมกับ quil A มีการแสดงออกของ Mx1, IRF3, IRF7, IFNa, OPN, IL-2, IL-13, IFNb และ IFNg เพิ่มขึ้นเมื่อเทียบกับ PBMC ของสุกรที่ได้รับวัคซีนเพียงอย่างเดียว

เมื่อสุกรได้รับการฉีดเชื้อพิษทับพบว่าสุกรที่ได้รับวัคซีนร่วมกับ quil A มีการแสดงออกของ IFNb, OAS1, OPN, IFNa, IFNg, IL-2, TNFa, IL-13 และ IRF3 เพิ่มขึ้น และมีปริมาณไวรัสพีอาร์อาร์เอสในกระแสเลือดลดลงเมื่อเทียบกับสุกรที่ได้รับวัคซีนเพียงอย่างเดียว แต่ทั้งสองกลุ่มไม่มีความแตกต่างกันของระดับแอนติบอดี ภาวะไข้ อาการทางคลินิก และประสิทธิภาพการเจริญเติบโต

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 **Charerntantanakul W**., Fabros Jr D. Saponin Quil A up-regulates type I interferon-regulated gene and type I and II interferon expressions which are suppressed by porcine reproductive and respiratory syndrome virus. *Vet Immuno Immunopathol*. 2018;195,76-83.

1.2 **Charerntantanakul W**., Pongjaroenkit S. Co-administration of saponin quil A and PRRSV-1 modified-live virus vaccine up-regulates gene expression of type I interferon-regulated gene, type I and II interferon, and inflammatory cytokines and reduces viremia in response to PRRSV-2 challenge. *Vet Immuno Immunopathol*. Submitted.

2. การนำผลงานวิจัยไปใช้ประโยชน์

2.1 เชิงวิชาการ

สร้างเครือข่ายความร่วมมือทางวิชาการระหว่างหน่วยงานของผู้วิจัย (สาขาเทคโนโลยีชีวภาพ คณะวิทยาศาสตร์ มหาวิทยาลัยแม่โจ้) และผู้ร่วมงานวิจัย (สาขาวิชาพันธุศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยแม่โจ้) ซึ่งไม่เคยได้ร่วมงานกันมาก่อน โดยผู้ร่วมวิจัยมีความรู้ความสามารถในการเตรียมรีคอมบิแนนท์พลาสมิดเพื่อใช้ในงาน Real-time PCR ทำให้ผู้วิจัยทำงานได้รวดเร็วมากขึ้น

3. การเสนอผลงานในที่ประชุมวิชาการ

3.1 **Charerntantanakul W***., Praphrute R. Effect of matrix on expression of type I interferon-regulated genes and inflammatory cytokine genes in porcine peripheral blood mononuclear cells. The 16th TRF annual meeting, Petchburi. Jan 11-13, 2017. *Poster*.

3.2 **Charerntantanakul W***., Fabros Jr. D.M. Saponin Quil A up-regulates type I interferon-regulated gene and type I and II interferon expressions which are suppressed by porcine reproductive and respiratory syndrome virus. The 17th TRF annual meeting, Petchburi. Jan 10-12, 2018. *Poster*.

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Short communication

Saponin Quil A up-regulates type I interferon-regulated gene and type I and II interferon expressions which are suppressed by porcine reproductive and respiratory syndrome virus

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ABSTRACT

Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses innate immune response following infection of myeloid antigen-presenting cells. Poor innate immune response results in weak and delayed PRRSV-specific adaptive immunity, and facilitates PRRSV replication, pathogenesis, and persistent infection. Numerous efforts have been made to enhance the effective innate and adaptive immune defenses to PRRSV, however, only a few attempts have so far elicited satisfactory results. The present study aims to evaluate in vitro the potential of saponin quil A to enhance the expression of type I interferon (IFN)-regulated gene, type I and II IFNs, and pro-inflammatory cytokines in PRRSV-inoculated peripheral blood mononuclear cells (PBMC). Naïve PBMC from four PRRSV-seronegative pigs were inoculated with PRRSV and subsequently stimulated with quil A in the absence or presence of either polyinosinic:polycytidylic acid (poly IC) or lipopolysaccharide (LPS). The mRNA expression levels of myxovirus resistance 1 (Mx1), interferon regulatory factor 3 (IRF3), IRF7, 2′-5′-oligoadenylatesynthetase 1 (OAS1), stimulator of interferon genes (STING), osteopontin (OPN), IFNα, IFNβ, IFNγ, interleukin-2 (IL-2), IL-10, IL-13, tumor necrosis factor alpha (TNFα), and transforming growth factor beta (TGFβ) were evaluated by real-time PCR. Compared with uninoculated PBMC, PRRSV significantly suppressed expression of all immune parameters except IL-2, IL-10, IL-13, and TGFβ. When compared with PRRSV-inoculated PBMC, stimulation with quil A significantly enhanced Mx1, IRF3, IRF7, OAS1, STING, IFNβ, and IFNγ mRNA expressions, and significantly reduced TGFβ mRNA expression. Our findings thus suggest that quil A has a potential to up-regulate the expression of type I IFN-regulated gene and type I and II IFNs which are suppressed by PRRSV. Therefore, it may serve as an effective immunostimulator for potentiating the innate immune defense to PRRSV.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) causes serious economic loss in swine industry worldwide. The virus is an enveloped positive-sense single-stranded linear RNA virus, belonging to the family Arteriviridae. PRRSV genome is approximately 15 kb in size, and comprises 10 open-reading frames (ORFs), designated ORF1a, 1b, 2a, 2b, 3, 4, 5a, 5, 6, and 7. PRRSV ORF1a and ORF1b encode 14 nonstructural proteins (nsp), while ORF2-7 encode eight structural proteins, i.e. glycoprotein 2a (GP2a), 2b protein, GP3, GP4, ORF5a protein, GP5, matrix (M), and nucleocapsid (N), respectively (Kappes and Faaberg, 2015). Both nsp and structural proteins play important roles in PRRSV evasion of host immune defense (Lunney et al., 2016).

PRRSV strains are divided into European (type 1) and North American (type 2) genotypes. Within the type 1 PRRSV genotype, three subtypes have been delineated. Type 1 PRRSV subtype 3 strains are more pathogenic than the other two subtypes. Type 2 PRRSV strains consist of recently emerged highly pathogenic strains called “HP-PRRSV” which caused serious outbreaks in China and Southeast Asian countries. PRRSV of all strains infects myelomonocytic cell lineage, including monocytes, macrophages, and dendritic cells. Following infection, the virus induces poor innate and adaptive immune responses. In PRRSV-infected cells, the expression of type I and II interferons (IFNs) was barely detected (Albina et al., 1998; Meier et al., 2003), and the expression of pro-inflammatory cytokines, e.g. tumor necrosis factor alpha (TNFα) and interleukin 1 (IL-1) was relatively low, compared to those seen after infection with other pathogens (Lopez-Fuertes et al., 2000; Van Reeth et al., 1999). Other innate immune activities including phagocytosis, production of reactive oxygen species, antigen

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presentation, T cell activation, and microbial killing activity of PRRSV-infected cells were reduced (Charerntantanakul and Kasinrerk, 2012; Sang et al., 2011). In in vivo studies, PRRSV-specific cytotoxic T lymphocytes (CTL) and T helper 1 (Th1) cells as well as neutralizing antibodies (NAb) appeared in peripheral blood and serum approximately three to four weeks after infection (Meier et al., 2003). These were considered delay compared to similar responses to other swine viral pathogens, e.g. swine influenza virus and transmissible gastroenteritis virus (Thacker, 2001). The population of CTL and Th1 cells and the titer of NAb were slowly increased over a period of months (Meier et al., 2003). Delayed and weak adaptive immune response to PRRSV was suggested to be attributed, at least in part, to a weak innate immune response to the virus (Loving et al., 2015).

In contrast to immune responses to most PRRSV strains, immune responses to type 1 PRRSV subtype 3 and HP-PRRSV are faster and stronger in terms of antibody and cell-mediated immunity (CMI) as well as inflammatory cytokine expressions e.g. IL-1, IL-12, TNFα, IFNα, and IFNγ. This is mainly due to the increased infectivity and replication efficiency of these virus strains in vivo. Other mechanisms involving in an improved immune response to these PRRSV strains have not been clearly identified. The stronger immune responses may have supported an enhanced or faster clearance of virus in tissues, compared to most PRRSV strains (Li et al., 2016; Weesendorp et al., 2014).

Mechanisms involving in poor innate immune response to PRRSV have been studied. These reportedly include PRRSV suppression of signaling molecule and transcription factor activation, e.g. retinoic acid induced gene-1 (RIG-1) (Luo et al., 2008), mitochondrial antiviral signaling protein (MAVS) (Sun et al., 2016), interferon regulatory factor 3 (IRF3) (Beura et al., 2010), nuclear factor kappa B (NFκB) (Wang et al., 2015), and signal transducer and activator of transcription 1 (STAT3) (Yang et al., 2017), and extracellular signal-regulated kinase (ERK) (Hou et al., 2012); PRRSV suppression of pattern recognition receptor (PRR) expression, e.g. Toll-like receptor 3 (TLR3) and TLR7 (Chaung et al., 2010); PRRSV-mediated degradation of signaling molecules, i.e. CREB-binding protein (CBP) (Kim et al., 2010); PRRSV inhibition of nuclear translocation of transcription factors, e.g. STAT1 and STAT2 (Yang and Zhang, 2016), and PRRSV up-regulation of anti-inflammatory cytokine, i.e. IL-10 (Charerntantanakul and Kasinrerk, 2010, 2012). These mechanisms have been reported to mediate, at least in part, through PRRSV nsp1 (Beura et al., 2010), nsp2 (Sun et al., 2010), nsp4 (Huang et al., 2016), nsp5 (Yang et al., 2017), nsp11 (Sun et al., 2016), GP5 (Zhixuan et al., 2015), and N proteins (Sagong and Lee, 2011).

Efforts to enhance immune response to PRRSV have been made. These include the use of various forms of PRRSV vaccines, e.g. modified-live virus, inactivated virus, gene-deleted, vectored, DNA, and subunit vaccines (Charerntantanakul, 2012), in combination with various types of adjuvants, for example, pro-inflammatory cytokines, e.g. IL-1, IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF); chemical reagents, e.g. polyinosinic:polycytidylic acid (poly IC), poly IC with polylysine and carboxymethylcellulose (poly ICLC), and poly(lactic-co-glycolic) acid (PLGA); bacterial components, e.g. CpG oligodeoxynucleotides (ODN), cholera toxin, and lipopolysaccharide (LPS); immunostimulatory proteins, e.g. C3d, CD40 ligand, and peptide nanofiber hydrogel; and commercial adjuvants, e.g. Montanide™ Gel 01 ST (Binjawadagi et al., 2014; Charerntantanakul, 2009; Li et al., 2013; Tabynov et al., 2016). Some of those efforts efficiently improve immune response and vaccine efficacy against homologous PRRSV challenge, but only a few of them, e.g. PLGA, CpG ODN, peptide nanofiber hydrogel, and Montanide™ Gel 01 ST reportedly improve immune response and vaccine efficacy against heterologous PRRSV challenge (Binjawadagi et al., 2014; Charerntantanakul, 2009; Li et al., 2013; Tabynov et al., 2016). This small number of achievement suggests the need of further investigations on other potential immunostimulators for PRRSV, particularly for cross-protection against heterologous challenge with varying field PRRSV isolates.

Quil A is a triterpenoid saponin from the bark of South American tree, *Quillaja saponaria* Molina. It has been reported to possess immunostimulatory effects, particularly on stimulation of CTL, Th1 cells, and antibody responses in human and veterinary experimental vaccines (Sun et al., 2009a). Its mechanism of immunostimulation is not well identified. In pigs, quil A has been reported to enhance serum and mucosal IgA production and protective efficacy of *Actinobacillus pleuropneumoniae* inactivated and subunit vaccines (Willson et al., 1995); NAb production and protective efficacy of inactivated swine influenza H3N2 virus vaccine (Bikour et al., 1996); antibody production and protective efficacy of *Taenia solium* TSOL18 vaccine (Assana et al., 2010), *Streptococcus suis* Sao vaccine (Li et al., 2007), and *Toxoplasma gondii* crude rhoptry vaccine (da Cunha et al., 2012); protective efficacy of *T. solium* TSOL16-TSOL18 fusion vaccine (Jayashi et al., 2012); and antibody production of foot-and-mouth disease virus (FMDV) vaccine (Xiao et al., 2007) and *S. suis* recombinant SsnA vaccine (Gomez-Gascon et al., 2016).

When combined with cholesterol and phospholipids to form immunostimulatory complexes (ISCOMs), quil A can activate both CMI and antibody responses to a broad range of viral, bacterial, and protozoal antigens (Sun et al., 2009b). In pigs, quil A-integrated ISCOMs have been reported to induce IFNβ, TNFα, and osteopontin (OPN) but not IFNα expression in porcine peripheral blood mononuclear cells (PBMC) (Fossum et al., 2014) and OPN expression in injected muscle (Ahlberg et al., 2012). In addition, quil A-integrated ISCOMs reportedly enhance lymphocyte proliferation, antibody production and protective efficacy of pseudorabies virus (PRV) subunit vaccine (Tulman and Garmendia, 1994) and live *Mycoplasma hyopneumoniae* vaccine (Maes, 2014); protective efficacy and antibody production of *T. gondii* crude rhoptry vaccine (Garcia et al., 2005) and FMDV recombinant C-terminal VP1 vaccine (Bayry et al., 1999); and antibody production of enterotoxigenic *Escherichia coli* fimbriae vaccine (Nagy et al., 1990).

The present study evaluates the immunostimulatory effects of quil A on the expression of type I IFN-regulated genes, type I and II IFNs, and inflammatory cytokines in porcine PBMC in response to PRRSV. Our findings provide useful evidence for further exploitation of quil A as immunostimulator for PRRSV and possibly for other swine vaccines.

2. Materials and methods

2.1. PRRSV

MARC-145 cells and HP-PRRSV (isolate CUVDL 3/1/4; isolated from lung lavage) were a courtesy of the veterinary diagnostic laboratory, faculty of veterinary science, Chulalongkorn university, Thailand. Both cells and viruses were propagated in MEM^{++} comprising MEM (Caisson Laboratories, Smithfield, UT), 10% heat-inactivated FBS (Capricorn Scientific GmbH, Germany), penicillin (100 IU/ml), streptomycin (100 μg/ml), and amphotericin B (250 ng/ml) (all from Gibco, Grand Island, NY). The virus cultures were frozen and thawed twice, centrifuged, and the supernatants were collected, filtered through 0.22 μM filter (Minisart®, Sartorius, France), and kept at −80 °C. The virus titer was determined by immunoperoxidase monolayer assay, using primary mouse mAbs specific for PRRSV N proteins of both type 1 and type 2 genotypes (IgG2b, clone 5C61) (Median Diagnostics, South Korea) and secondary horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (CiteAB, UK). The virus titer was adjusted to 10^6 $TCID_{50}$/ml. The passage number of PRRSV used in this study was at passage ninth in MARC-145 cells.

2.2. Quil A

Quil A (cat# vac-quil, Invivogen, San Diego, CA) was resuspended with sterile water to 10 mg/ml. The solution was filtered through 0.22 μM filter (Minisart®), aliquoted, kept at −20 °C, and protected from light until use. The presence of LPS in the resuspended quil A

solution was less than 0.005 EU/ml as determined by limulus amebocyte lysate assay (Chromo LAL®, Associates of Cape Cod, Inc., East Falmouth, MA).

2.3. Animals

Four eight-week-old crossbred pigs (Large White/Landrace x Duroc) were used as sources of PBMC. The animals were seronegative to PRRSV and *M. hyopneumoniae* as determined by commercial ELISA test kits (IDEXX Laboratories, Westbrook, ME). They were reared in the swine research farm, faculty of animal science and technology, Maejo university (MJU). The animal experiment was approved by the MJU animal care and use committee.

2.4. Optimization of real-time PCR condition

2.4.1. PBMC isolation and stimulation

Whole blood was collected from jugular vein of the pigs and was placed into EDTA-containing tubes. PBMC were isolated by Ficoll-Hypaque gradient centrifugation (Histopaque®-1077, Sigma, St. Louis, MO) as described previously (Charerntantanakul et al., 2006). Contaminating red blood cells were lysed with a cold hypotonic solution comprising of 0.156 M ammonium chloride, 10 mM sodium bicarbonate, and 1 mM EDTA. The isotonicity was restored with 3 x PBS. PBMC were centrifuged at 1250 rpm at 4 °C for 15 min and washed once with PBS prior to resuspension in $RPMI^{++}$ (RPMI-1640 with L-glutamine (Caisson Laboratories, Smithfield, UT), 10% heat-inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (250 ng/ml)) to 5×10^5 cells/ml.

Two hundred microliters of PBMC suspension was pipetted into 96-well flat-bottom plates (Nunc, Denmark). The cells then received 50 µl of either poly IC (5 µg/ml final) or LPS from *E. coli* O111:B4 (5 µg/ml final) (all from Sigma, St. Louis, MO) for induction of immune gene expression. The cultures were incubated at 37 °C in a humidified 5% CO_2 incubator for 18 h. The cells were then harvested and subjected to RNA isolation.

2.4.2. Generation of cDNA stocks

Isolation of total RNA and elimination of contaminating DNA were performed using NucleoSpin® Blood kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instructions. Complete elimination of genomic DNA in the total RNA preparation was confirmed by real-time PCR using total RNA preparation as template and primers for RPL32 (ribosomal protein L32) (Charerntantanakul et al., 2013). Reverse transcription was carried out, using RevertAid™ First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). The concentration and purity of RNA and the concentration of cDNA were determined by spectrophotometry (Nanodrop ND-1000; NanoDrop Technologies). All RNA samples had absorbance values at 260 and 280 nm (A260/280) and 260 and 230 nm (A260/230) between 2.0-2.2 and 1.8-2.2, respectively.

2.4.3. Real-time PCR

Published primer pairs specific for myxovirus resistance 1 (Mx1), IRF3, IRF7, 2′-5′-oligoadenylatesynthetase 1 (OAS1), stimulator of interferon genes (STING), OPN, IFNα, IFNβ, IFNγ, IL-2, IL-10, IL-13, TNFα, transforming growth factor beta (TGFβ), RPL32 and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) were used (Dobrescu et al., 2014; Dong et al., 2015; Fossum et al., 2014; Royaee et al., 2004; Wikstrom et al., 2011) (Supplementary Table 1). All primer pairs were evaluated using primer concentration of 200, 300, 400, and 500 nM with annealing temperatures between 55 and 60 °C.

Real-time PCR was performed on the MJ Research PTC-200 thermal cycler in a total reaction volume of 20 µl, consisting of 2 µl of serial 4-fold dilutions of cDNA template (starting at 500 ng of cDNA), and 10 µl SYBR® Green real time PCR master mix (Toyobo, Japan). All reactions were set up in duplicates. The PCR condition was 95 °C (15 min); and 40 cycles of 94 °C (15s), indicated annealing temperature (30s), and 72 °C (30s). Melting curve analysis was conducted following the completion of PCR cycles for product verification. Agarose gel electrophoresis was performed to confirm a single product of the expected size. In each run, nuclease-free water was included as no template control. PCR efficiencies between 90 and 103% and r^2 between 0.990–0.999 were obtained (Supplementary Table 1).

2.5. Determination of optimal concentration of quil a

Two hundred microliters of PBMC suspension was pipetted into 96-well flat-bottom plates in duplicate settings. The cells then received 50 µl of serial 2-fold dilutions of quil A in $RPMI^{++}$ (starting at 2000 µg/ml final). The cultures were incubated at 37 °C in a humidified 5% CO_2 incubator for 36 h. Cytotoxicity was determined by trypan blue dye exclusion and methylthiazolyldiphenyl-tetrazolium bromide (MTT) assays. For MTT assay, PBMC suspension were transferred correspondingly to 96-well round-bottom plates, and were centrifuged at 1200 rpm at 4 °C for 1 min. All supernatants were gently removed and replaced with 90 µl of $RPMI^{++}$ and 10 µl of MTT solution in PBS (5 mg/ml). PBMC were incubated in the dark at 37 °C for 4 h, then received 100 µl of MTT solvent (4 mM HCl, 0.1% NP40 in isopropanol). The cells were incubated further at 37 °C for 15 min, prior to determination of optical density at 560 nm. PBMC incubated with only $RPMI^{++}$ served as control. The cytotoxicity tests were conducted in three independent experiments. The highest concentration of quil A (4 µg/ml) that showed least cytotoxic effects to PBMC, compared to unstimulated PBMC control, as well as its 10-fold (0.4 µg/ml) and 100-fold (0.04 µg/ml) dilutions were chosen for subsequent studies.

2.6. Optimization of incubation period for quil A-induced immune gene expression

Two hundred microliters of PBMC suspension was pipetted into 96-well flat-bottom plates in duplicate settings. The cells then received 50 µl of quil A at indicated concentrations. The cultures were incubated at 37 °C in a humidified 5% CO_2 incubator for 12, 24, and 36 h. Then the cells were harvested and subjected to RNA isolation, cDNA synthesis, and real-time PCR as described above. The real-time PCR reaction comprised 2 µl cDNA template, optimal concentration of each primer (Supplementary Table 1), and 10 µl SYBR® Green real time PCR master mix (Toyobo, Japan). All reactions were set up in duplicates. The threshold cycles (C_T) of all genes were collected and used for calculation of immune parameter gene expression by $2^{\wedge}(-\Delta\Delta C_T)$ method (Pfaffl, 2001). The RPL32 and YWHAZ mRNA of the same animal was used for normalization of immune parameter gene expression. The expression values were transformed into log2 scale in order that the expression was relative to 0. Melting curve analysis was conducted following the completion of PCR cycles. In each run, nuclease-free water was included as no template control. PBMC incubated with only $RPMI^{++}$ served as unstimulated control, while PBMC incubated with either poly IC or LPS served as positive control.

2.7. Effects of quil a on immune gene expression in PRRSV-inoculated PBMC

Two hundred microliters of PBMC suspension was pipetted into 96-well flat-bottom plates in duplicate settings. The cells then received 100 µl of either $RPMI^{++}$ or PRRSV (approximately 10 multiplicity of infection) and were incubated at 37 °C in a humidified 5% CO_2 incubator for 48 h. Cell viability was determined by trypan blue dye exclusion. Subsequently, 100 µl of supernatant was gently removed. Cells that were incubated with $RPMI^{++}$ then received 50 µl of quil A at 4 µg/ml final (referred to as quil A-stimulated PBMC). Cells that were

inoculated with PRRSV then received 50 µl of either RPMI^{++} (referred to as PRRSV-inoculated PBMC) or quil A at 4 µg/ml final (referred to as PRRSV-inoculated/quil A-stimulated PBMC). The cultures were incubated at 37 °C in a humidified 5% CO_2 incubator for 12 h. The cells then were harvested, determined for cell viability by trypan blue dye exclusion, and subjected to RNA isolation as described above. In every real-time PCR run, nuclease-free water was included as no template control. PBMC incubated with only RPMI^{++} served as uninoculated/unstimulated control.

2.8. Effects of quil a on PRRSV-suppressed immune gene expression in PBMC stimulated with poly IC or LPS

Two hundred microliters of PBMC suspension was pipetted into 96-well flat-bottom plates in duplicate settings. The cells then received 100 µl of either RPMI^{++} or PRRSV (approximately 10 multiplicity of infection) and were incubated at 37 °C in a humidified 5% CO_2 incubator for 48 h. For cells that received RPMI^{++}, 150 µl of supernatant was gently removed and the cultures received 50 µl of either poly IC or LPS and 50 µl of RPMI^{++} (referred to as inducer-stimulated PBMC). For cells that received PRRSV, 150 µl of supernatant was gently removed and the cultures received 50 µl of either poly IC or LPS and 50 µl of RPMI^{++} (referred to as PRRSV-inoculated/inducer-stimulated PBMC) or 50 µl of either poly IC or LPS and 50 µl of quil A (4 µg/ml final) (referred to as PRRSV-inoculated/quil A + inducer-stimulated PBMC). The cultures were incubated further at 37 °C in a humidified 5% CO_2 incubator for 12 h. The cells then were harvested, determined for cell viability by trypan blue dye exclusion, and subjected to RNA isolation as described above. In every real-time PCR run, nuclease-free water was included as no template control. PBMC incubated with only RPMI^{++} served as uninoculated/unstimulated control.

2.9. Data analysis

Statistical analysis was performed using the SPSS software version 17 (IBM, Armonk, NY). Mean fold differences of all immune parameter gene expressions as well as mean difference of%viable cells were tested by one-way analysis of variance, followed by Dunnett's test. $P < 0.05$ was set as a statistically significant level throughout this study.

3. Results and discussion

3.1. Determination of optimal quil a concentration and incubation period for quil A-induced immune gene expression

Among all concentrations tested, quil A concentration at 4 µg/ml was the highest concentration that was least cytotoxic to PBMC after 36 h of incubation (data not shown). This concentration yielded greater than 90% PBMC viability. Therefore, it was chosen for subsequent studies. In addition, its 10-fold and 100-fold dilutions, i.e. 0.4 and 0.04 µg/ml, were included in subsequent studies.

As shown in Table 1, stimulation of PBMC with quil A at 4 µg/ml for 12 h resulted in highest expression of all immune gene parameters, compared with stimulation at 0.4 and 0.04 µg/ml and with other incubation periods. These results indicate that quil A potentially up-regulates mRNA expression of type I IFN-regulated genes, type I and II IFNs, and inflammatory cytokines. Previous studies regarding immunostimulatory effect of quil A in the form of ISCOMs showed that quil A-integrated ISCOMs up-regulated IFNβ, TNFα, and OPN mRNA expression in PBMC collected from pigs injected intramuscularly with quil A-integrated ISCOMs (Fossum et al., 2014). Also, quil A-integrated ISCOMs up-regulated OPN mRNA expression in injected muscle of the pigs (Ahlberg et al., 2012).

Table 1
Immune gene expressions in PBMC (n = 4 pigs) stimulated with different concentrations of quil A after incubation for 12, 24, and 36 h*.

Gene	12 h				24 h				36 h			
	Pos Ctrl**	4 µg/ml	0.4 µg/ml	0.04 µg/ml	Pos Ctrl**	4 µg/ml	0.4 µg/ml	0.04 µg/ml	Pos Ctrl**	4 µg/ml	0.4 µg/ml	0.04 µg/ml
Mx1	2.8 ± 0.2	1.5 ± 0.8^{a}	0.0 ± 0.3^{b}	0.0 ± 0.1^{b}	2.5 ± 0.0	0.5 ± 0.2^{a}	0.4 ± 0.1^{a}	-0.7 ± 0.1^{b}	0.9 ± 0.1	-0.1 ± 0.2^{a}	-0.3 ± 0.3^{a}	-0.3 ± 0.2^{a}
IRF3	2.7 ± 0.1	1.1 ± 0.1^{a}	0.7 ± 0.1^{b}	−0.7 ± 0.1^{c}	0.8 ± 0.1	0.3 ± 0.1^{a}	−0.3 ± 0.1^{b}	−0.7 ± 0.1^{c}	0.4 ± 0.0	−0.2 ± 0.1^{a}	−0.3 ± 0.3^{b}	−0.3 ± 0.5^{a,b}
IRF7	2.5 ± 0.0	1.4 ± 0.5^{a}	0.5 ± 0.2^{b}	0.1 ± 0.0^{c}	0.8 ± 0.1	0.3 ± 0.2^{a}	−0.3 ± 0.1^{b}	−0.3 ± 0.1^{b}	0.5 ± 0.2	−0.2 ± 0.3^{a}	−0.3 ± 0.4^{a}	−0.3 ± 0.5^{a}
OAS1	4.3 ± 0.1	2.5 ± 0.6^{a}	0.8 ± 0.2^{b}	−0.7 ± 0.1^{c}	2.4 ± 0.1	0.3 ± 0.2^{a}	−0.3 ± 0.1^{b}	−0.3 ± 0.1^{b}	1.5 ± 0.4	−0.3 ± 0.5^{a}	−0.3 ± 0.5^{a}	−0.3 ± 0.6^{a}
STING	3.2 ± 0.1	1.6 ± 0.7^{a}	0.6 ± 0.2^{b}	−0.3 ± 0.1^{c}	2.4 ± 0.0	0.7 ± 0.3^{a}	0.0 ± 0.2^{b}	−0.2 ± 0.1^{b}	0.7 ± 0.3	−0.3 ± 0.3^{a}	−0.3 ± 0.4^{a}	−0.3 ± 0.5^{a}
OPN	1.6 ± 0.1	0.8 ± 0.3^{a}	−0.2 ± 0.2^{b}	−0.5 ± 0.1^{b}	0.9 ± 0.1	0.2 ± 0.3^{a}	−0.3 ± 0.2^{a}	−0.3 ± 0.1^{b}	0.7 ± 0.2	−0.2 ± 0.3^{a}	−0.3 ± 0.3^{a}	−0.3 ± 0.6^{a}
IFNα	4.0 ± 0.1	1.5 ± 0.1^{a}	0.6 ± 0.1^{b}	−0.5 ± 0.1^{c}	3.5 ± 0.1	0.8 ± 0.1^{a}	0.3 ± 0.1^{b}	−0.1 ± 0.2^{c}	2.0 ± 0.3	−0.1 ± 0.3^{a}	−0.3 ± 0.3^{a}	−0.7 ± 0.3^{a}
IFNβ	3.6 ± 0.3	1.4 ± 0.8^{a}	0.8 ± 0.4^{a}	0.4 ± 0.1^{b}	2.2 ± 0.1	0.1 ± 0.3^{a}	−0.3 ± 0.1^{a}	−0.3 ± 0.5^{a}	1.1 ± 0.2	−0.3 ± 0.6^{a}	−0.3 ± 0.5^{a}	−0.3 ± 0.3^{a}
IFNγ	2.1 ± 0.1	1.3 ± 0.8^{a}	0.8 ± 0.2^{a}	−0.3 ± 0.1^{b}	1.8 ± 0.0	0.8 ± 0.4^{a}	0.4 ± 0.1^{a}	−0.7 ± 0.3^{b}	1.5 ± 0.3	−0.3 ± 0.3^{a}	−0.3 ± 0.3^{a}	−0.7 ± 0.3^{a}
IL−2	2.3 ± 0.2	1.2 ± 0.2^{a}	0.3 ± 0.2^{b}	−0.5 ± 0.1^{c}	2.0 ± 0.1	0.1 ± 0.2^{a}	−0.3 ± 0.1^{b}	−0.8 ± 0.2^{c}	1.3 ± 0.1	0.3 ± 0.1^{a}	−0.4 ± 0.2^{b}	−0.7 ± 0.3^{b}
IL−10	2.2 ± 0.5	1.0 ± 0.1^{a}	0.6 ± 0.2^{b}	-0.2 ± 0.1^{c}	1.8 ± 0.3	0.8 ± 0.2^{a}	0.4 ± 0.1^{b}	−0.3 ± 0.1^{c}	1.4 ± 0.2	0.4 ± 0.1^{a}	0.3 ± 0.1^{a}	−0.7 ± 0.2^{b}
IL−13	2.5 ± 0.3	0.8 ± 0.6^{a}	0.1 ± 0.2^{a,b}	0.0 ± 0.1^{b}	2.1 ± 0.1	0.3 ± 0.4^{a}	−0.3 ± 0.1^{b}	−0.7 ± 0.2^{c}	1.7 ± 0.3	−0.5 ± 0.2^{a}	−0.3 ± 0.2^{a}	−0.3 ± 0.5^{a}
TNFα	2.5 ± 0.1	0.7 ± 0.1^{a}	−1.0 ± 0.2^{b}	−0.3 ± 0.2^{c}	2.1 ± 0.1	0.2 ± 0.4^{a}	−0.5 ± 0.1^{b}	−0.5 ± 0.1^{b}	1.8 ± 0.2	−0.3 ± 0.4^{a}	−0.3 ± 0.3^{a}	−0.3 ± 0.3^{a}
TGFβ	2.0 ± 0.4	0.4 ± 0.2^{a}	0.0 ± 0.3^{a}	−0.3 ± 0.1^{b}	1.5 ± 0.0	0.3 ± 0.4^{a}	−0.2 ± 0.1^{a}	−0.5 ± 0.2^{b}	1.1 ± 0.2	−0.3 ± 0.2^{a}	−0.3 ± 0.2^{a}	−0.3 ± 0.4^{a}

*Immune gene expressions are presented in log2 scale as mean fold change ± standard error of mean (SEM), according to $2^{-}(-\Delta\Delta C_T)$ method. The expressions are relative to those in unstimulated PBMC. The immune gene expressions were normalized with mRNA expressions of two housekeeping genes, i.e. RPL32 and YWHAZ of the same pigs. **Gene expression in positive control (Pos Ctrl) was stimulated by Poly IC (for Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNα, IFNβ, IL-10, TGFβ) and LPS (for IFNγ, IL-2, IL-13, TNFα). ^{a,b,c}Different letters indicate significant mean fold difference as determined by one-way analysis of variance, followed by Dunnett's test ($p < 0.05$).

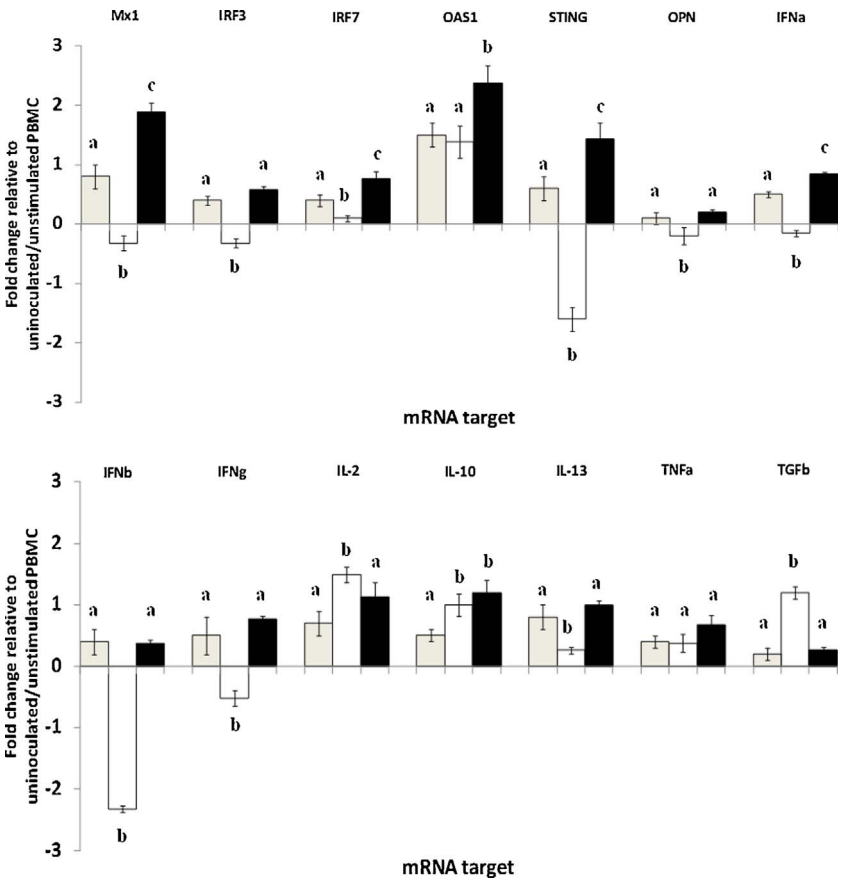

Fig. 1. Effects of quil A on immune gene expressions in PRRSV-inoculated PBMC (n = 4 pigs). PBMC were incubated with RPMI^{++} for 48 h, then received quil A at 4 μg/ml (quil A-stimulated; grey bar). In parallel settings, PBMC were inoculated with PRRSV for 48 h, then received an equal volume of either RPMI^{++} (PRRSV-inoculated PBMC; white bar) or quil A at 4 μg/ml (PRRSV-inoculated/quil A-stimulated PBMC; black bar). The cultures were incubated further for 12 h prior to determination of immune gene expression by real-time PCR. Uninoculated PBMC receiving only RPMI^{++} were prepared in the same fashion and served as uninoculated/unstimulated control. mRNA expressions of immune genes were normalized with mRNA expressions of two housekeeping genes, i.e. RPL32 and YWHAZ of the same pigs. Expressions of immune genes in all groups were transformed to log2 scale and were presented in fold change, according to $2^{-(\Delta\Delta C_T)}$ method, relative to those in uninoculated/unstimulated PBMC. One-way analysis of variance (ANOVA), followed by Dunnett's test was used to determine significant difference of mean fold expressions. Error bar indicates the standard error of the mean (SEM). Different alphabets represent a significant mean difference among groups ($p < 0.05$).

3.2. Effects of quil a on immune gene expression in PRRSV-inoculated PBMC

When evaluated the potential of quil A in response to PRRSV, PRRSV-inoculated/quil A-stimulated PBMC significantly up-regulated mRNA expression of Mx1 (1.9 ± 0.2 vs −0.3 ± 0.1), IRF3 (0.6 + 0.1 vs −0.3 ± 0.1), IRF7 (0.8 ± 0.1 vs 0.1 ± 0.1), OAS1 (2.4 ± 0.7 vs 1.4 ± 0.3), STING (1.4 ± 0.3 vs −1.6 ± 0.2), OPN (0.2 ± 0.1 vs −0.2 ± 0.1), IFNα (0.8 ± 0.0 vs −0.2 ± 0.1), IFNβ (0.4 ± 0.1 vs −2.3 ± 0.1), IFNγ (0.8 ± 0.1 vs −0.5 ± 0.1), and IL-13 (1.0 ± 0.1 vs 0.3 ± 0.1), and significantly down-regulated mRNA expressions of IL-2 (1.1 ± 0.2 vs 1.5 ± 0.1) and TGFβ (0.3 ± 0.1 vs 1.2 ± 0.1), as compared to PRRSV-inoculated PBMC (Fig. 1). When compared to quil A-stimulated PBMC, PRRSV-inoculated/quil A-stimulated PBMC significantly up-regulated mRNA expression of Mx1 (1.9 ± 0.2 vs 0.8 ± 0.2), IRF7 (0.8 ± 0.1 vs 0.4 ± 0.1), OAS1 (2.4 ± 0.7 vs 1.5 ± 0.2), STING (1.4 ± 0.3 vs 0.6 ± 0.2), IFNα (0.8 ± 0.0 vs 0.5 ± 0.1), and IL-10 (1.2 ± 0.2 vs 0.5 ± 0.1). The viability of PBMC after inoculation with PRRSV for 48 h was greater than 70%. Majority of cell death belonged to monocytes and some bystander lymphocytes.

PRRSV has been reported to induce minimal IFNα and IFNβ mRNA expression following infection both in vitro and in vivo (Albina et al., 1998; Buddaert et al., 1998). Minimal type I IFN expression facilitates PRRSV replication as these cytokines potentially suppress PRRSV replication (Albina et al., 1998). In in vivo studies, PRRSV-infected pigs receiving an injection of recombinant human adenovirus 5 expressing IFNα showed delayed viremia and reduced viral loads in sera (Brockmeier et al., 2009; Brockmeier et al., 2012). Possible mechanism of type I IFN to suppress PRRSV replication might be through induction of antiviral protein Mx1 and OAS1 (Huang et al., 2012; Zhao et al., 2016). Poor type I IFN response to PRRSV has been demonstrated to be attributed, at least in part, to PRRSV interference with immune signaling pathway. These include virus suppression of RIG-1 (Luo et al., 2008), MAVS (Sun et al., 2016), IRF3 (Beura et al., 2010), NFκB (Wang et al., 2015), STAT1 and STAT2 (Yang and Zhang, 2016), STAT3 (Yang et al., 2017), TLR3 and TLR7 (Chaung et al., 2010), and CBP (Kim et al., 2010). Significant up-regulation of IFNα and IFNβ mRNA expression induced by quil A suggests the potential of quil A to potentiate antiviral and innate immune response to PRRSV. Mechanisms of quil A involving in up-regulation of type I IFN expression are not known, but could be partly through up-regulation of IRF3 and STING expression as expression of these transcription factors is upstream of type I IFN and is essential for type I IFN expression (Zevini et al., 2017).

The roles of IL-13 and TGFβ in response to PRRSV infection have not been well studied. Both cytokines possess anti-inflammatory activities and promote immunoglobulin production (Bauche and Marie, 2017; Seyfizadeh et al., 2015). Their expression is reportedly up-regulated in PRRSV-infected cells and in PRRSV-specific $CD4^+CD8^+CD25^+FoxP3^+$ regulatory T cells (Treg), respectively (Silva-Campa et al., 2012; Wongyanin et al., 2010). In pigs, IL-13 favors Th2 differentiation (Raymond and Wilkie, 2004). It acts together with GM-CSF to induce the differentiation of monocytes to dendritic cells (Bautista et al., 2007). TGFβ, on the other hand, favors Treg differentiation (Kaser et al., 2015). Its expression along with increased Treg response is observed during early PRRSV infection, which correlates positively with PRRSV viremia but correlates negatively with the presence of PRRSV-specific CTL and Th1, and pro-inflammatory cytokine expression (Silva-Campa et al., 2012; Wongyanin et al., 2010). Based on reported evidence, it is likely that TGFβ might play a role in suppressing innate immune response to PRRSV (Cecere et al., 2012). In murine experiments, TGFβ reportedly suppresses CTL and Th1 cell proliferation and activation, induces Th17 and Treg differentiation, and promotes IgA production (Bauche and Marie, 2017). Further studies regarding the roles of IL-13 and TGFβ in PRRSV infection are required. Whether significant up-regulation of IL-13 and significant down-regulation of

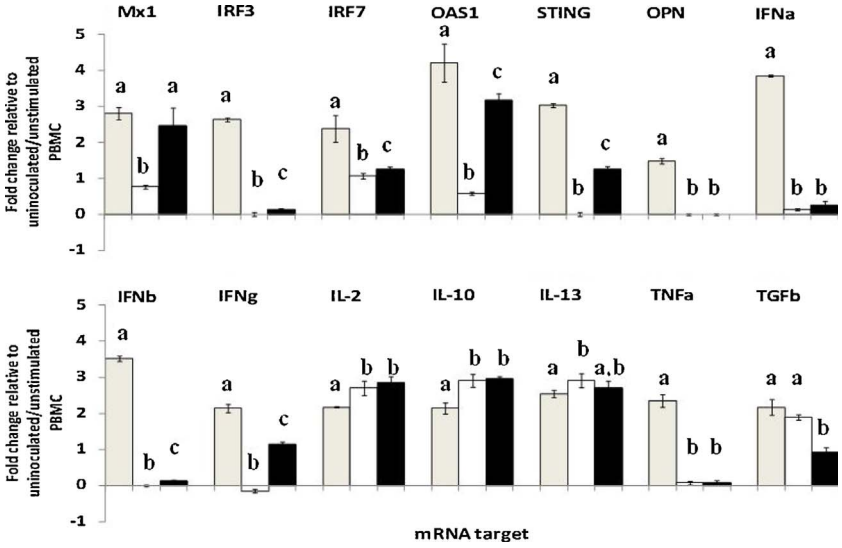

Fig. 2. Effects of quil A on immune gene expressions in PRRSV-inoculated PBMC stimulated with individual inducer, i.e. poly IC or LPS (n = 4 pigs). Poly IC was used to stimulate Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNα, IFNβ, IL-10 and TGFβ gene expressions, while LPS was used to stimulate IFNγ, IL-2, IL-13, and TNFα gene expressions. PBMC were incubated with RPMI^{++} for 48 h, then received inducer (inducer-stimulated; grey bar). In parallel settings, PBMC were inoculated with PRRSV for 48 h, then received an equal volume of either inducer (PRRSV-inoculated/inducer-stimulated PBMC; white bar) or quil A at 4 μg/ml simultaneously with inducer (PRRSV-inoculated/quil A + inducer-stimulated PBMC; black bar). The cultures were incubated further for 12 h prior to determination of immune gene expression by real-time PCR. Uninoculated PBMC receiving only RPMI^{++} were prepared in the same fashion and served as uninoculated/unstimulated control. mRNA expressions of immune genes were normalized with mRNA expressions of two housekeeping genes, i.e. RPL32 and YWHAZ of the same pigs. Expressions of immune genes in inducer-stimulated PBMC, PRRSV-inoculated/inducer-stimulated PBMC, and PRRSV-inoculated/quil A + inducer-stimulated PBMC were transformed to log2 scale and were presented in fold change, according to $2^{-\Delta\Delta C_T}$ method, relative to those in uninoculated/unstimulated PBMC. Significant difference of mean fold expressions was tested by one-way ANOVA, followed by Dunnett's test. Error bar indicates the SEM. Different alphabets represent a significant mean difference among groups ($p < 0.05$).

TGFβ expressions induced by quil A contribute to immune protection from PRRSV needs further study.

3.3. Effects of quil a on PRRSV-suppressed immune gene expression in PBMC stimulated with poly IC or LPS

In order to understand better the potential of quil A on up-regulation of PRRSV-mediated immune suppression, two inducers, i.e. poly IC and LPS which selectively induce immune gene expression were added to the cultures. Compared to inducer-stimulated PBMC, PRRSV-inoculated/inducer-stimulated PBMC demonstrated significantly reduced Mx1 (0.8 ± 0.1 vs 2.8 ± 0.2), IRF3 (0.0 ± 0.1 vs 2.6 ± 0.1), IRF7 (1.1 ± 0.1 vs 2.4 ± 0.4), OAS1 (0.6 ± 0.1 vs 4.2 ± 0.5), STING (0.0 ± 0.1 vs 3.0 ± 0.1), OPN (0.0 ± 0.0 vs 1.5 ± 0.1), IFNα (0.1 ± 0.0 vs 3.8 ± 0.0), IFNβ (0.0 ± 0.0 vs 3.5 ± 0.1), IFNγ (−0.2 ± 0.1 vs 2.1 ± 0.1), and TNFα (0.1 ± 0.1 vs 2.4 ± 0.2) expressions (Fig. 2). On the other hand, the virus-inoculated cultures demonstrated significantly increased IL-2 (2.7 ± 0.2 vs 2.2 ± 0.0), IL-10 (2.9 ± 0.2 vs 2.1 ± 0.2), and IL-13 (2.9 ± 0.2 vs 2.5 ± 0.1) expressions. Significant changes of immune gene expressions between inducer-stimulated PBMC and PRRSV-inoculated/inducer-stimulated PBMC may be attributed partly to differential viable subpopulations of PBMC after PRRSV inoculation as PRRSV causes monocyte and bystander lymphocyte cell death. Differential survival cell subpopulations may respond to inducer and later to quil A to different degrees. Nonetheless, PRRSV suppression of IFNα, IFNβ, IFNγ, and TNFα gene expressions has been reported (Charerntantanakul and Kasinrerk, 2012). Stimulation of IL-2 and IL-10 gene expressions following PRRSV infection in vitro and in vivo also has been evidenced (Charerntantanakul and Kasinrerk, 2010, 2012; Charerntantanakul et al., 2006). Significant up-regulation of IL-10 gene expression following PRRSV infection has been demonstrated to contribute to significant suppression of IFNγ and TNFα production, as knockdown of IL-10 expression results in significantly increased expression of both cytokines (Charerntantanakul and Kasinrerk, 2012).

When quil A was added to the PRRSV-inoculated/inducer-stimulated cultures, the expressions of Mx1 (2.5 ± 0.5 vs 0.8 ± 0.1), IRF3 (0.1 ± 0.0 vs 0.0 ± 0.1), IRF7 (1.3 ± 0.1 vs 1.1 ± 0.1), OAS1 (3.2 ± 0.2 vs 0.6 ± 0.1), STING (1.3 ± 0.1 vs 0.0 ± 0.1), IFNβ (0.1 ± 0.0 vs 0.0 ± 0.0), and IFNγ (1.1 ± 0.1 vs 0.2 ± 0.1) mRNA were significantly up-regulated, while the expression of TGFβ (0.9 ± 0.1 vs 1.9 ± 0.1) mRNA was significantly down-regulated, compared to PRRSV-inoculated/inducer-stimulated PBMC (Fig. 2). These findings again indicate that quil A potentially stimulates expression of type I IFN-regulated gene, and type I and II IFNs and potentially down-regulates expression of TGFβ. Mechanisms involving in quil A alteration of gene expression are not known. However, the findings that quil A did not affect IL-10 expression suggests that the increased expressions of type I IFN-regulated gene and type I and II IFNs were not due to a quil A-mediated reduction of IL-10 expression (Fig. 2).

It is important to note that in PBMC culture, mixed cell population exists and cells that respond to quil A may not only be cells that are infected by PRRSV. Thus, in order to precisely evaluate the ability of quil A to overcome PRRSV-mediated immune suppression of PRRSV-infected cells, further study may employ a pure culture system, e.g. monocytes and alveolar macrophages which are susceptible to PRRSV infection.

4. Conclusion

The present study evaluates the potential of quil A to enhance innate immune response to PRRSV, i.e. the expression of type I and II IFNs, type I IFN-regulated genes, and inflammatory cytokines. Our findings suggest that quil A is a potent immunostimulator for potentiating expression of type I IFN-regulated genes, and type I and II IFNs which are suppressed by PRRSV. It may be exploited as an adjuvant for PRRSV vaccines to enhance innate immune response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2017.11.009.

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September 12, 2018

Dr. Elizabeth Glass
Editor in Chief, Veterinary Immunology and Immunopathology
The Roslin Institute and Royal (Dick) School of Veterinary Studies,
The University of Edinburgh, Easter Bush, Midlothian, Scotland
United Kingdom

Dear Dr. Glass,

We are submitting a revised version of our manuscript ID VETIMM_2018_196. We found the editor and reviewers' comments to be quite helpful and have responded to them as outlined below.

Editor:

1. Information regarding the criteria that the Animal Care and Use Committee considered for the study and the monitoring of animals during experimentation as well as the method of euthanasia have been provided as suggested (see page 10, line 231-236).

Reviewer 1:

1. The title has been changed as suggested.

2. We agree with the reviewer's suggestion on pathological and posttranslational evaluation. However, according to our resources, we could not conduct those analyses in this study.

3. The day of challenge and the treatment group legend have been added to the figures as suggested.

4. Individual titles in the result section have been edited as suggested.

Reviewer 2:

1. The title has been changed as suggested.

2. The introduction section has been shortened as suggested.

3. The genus and family names of PRRSV have been edited as suggested.

4. A subtype 4 of PRRSV-1 has been added as suggested (see page 3, line 70-71).

5. The presence of HP-PRRSV within PRRSV-1 has been added to the revised manuscript as

suggested (see page 3, line 71-74).

6. The sentence stating inconsistent cross protective efficacy of commercial PRRSV vaccines has been removed as suggested.

7. The format of in-text citation has been edited as suggested.

8. The statement has been revised as suggested (see page 5-6, line 122-131).

9. The results regarding the section 2.3.1 of Materials and Methods have been added to the revised manuscript as suggested (see page 15, line 355-361).

10. The statement “of the same size” has been removed.

11. The concentration of quil A used for in vivo injection was according to manufacturer’s recommendation.

12. “Day post vaccination” and “Day post inoculation” have been specified as suggested.

13. The approval number of the experiment has been added as suggested.

14. The subsection “Clinical evaluation and growth performance” in Materials and methods has been moved as suggested (see page 10, line 238-242).

15. A figure describing effect of quil A on PRRSV infectivity and replication has been made as suggested (see Figure 2).

16. Proportion of viremic pigs has been added to the revised manuscript as suggested (see page 17-18, line 422-425).

17. Details regarding antibody response following PRRSV challenge have been added to the revised manuscript as suggested (see page 19-20, line 468-483).

18. Increased IL-10 gene expression in group 1-3 pigs following PRRSV-2 challenge and possible impact on other immune gene expressions have been discussed as suggested (see page 22-23, line 543-549).

19. Differential TGFβ gene expressions among tissues in response to PRRSV infection have been discussed as suggested (see page 23, line 550-557).

We hope that you will find the revised manuscript acceptable for publication. We would be happy to address any additional concerns or questions that you or the reviewers may have.

Sincerely,

Wasin Charerntantanakul, DVM, PhD

Figure legend

Figure 1. Determination of non-cytotoxic concentration of quil A to MARC-145 cells. MARC-145 cells were incubated with 2-fold serially diluted quil A starting at a final concentration of 500 µg/ml for 96 h. Then the cells were fixed with acetone:methanol solution, and stained with 0.5% crystal violet solution and Sorenson's buffer. MARC-145 cells incubated with only MEM^{++} served as untreated control. The O.D. values obtained from quil A-treated cells were compared with those from untreated control and expressed as %viability of MARC-145 cells. Error bar indicates the standard error of the mean (SEM).

Figure 2. Effect of quil A on PRRSV-1 infectivity and replication in vitro. For effect on PRRSV-1 infectivity, quil A was incubated with serially diluted PRRSV-1 for 1 h prior to subsequent inoculation onto adherent MARC-145 cells and a further incubation for 96 h (a). For effect on PRRSV-1 replication, MARC-145 cells were inoculated with serially diluted PRRSV-1 for 1 h, then the supernatants were discarded, and the cultures were washed and further incubated with quil A for 96 h (b). PRRSV titers were determined by IPMA test. MARC-145 cells receiving only serially diluted mock antigens or PRRSV served as mock and PRRSV controls, respectively. Error bar indicates the SEM.

Figure 3. PRRSV-specific antibody response tested over time by ELISA. Pigs (n=6) were injected i.m. with Amervac® PRRS MLV (0 dpv) (MLV), Amervac® PRRS MLV (0 dpv) and quil A (-1, 0, 1 dpv) (MLV+Quil A), or vaccine solvent used for resuspension of lyophilized Amervac® PRRS MLV (0 dpv) (Vaccine solvent). The animals were challenged i.n. with PRRSV isolate 01NP1 at 28 dpv. Strict control pigs (Strict CTRL) received no treatment. Sera were collected weekly from all pigs at 0 to 49 dpv. The positive antibody response was determined at s/p ratio of 0.4 or higher. One-way ANOVA, followed by

Dunnett's test was used to determine significant difference of mean s/p ratios. Error bar indicates the SEM. Different alphabets represent a significant mean difference among groups ($p<0.05$).

Figure 4. Immune gene expression in PBMC re-stimulated in vitro with PRRSV isolate 01NP1. Pigs (n=6) were injected i.m. with Amervac® PRRS MLV (0 dpv) (MLV), Amervac® PRRS MLV (0 dpv) and quil A (-1, 0, 1 dpv) (MLV+Quil A), or vaccine solvent used for resuspension of lyophilized Amervac® PRRS MLV (0 dpv) (Vaccine solvent). The animals were challenged i.n. with PRRSV isolate 01NP1 at 28 dpv. Strict control pigs (Strict CTRL) received no treatment. PBMC were collected weekly from all pigs on at 0 to 49 dpv. Harvested PBMC were incubated with PRRSV isolate 01NP1 for 72 h prior to determination of immune gene expression by real-time PCR. PBMC receiving mock antigens served as mock control. PBMC stimulated with poly IC (for induction of Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNα, IFNβ, IL-10 and TGFβ gene expressions) or LPS (for induction of IFNγ, IL-2, IL-13, and TNFα gene expressions) served as positive control. mRNA expressions of immune genes were normalized with mRNA expressions of two housekeeping genes, i.e. RPL32 and YWHAZ of the same pigs. Expressions of immune genes in all groups were transformed to $\log_2$ scale and were presented in fold change, according to $2^{(-\Delta\Delta C_T)}$ method, relative to those in mock control. One-way ANOVA, followed by Dunnett's test was used to determine significant difference of mean fold expressions. Error bar indicates the SEM. Different alphabets represent a significant mean difference among groups ($p<0.05$).

Figure 5. Number of viremic pigs and $\log_{10}$ PRRSV ORF7 copy numbers in serum following PRRSV challenge. Pigs (n=6) were injected i.m. with Amervac® PRRS MLV (0

dpv) (MLV), Amervac® PRRS MLV (0 dpv) and quil A (-1, 0, 1 dpv) (MLV+Quil A), or vaccine solvent used for resuspension of lyophilized Amervac® PRRS MLV (0 dpv) (Vaccine solvent). The animals were challenged i.n. with PRRSV isolate 01NP1 at 28 dpv (0 dpi). Strict control pigs (Strict CTRL) received no treatment. Serum samples were collected weekly from all pigs at 0 to 21 dpi and were determined for PRRSV ORF7 copy numbers by real-time PCR. The C_T obtained from each sample was compared with C_T standard curve generated from 10^1-10^8 copies of recombinant PRRSV ORF7 plasmids. The calculated copy numbers were transformed to $\log_{10}$ scale. Data were presented per ml of serum sample. One-way ANOVA, followed by Dunnett's test was used to determine significant difference of mean $\log_{10}$ PRRSV ORF7 copy numbers. Error bar indicates the SEM. Different alphabets represent a significant mean difference among groups ($p<0.05$).

Figure 6. Rectal temperature, clinical score, and average daily weight gain (ADWG) of pigs following PRRSV challenge. Pigs (n=6) were injected i.m. with Amervac® PRRS MLV (0 dpv) (MLV), Amervac® PRRS MLV (0 dpv) and quil A (-1, 0, 1 dpv) (MLV+Quil A), or vaccine solvent used for resuspension of lyophilized Amervac® PRRS MLV (0 dpv) (Vaccine solvent). The animals were challenged i.n. with PRRSV isolate 01NP1 at 28 dpv (0 dpi). Strict control pigs (Strict CTRL) received no treatment. Rectal temperature and clinical score were recorded daily. ADWG was calculated from day 0 to 21 dpi. One-way ANOVA, followed by Dunnett's test was used to determine significant difference of mean rectal temperature, clinical score, and ADWG. Error bar indicates the SEM. Different alphabets represent a significant mean difference among groups ($p<0.05$).

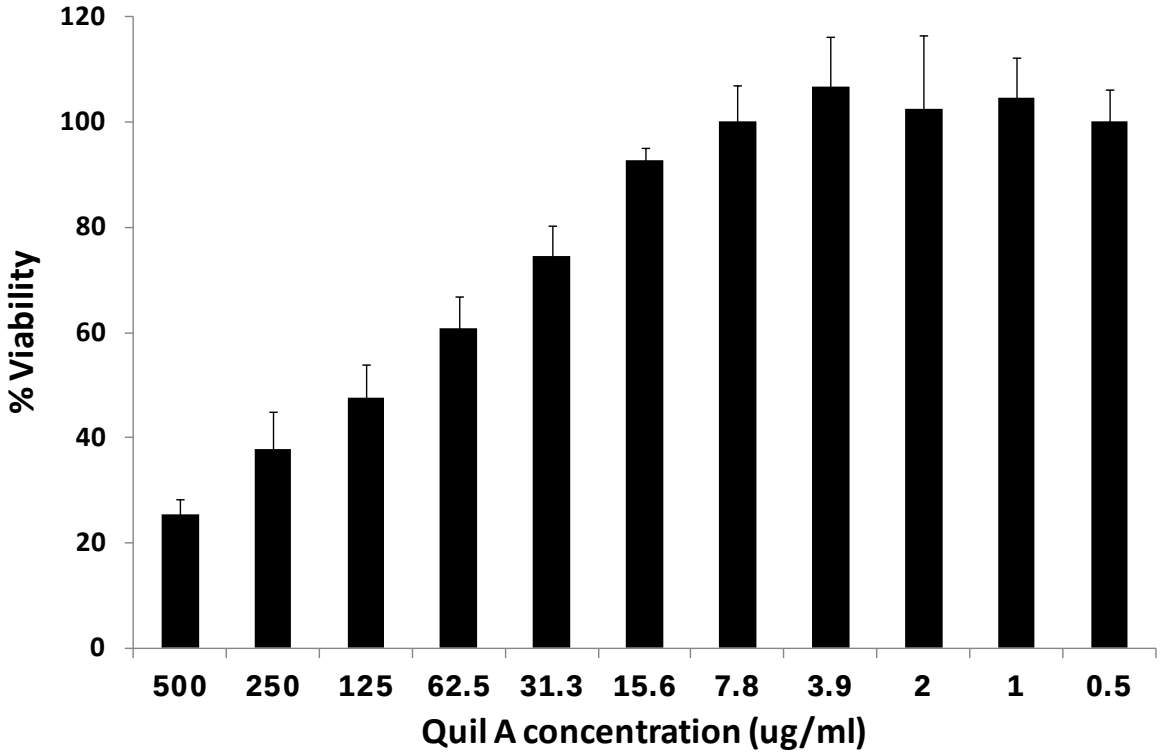
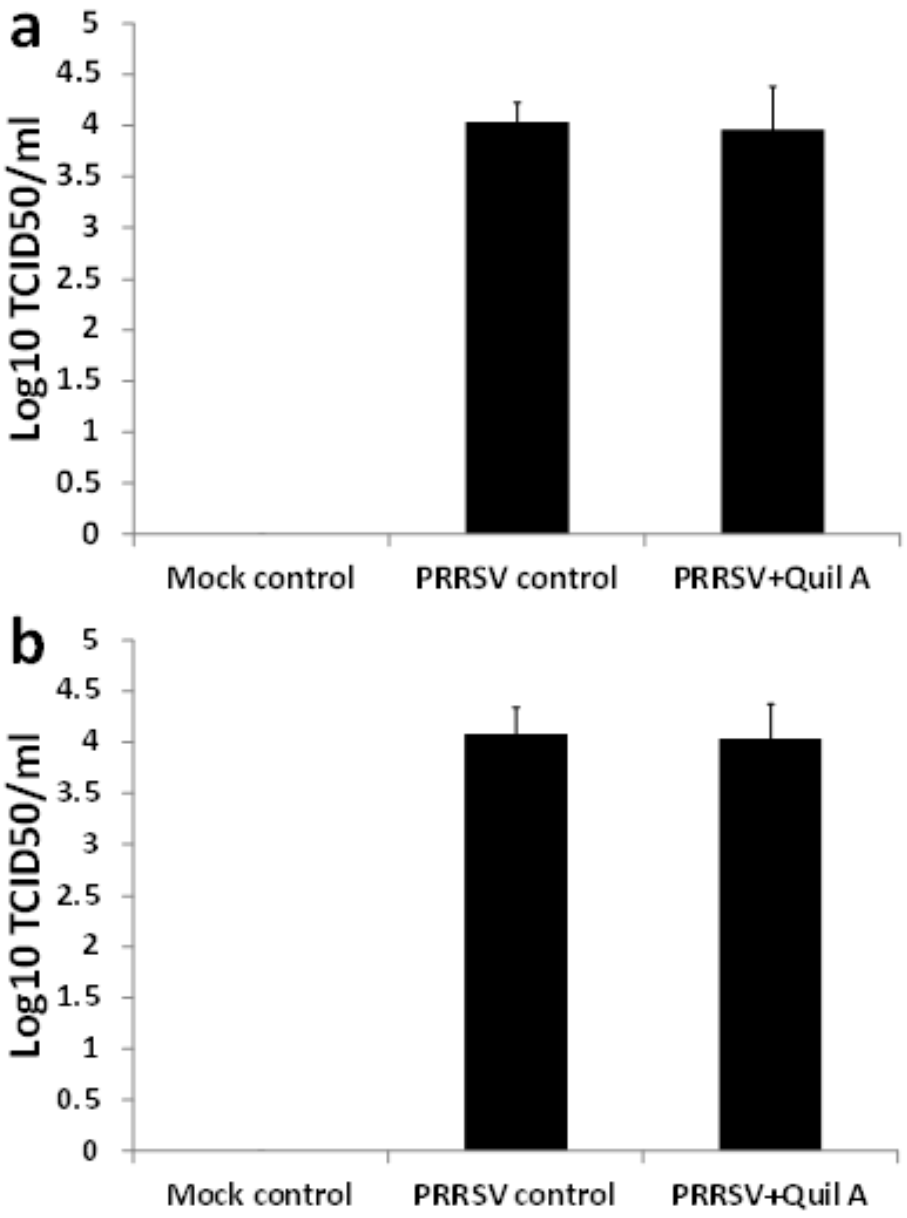

120
100
80
60
40
20
0
% Viability
500
250
125
62.5
31.3
15.6
7.8
3.9
2
1
0.5
Quil A concentration (ug/ml)

Figure 1.


a
Log10 TCID50/ml
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Mock control
PRRSV control
PRRSV+Quil A
b
Log10 TCID50/ml
5
4.5
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2
1.5
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0
Mock control
PRRSV control
PRRSV+Quil A

Figure 2.

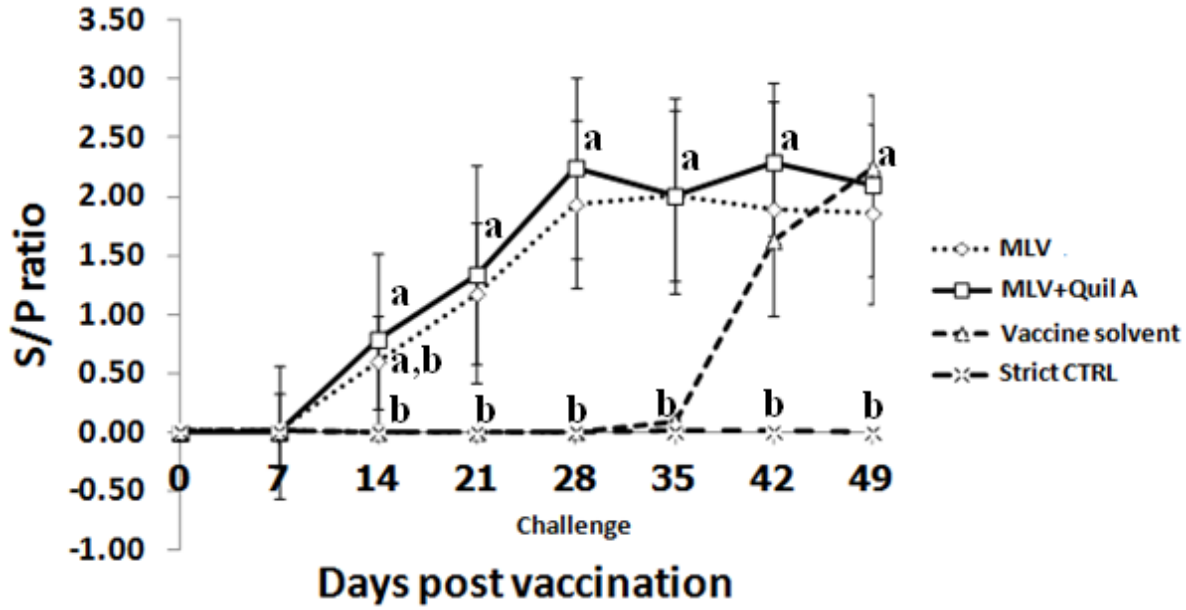

3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
S/P ratio
0
7
14
21
28
35
42
49
Challenge
Days post vaccination
a
a,b
b
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL

Figure 3.

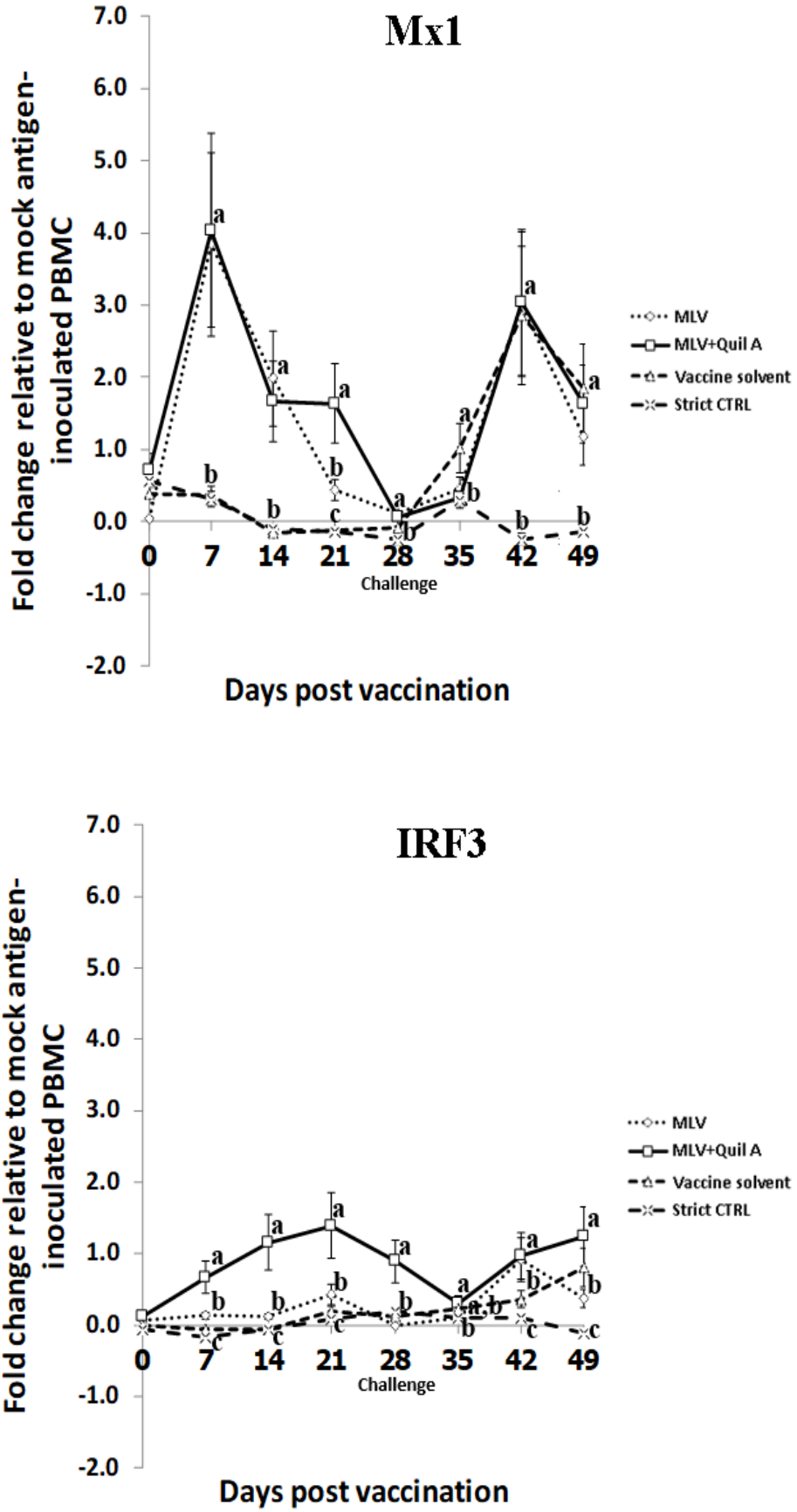
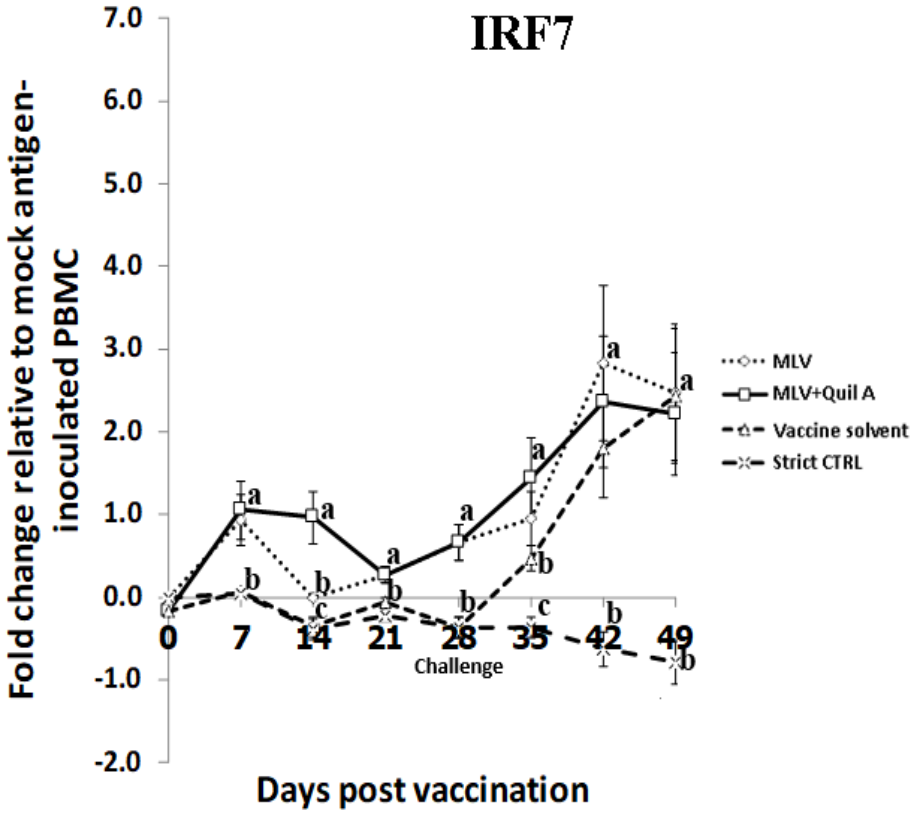
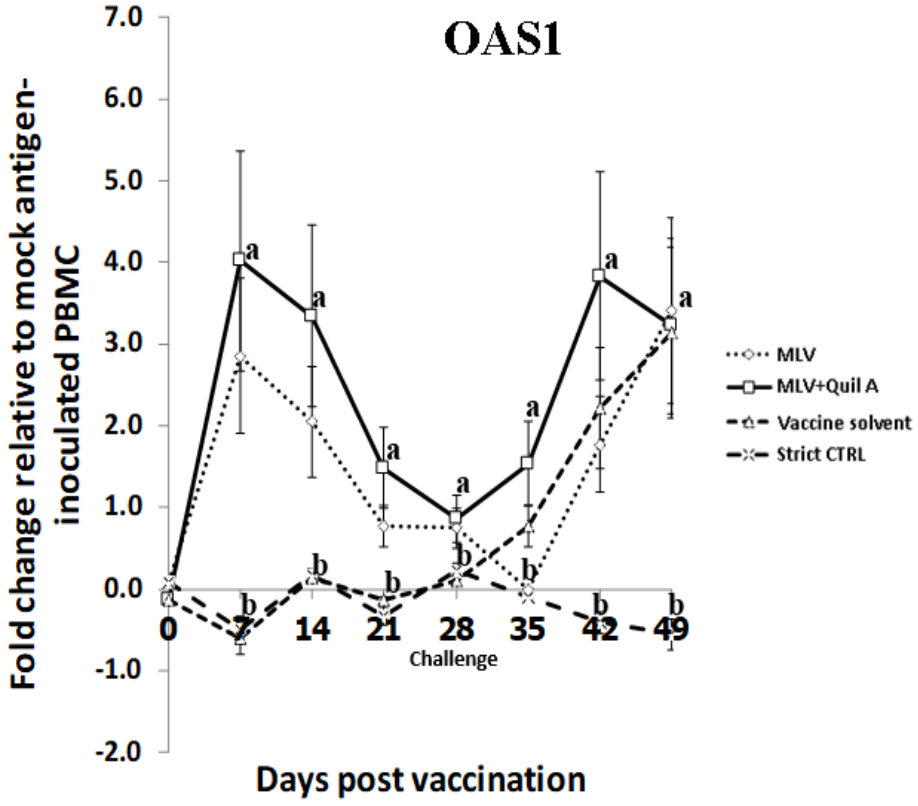

Mx1
Fold change relative to mock antigen-inoculated PBMC
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
0
7
14
21
28
35
42
49
Challenge
Days post vaccination
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL
IRF3
Fold change relative to mock antigen-inoculated PBMC
Days post vaccination
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL

Figure 4.





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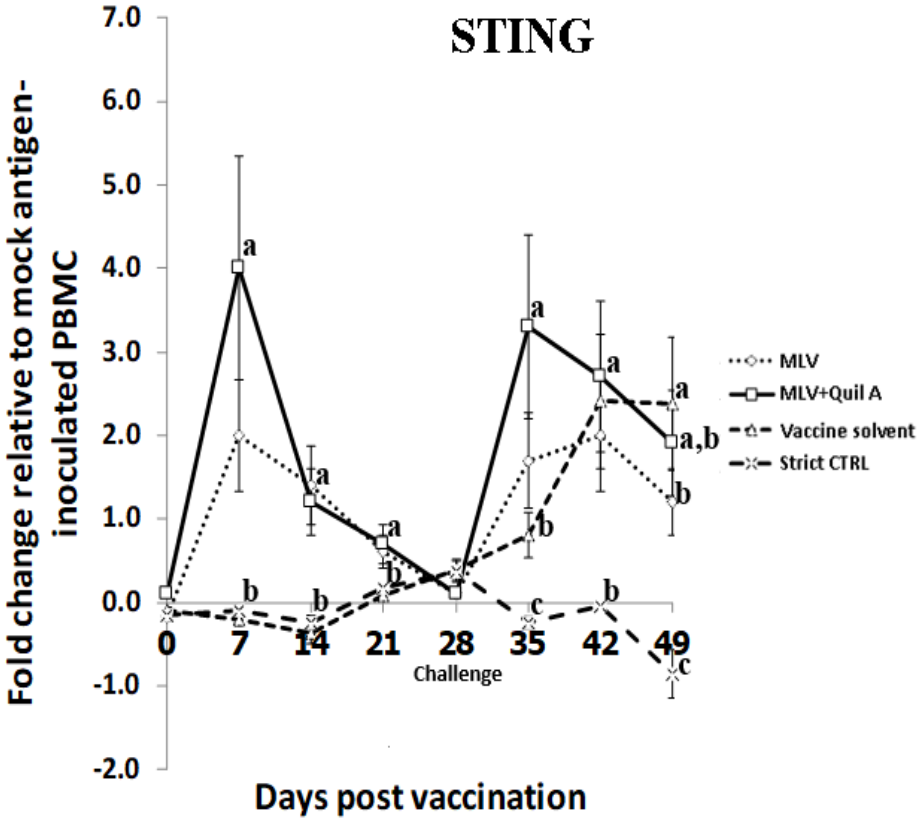

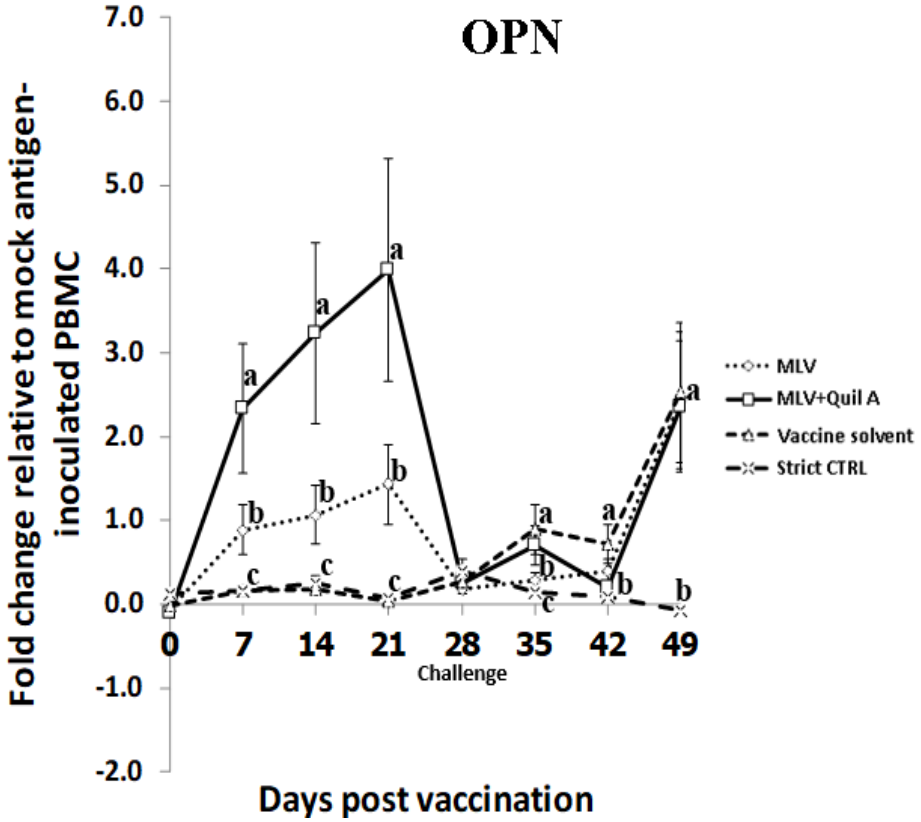

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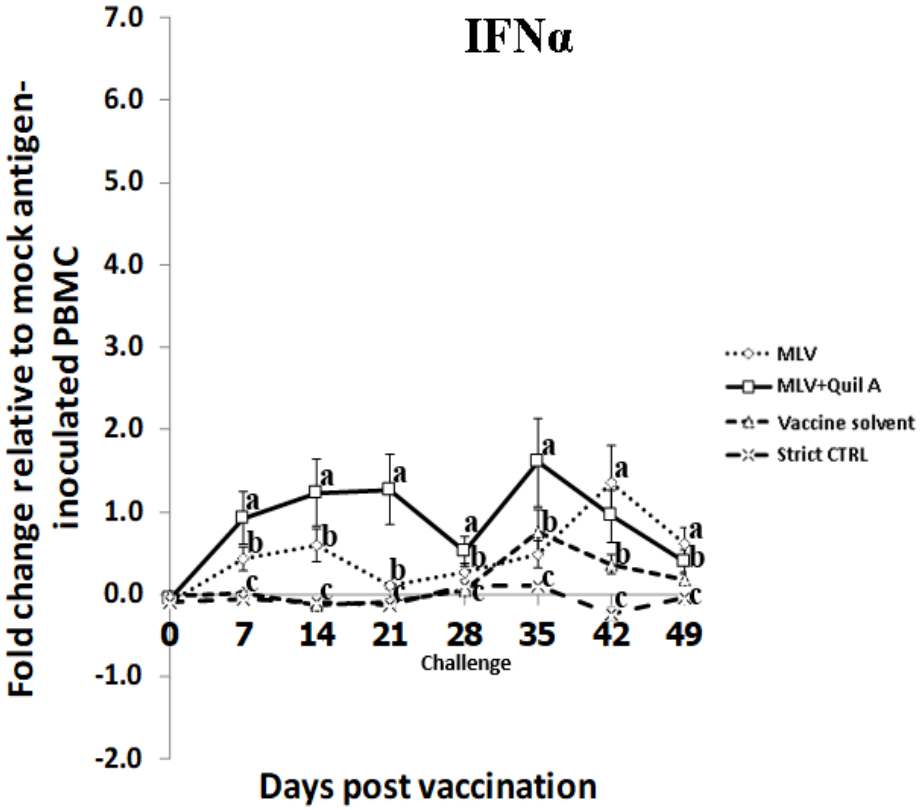


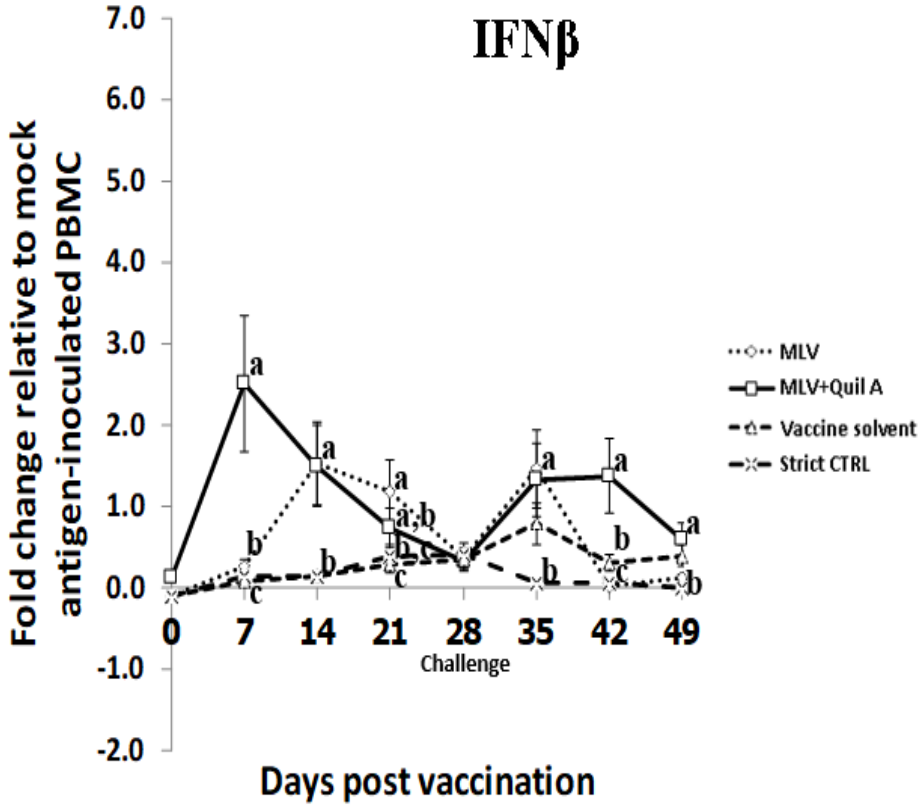
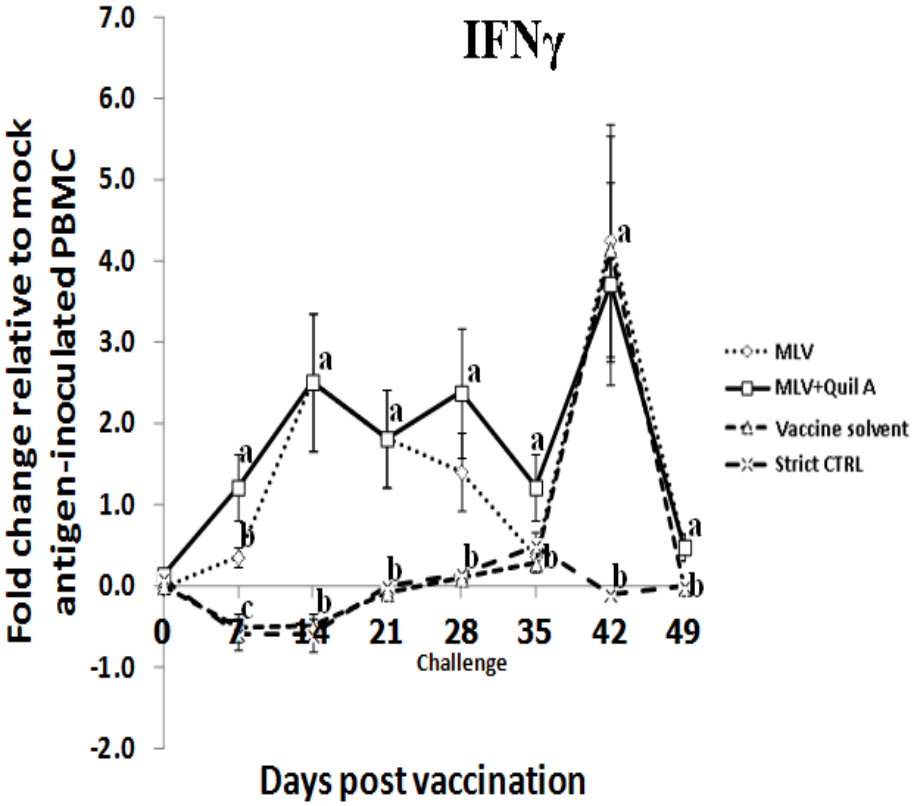


Figure 4 (Cont).



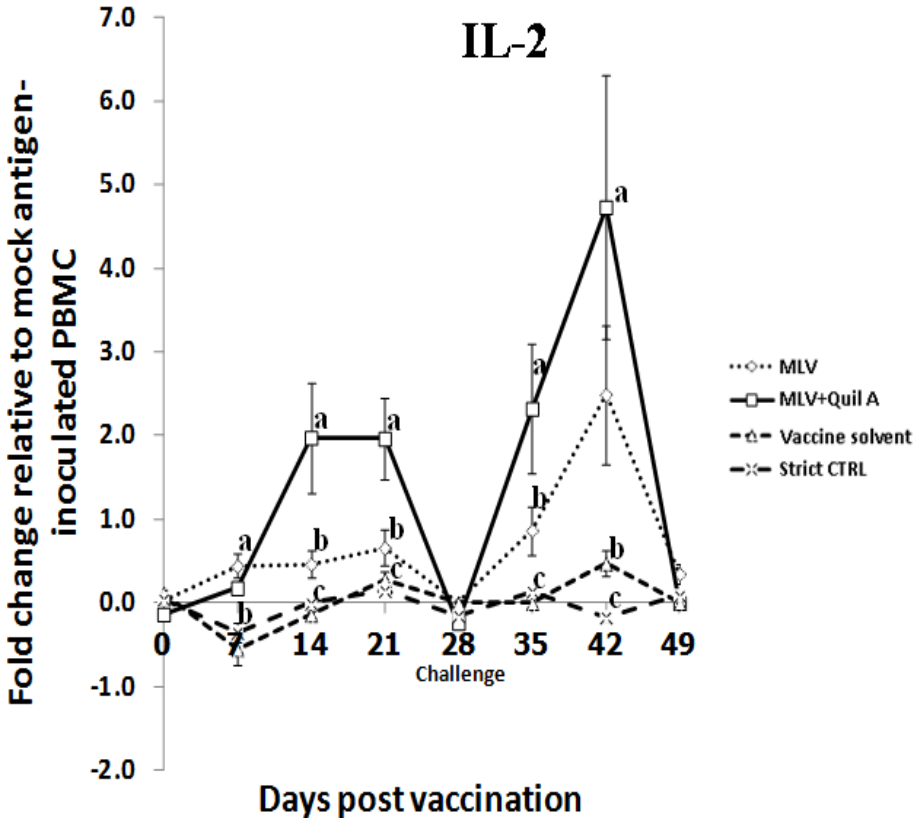
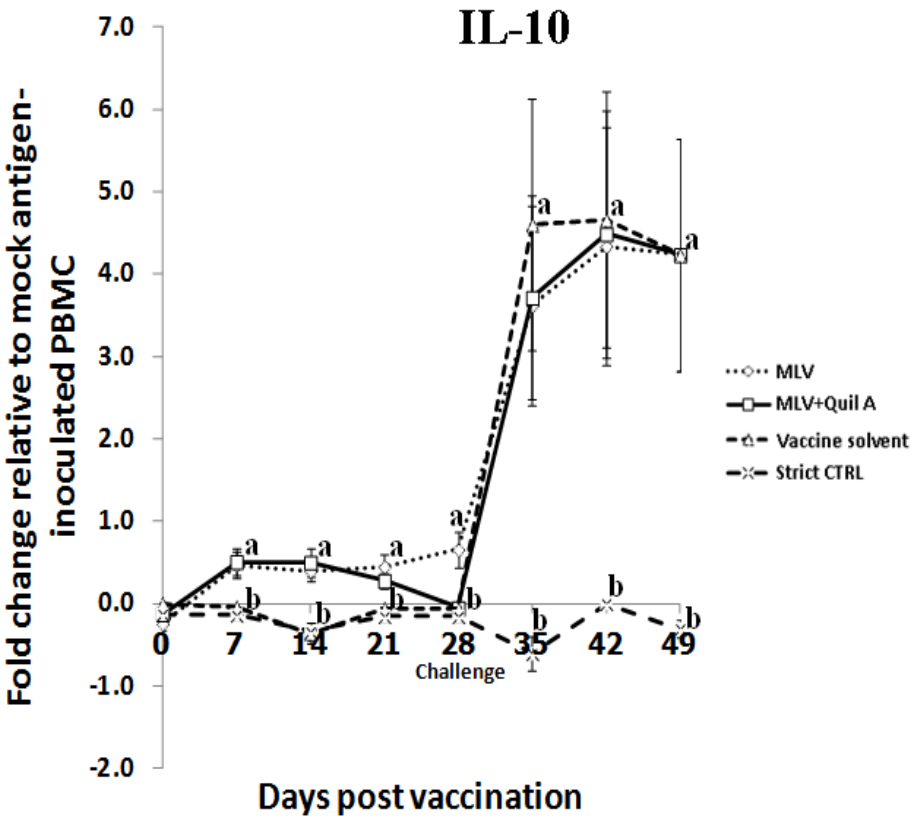


Figure 4 (Cont).



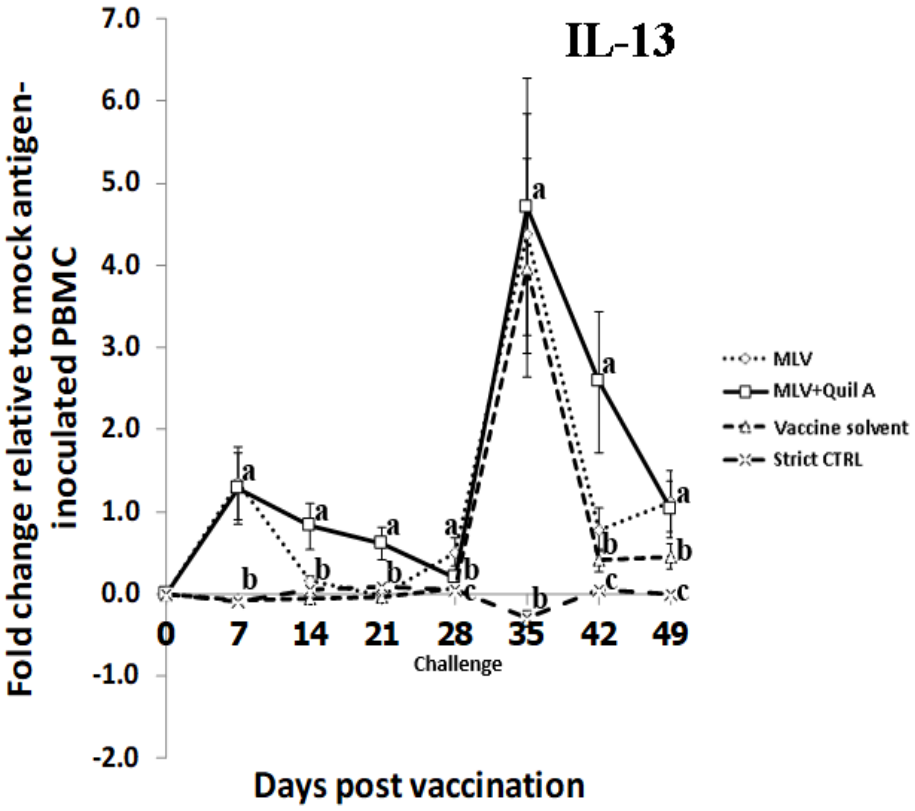
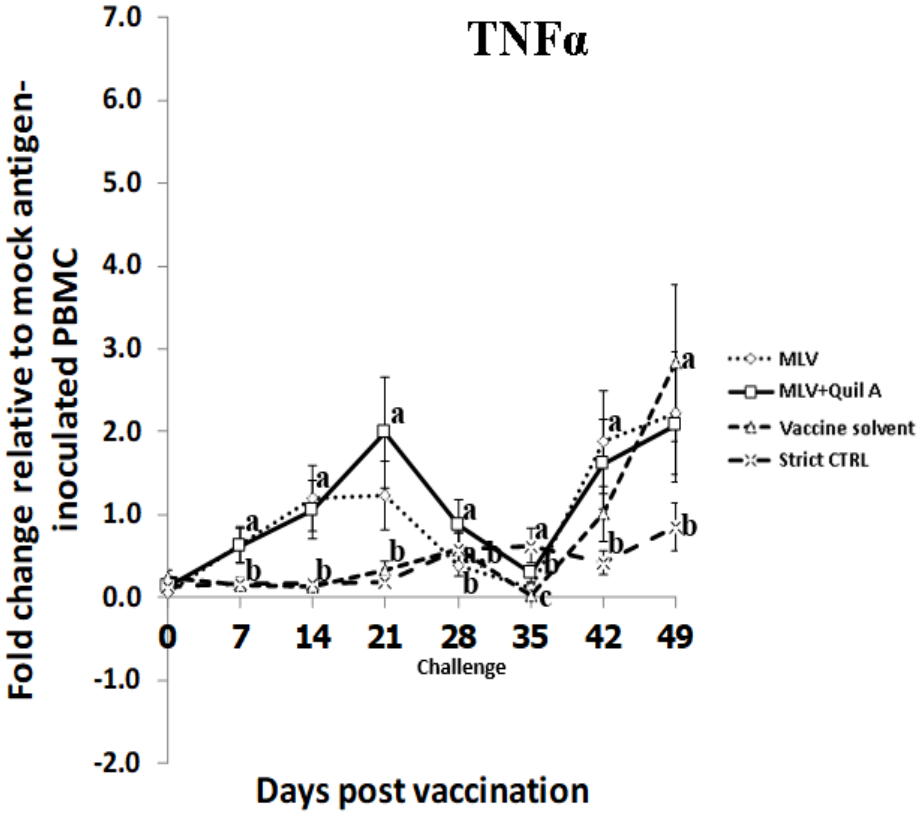


Figure 4 (Cont).



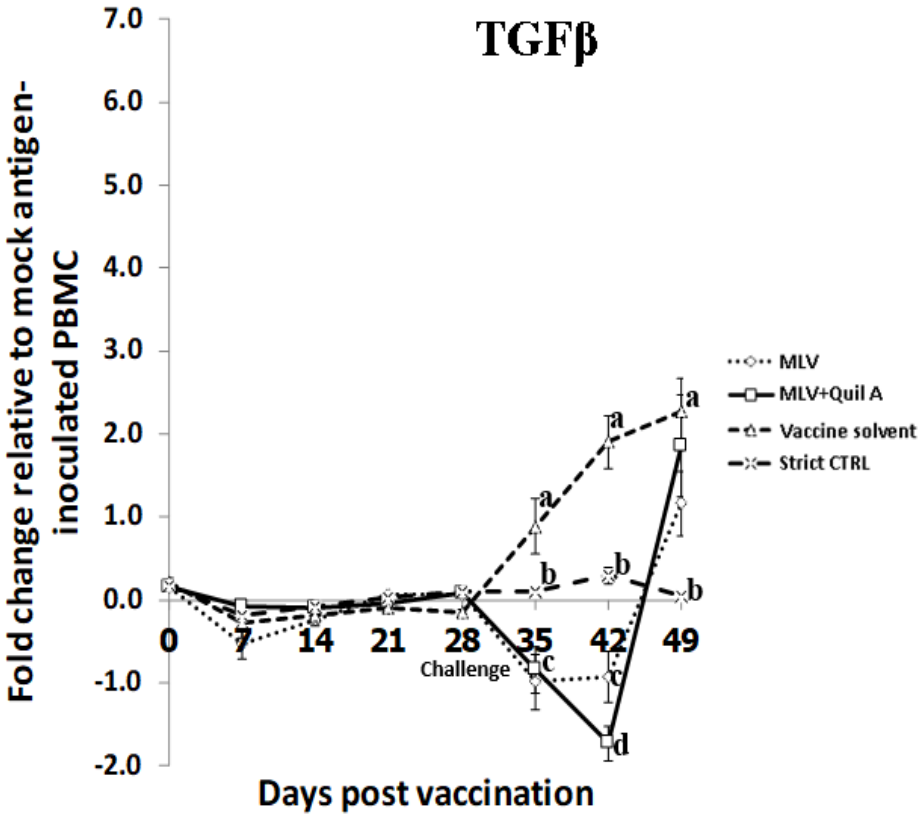
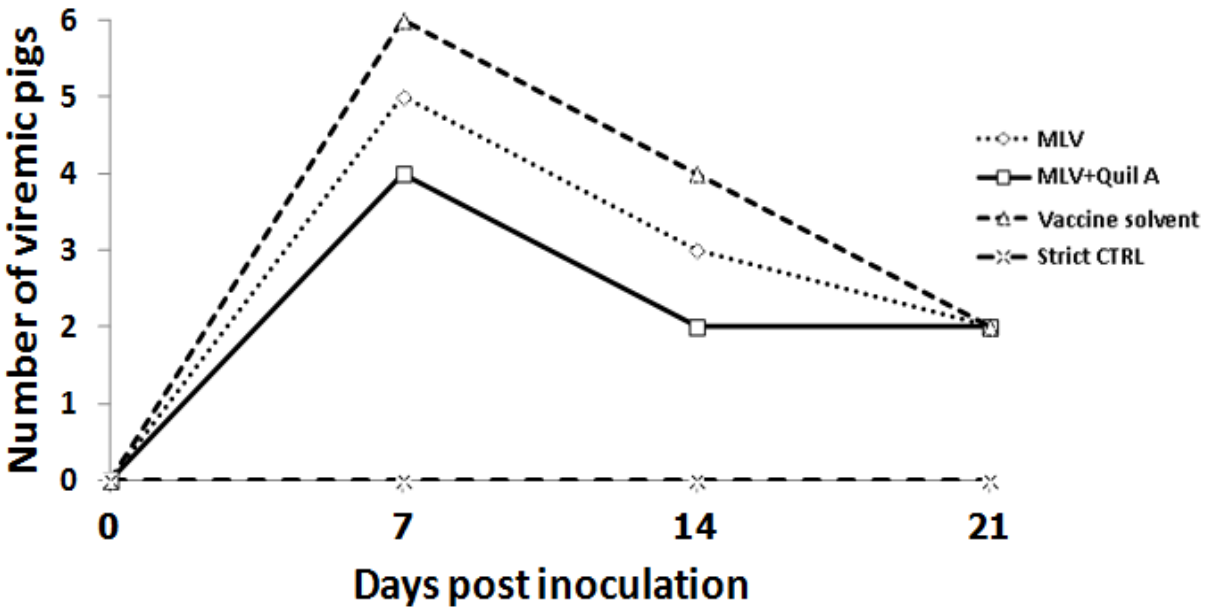


Figure 4 (Cont).


Number of viremic pigs
0
1
2
3
4
5
6
0
7
14
21
Days post inoculation
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL

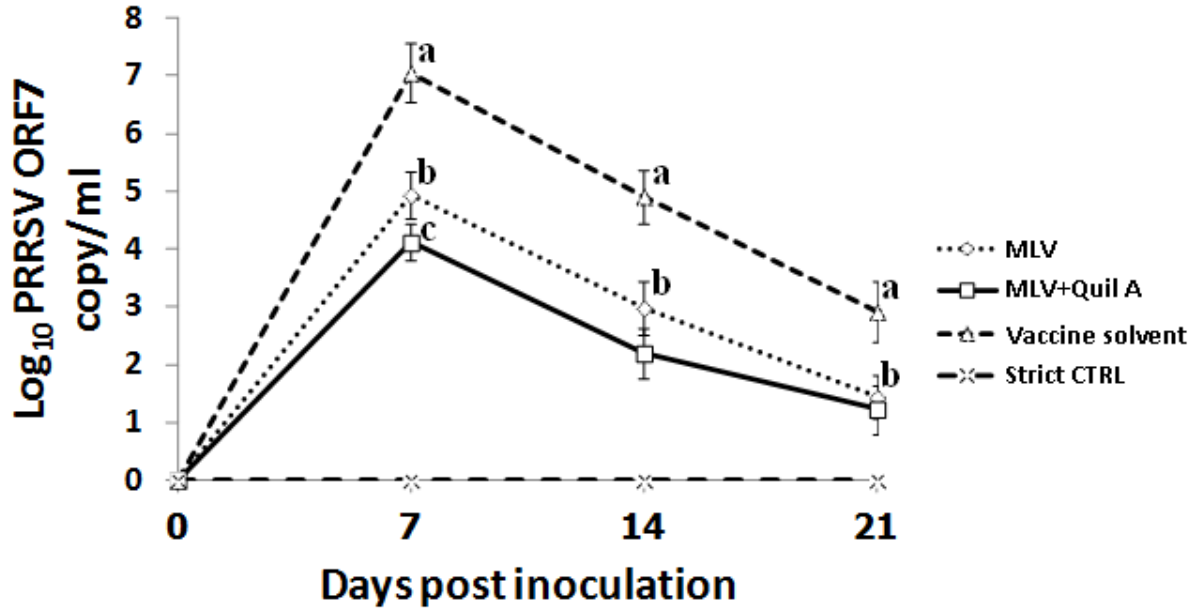

Log10 PRRSV ORF7 copy/ml
0
1
2
3
4
5
6
7
8
a
b
c
a
b
a
b
0
7
14
21
Days post inoculation
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL

Figure 5.

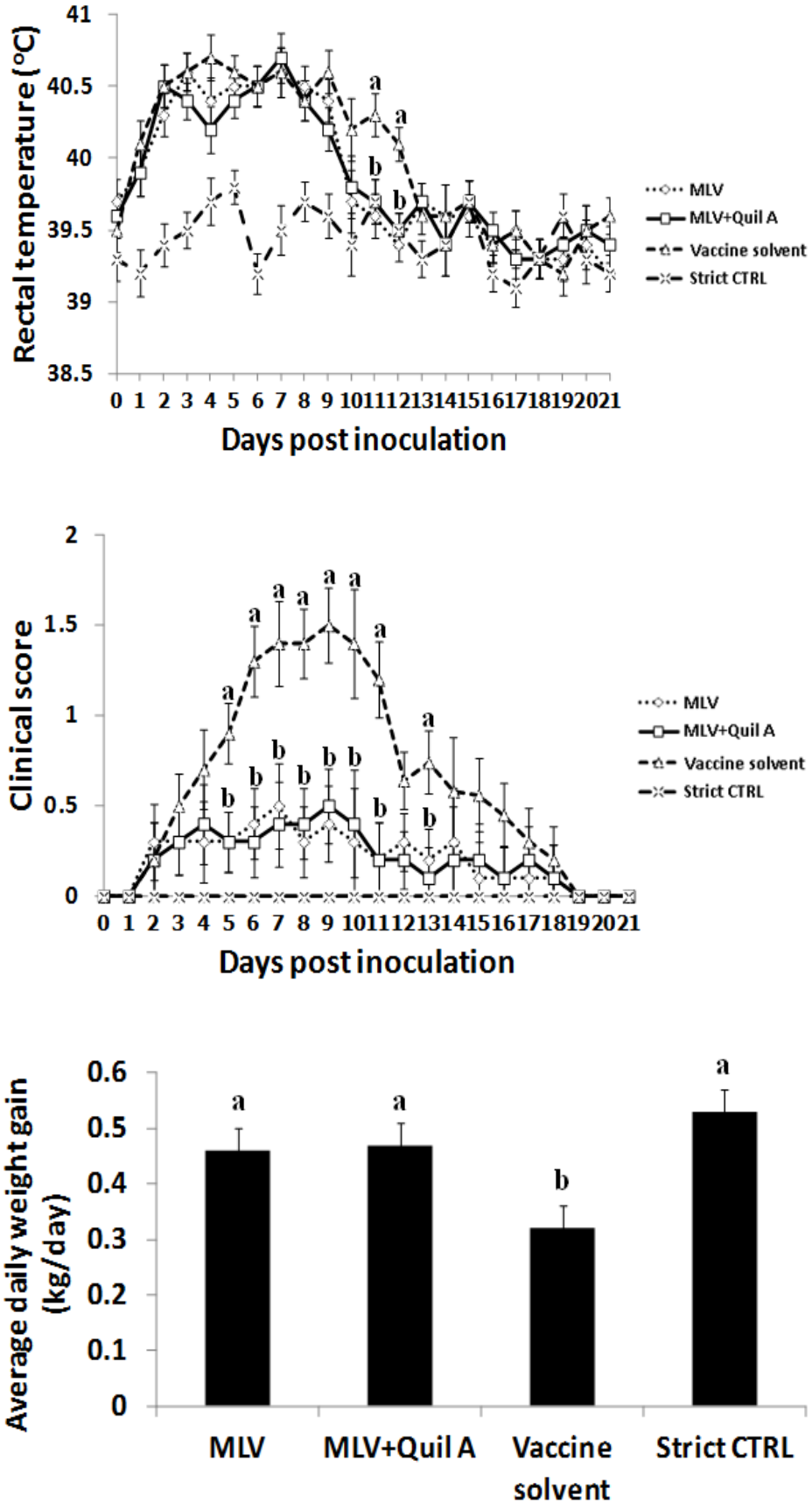

Rectal temperature (°C)
41
40.5
40
39.5
39
38.5
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days post inoculation
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL
Clinical score
2
1.5
1
0.5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days post inoculation
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL
Average daily weight gain (kg/day)
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
b
a
MLV
MLV+Quil A
Vaccine solvent
Strict CTRL

Figure 6.

Co-administration of saponin quil A and PRRSV-1 modified-live virus vaccine up-regulates gene expression of type I interferon-regulated gene, type I and II interferon, and inflammatory cytokines and reduces viremia in response to PRRSV-2 challenge

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Abstract

Porcine reproductive and respiratory syndrome virus (PRRSV) is a devastating virus which suppresses the expression of type I and II interferons (IFNs) as well as several pro-inflammatory cytokines. Our previous study reported that saponin quil A had a potential to up-regulate the expression of type I IFN-regulated genes and type I and II IFNs in porcine peripheral blood mononuclear cells (PBMC) inoculated with PRRSV. The present study evaluated the immunostimulatory effect of quil A on potentiating cross protective immunity of PRRSV-1 modified-live virus (MLV) vaccine against PRRSV-2 challenge. Twenty-four 4-week-old PRRSV-seronegative pigs were divided into four groups of six pigs. Group 1 and group 2 pigs were vaccinated with PRRSV-1 MLV vaccine at 0 dpv (day post vaccination), and additionally group 2 pigs were injected intramuscularly with quil A at -1, 0, 1 dpv. Group 3 pigs were injected with PRRSV-1 MLV vaccine solvent at 0 dpv and served as challenge control, while group 4 pigs served as strict control. Group 1-3 pigs were challenged intranasally with PRRSV-2 at 28 dpv and immune and clinical parameters were observed from 0 until 49 dpv. Group 1 pigs showed significantly reduced PRRSV viremia, number of viremic pigs, and clinical scores, and significantly improved average daily weight gain (ADWG), compared to group 3 pigs. Group 2 pigs showed significantly increased mRNA expressions of interferon regulatory factor 3, 2'-5'-oligoadenylatesynthetase 1, osteopontin, IFNα, IFNβ, IFNγ, interleukin-2 (IL-2), IL-13 and tumor necrosis factor alpha, compared to group 1 pigs. The animals demonstrated significantly reduced PRRSV viremia and number of viremic pigs, but did not demonstrate any further improved PRRSV-specific antibody levels, neutralizing antibody titers, rectal temperature, clinical scores, and ADWG as compared to group 1 pigs. Our findings suggest that quil A up-regulates type I IFN-regulated gene, type I and II IFNs, and inflammatory cytokine expressions which may contribute to further reducing PRRSV viremia and number of viremic pigs which were

conferred by PRRSV-1 MLV vaccine. Our findings also suggest that quil A may serve as an effective immunostimulator for potentiating cell-mediated immune defense to PRRSV.

Keywords: Porcine reproductive and respiratory syndrome virus, Quil A, Modified-live virus vaccine, Interferon, Cross protection

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most economically significant pathogens of swine industry worldwide. The virus causes reproductive failures in breeding swine and respiratory diseases, poor growth performance and probably death in growing pigs (Lunney et al., 2010a).

PRRSV is an enveloped positive-sense single-stranded RNA virus. According to recent taxonomy, PRRSV is divided into two species based on its 3′-terminal structural genes, i.e. PRRSV-1 (formerly known as genotype 1 or European-like PRRSV) and PRRSV-2 (formerly known as genotype 2 or North American PRRSV) (Adams et al., 2016; Kuhn et al., 2016). Both species of PRRSV belong to the genus *Porarterivirus*, family *Arteriviridae* and order *Nidovirales*. The virus genome consists of 10 open-reading frames (ORF), i.e. ORF1a, 1b, 2a, 2b, 3, 4, 5a, 5, 6, and 7, of which PRRSV ORF1a and ORF1b encode 14 nonstructural proteins (nsp), while the other ORFs encode eight structural proteins (Kappes and Faaberg, 2015). PRRSV-1 is further divided into three subtypes, i.e. pan-European subtype 1 and East European subtypes 2 and 3 (Stadejek et al., 2008). However, recent evidence suggests that an additional subtype 4 may be present (Stadejek et al., 2013). Both PRRSV-1 and PRRSV-2 comprise recently emerged highly pathogenic strains called "HP-PRRSV" which are highly virulent and cause serious devastation of pigs in Europe, China and Southeast Asian countries (Tian et al., 2007; Canelli et al., 2017; Han et al., 2017). Both

75 species of PRRSV share approximately 60% nucleotide sequence homology to each other
76 (Dokland, 2010).

77 Modified-live virus (MLV) and killed virus vaccines have been used to reduce clinical
78 losses caused by PRRSV. Vaccine produced from the same PRRSV species as infecting
79 PRRSV is recommended for PRRSV control as it confers better clinical protection than
80 vaccine produced from different PRRSV species (Charerntantanakul, 2012; Nan et al., 2017).
81 However, in many countries including Thailand, co-infection of both species of PRRSV does
82 exist. In Thailand, co-infection is present in the same herds, and even in the same pigs
83 (Amonsin et al., 2009; Nilubol et al., 2012; Nilubol et al., 2013). Therefore, cross protective
84 efficacy of vaccine from different PRRSV species becomes current major clinical issue.

85 Vaccine induction of robust immune response is one of the key factors that lead to
86 effective clinical protection against PRRSV (Charerntantanakul, 2012; Nan et al., 2017).
87 Currently available commercial PRRSV MLV vaccines elicit delayed and weak immune
88 response following vaccination (Loving et al., 2015). PRRSV-specific antibody and
89 neutralizing antibody (Nab) appear approximately two and four weeks after vaccination,
90 respectively (Charerntantanakul et al., 2006a; Zuckermann et al., 2007). The Nab titer is
91 relatively low, approximately 2^2-2^5 for at least six months after vaccination (Wills et al.,
92 1997). PRRSV-specific cell-mediated immune (CMI) response appears approximately two to
93 four weeks after vaccination (Charerntantanakul et al., 2006a; Zuckermann et al., 2007). The
94 response as determined by lymphocyte proliferation and cytokine expressions, i.e. interferon
95 gamma (IFNγ) and interleukin-2 (IL-2) increases rather slowly as compared to CMI response
96 to other swine viral pathogens, i.e. pseudorabies virus and swine influenza virus (Thacker,
97 2001; Meier et al., 2003). Both PRRSV-specific antibodies and CMI responses play
98 protective role in homologous and heterologous PRRSV challenge (Loving et al., 2015; Nan
99 et al., 2017).

Several types of vaccine adjuvants have been evaluated for their potential to enhance immune response to PRRSV vaccine. These include cytokines, e.g. IL-1, IL-2, and IL-6; chemical reagents, e.g. polyinosinic:polycytidylic acid (poly IC), poly IC with polylysine and carboxymethylcellulose (poly ICLC), and poly(lactic-co-glycolic) acid (PLGA); bacterial products, e.g. CpG oligodeoxynucleotides (ODN), lipopolysaccharide (LPS), and cholera toxin; immunostimulatory proteins, e.g. C3d, CD40 ligand, and peptide nanofiber hydrogel; and commercial adjuvants, e.g. Montanide™ Gel 01 ST (Charerntantanakul, 2009; Li et al., 2013; Binjawadagi et al., 2014; Tabynov et al., 2016). Only a few of these products, i.e. PLGA, CpG ODN, peptide nanofiber hydrogel, and Montanide™ Gel 01 ST can improve immune response and protective efficacy of PRRSV vaccine against a challenge by different PRRSV species (Charerntantanakul, 2009; Li et al., 2013; Binjawadagi et al., 2014; Tabynov et al., 2016). This small number of potential adjuvants suggests further exploration of other potential immunostimulator candidates for effective heterologous PRRSV control.

Our previous study on immunostimulatory effects of quil A, a triterpenoid saponin from South American tree bark, *Quillaja saponaria* Molina, in PRRSV-inoculated peripheral blood mononuclear cells (PBMC) demonstrated that quil A significantly enhanced mRNA expression levels of myxovirus resistance 1 (Mx1), interferon regulatory factor 3 (IRF3), IRF7, 2'-5'-oligoadenylatesynthetase 1 (OAS1), stimulator of interferon genes (STING), IFNβ, IFNγ, and significantly reduced mRNA expression of transforming growth factor beta (TGFβ) in PRRSV-inoculated/quil A-stimulated PBMC, as compared to PRRSV-inoculated PBMC control (Charerntantanakul and Fabros, 2018). Quil A has been reported to stimulate cytotoxic T cells, T helper 1 cells, and antibody responses to human and veterinary experimental vaccines (Sun et al., 2009). In pigs, quil A has been reported to enhance serum and mucosal IgA production and protective efficacy of *Actinobacillus pleuropneumoniae* inactivated and subunit vaccines (Loftager et al., 1993; Loftager et al., 1995; Willson et al.,

1995); Nab production and protective efficacy of inactivated swine influenza H3N2 virus vaccine (Bikour et al., 1996); antibody production and protective efficacy of *Streptococcus suis* Sao vaccine (Li et al., 2007), *Taenia solium* TSOL18 vaccine (Assana et al., 2010), and *Toxoplasma gondii* crude rhoptry vaccine (da Cunha et al., 2012); antibody production of foot-and-mouth disease virus (FMDV) vaccine (Xiao et al., 2007) and *S. suis* recombinant SsnA vaccine (Gomez-Gascon et al., 2016); and protective efficacy of *T. solium* TSOL16-TSOL18 fusion vaccine (Jayashi et al., 2012). Quil A, incombination with phospholipids and cholesterol to form immunostimulatory complexes (ISCOMs), reportedly induced IFNβ, tumor necrosis factor alpha (TNFα), and osteopontin (OPN) expressions in porcine PBMC (Fossum et al., 2014), OPN expression in injected muscle of the pig (Ahlberg et al., 2012), and lymphocyte proliferation, antibody production and/or protective efficacy of several vaccines, e.g. pseudorabies virus subunit, *T. gondii* crude rhoptry, FMDV recombinant C-terminal VP1, enterotoxigenic *Escherichia coli* fimbriae, and live *Mycoplasma hyopneumoniae* vaccines (Nagy et al., 1990; Tulman and Garmendia, 1994; Bayry et al., 1999; Garcia et al., 2005; Maes, 2014).

The objective of this study was to evaluate the potential of quil A as an adjuvant for PRRSV-1 MLV vaccine on improving immune response and protective efficacy of the vaccine against PRRSV-2 challenge in growing pigs. Our findings suggest that quil A serves as an effective immunostimulator for potentiating CMI defense against different PRRSV species.

2. Materials and methods

2.1 PRRSV

PRRSV-1 VP046 Bis strain (from commercial MLV vaccine) and PRRSV-2 isolate 01NP1 (isolated from lung lavage) (Thanawongnuwech et al., 2004) were cultured in

confluent MARC-145 cells grown in MEM^{++} (MEM (Caisson Laboratories, Smithfield, UT), 10% heat-inactivated FBS (Capricorn Scientific GmbH, Germany), penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml) (all from Gibco, Grand Island, NY)). The cultures were incubated in a humidified 5% CO_2 atmosphere at 37°C until 60-80% of cytopathic effects (CPE) were observed. Infected cultures were frozen and thawed twice, centrifuged, and the supernatant was collected, filtered through 0.22 µM filter (Minisart®, Sartorius, France), aliquoted, and stored at -80°C. The virus titers were determined by immunoperoxidase monolayer assay (IPMA), using primary mouse mAbs specific for PRRSV nucleocapsid (N) proteins of both PRRSV-1 and PRRSV-2 (1:800 dilution in BSA-PBST, IgG2b, clone 5C61; Median Diagnostics, South Korea) and secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies (1:600 dilution in BSA-PBST; CiteAB, UK). The virus titers were adjusted to 10^5 tissue culture infectious dose 50 ($TCID_{50}$)/ml. PRRSV-1 and PRRSV-2 used in this study were at their third and tenth passage in MARC-145 cells, respectively. Mock antigens were prepared in the same fashion as virus antigens except that no viruses were inoculated.

2.2 Quil A

Quil A (cat# vac-quil, Invivogen, San Diego, CA) was resuspended with sterile water to 10 mg/ml. The solution was filtered through 0.22 µM filter (Minisart®), aliquoted, kept at -20°C, and protected from light until use. The presence of LPS in the resuspended quil A solution was less than 0.005 EU/ml as determined by limulus amebocyte lysate assay (Chromo LAL®, Associates of Cape Cod, Inc., East Falmouth, MA). When used for i.m. injection, quil A solution was diluted with sterile water to 300 µg/ml.

2.3 Determination of effect of quil A on PRRSV-1 infectivity and replication in vitro

2.3.1 Optimization of quil A concentration

One hundred microliters of MARC-145 cell suspension (10^4 cells) were seeded into 96-well flat-bottom plates (Nunc, Denmark). The wells then received 100 µl of 2-fold serially diluted quil A in MEM^{++} in quadruplicate settings. The cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 96 h. The media were then removed and the cells were washed three times with PBS and fixed with 100 µl of ice-cold acetone:methanol (60:40) solution for 30 min. Following fixation, the fixing solution was discarded and 20 µl of 0.5% crystal violet solution was added. The plates were incubated at room temperature for 5 min, followed by three washes with water. The plates then received 100 µl of Sorenson's buffer and were incubated at room temperature for 15 min prior to determination of optical density (O.D.) values at 590 nm. MARC-145 cells that received only MEM^{++} served as untreated control. The highest concentration of quil A that was not toxic to MARC-145 cells as determined by %viable cells (calculated from O.D. value of quil A-treated cells*100/O.D. value of untreated cells) was used for subsequent anti-PRRSV studies. The cytotoxic assays were performed in three independent studies.

2.3.2 Effect of quil A on PRRSV-1 infectivity

Fifty microliters of quil A (7.8 µg/ml final) were incubated with 100 µl of 10-fold serially diluted PRRSV-1 VP046 Bis strain starting at a multiplicity of infection (m.o.i.) of 1 at 37°C for 1 h. The mixtures were then added into wells containing 100 µl of MARC-145 cells (10^4 cells) at 16-h old in two quadruplicate settings, and were incubated at 37°C in a humidified 5% CO_2 atmosphere for 1 h. Subsequently the supernatants were removed and replaced with 250 µl of MEM^{++}. The cultures were incubated further for 96 h prior to determination of PRRSV titers by IPMA test. MARC-145 cells that received only serially diluted PRRSV-1 VP046 Bis strain or serially diluted mock antigens served as PRRSV and mock controls, respectively. The assays were performed in three independent studies.

2.3.3 Effect of quil A on PRRSV-1 replication

One hundred microliters of MARC-145 cells at 16-h old (10^4 cells) were incubated at 37^oC in a humidified 5% CO_2 atmosphere for 1 h with 100 µl of 10-fold serially diluted PRRSV-1 VP046 Bis strain starting at a m.o.i. of 1. The assay was set in two quadruplicate settings. Subsequently the supernatants were removed, and the cells were washed and cultured in 200 µl of MEM^{++} and 50 µl of quil A (7.8 µg/ml final). The cultures were incubated further for 96 h prior to determination of PRRSV titers by IPMA test. MARC-145 cells that received only serially diluted PRRSV-1 VP046 Bis strain or serially diluted mock antigens served as PRRSV and mock controls, respectively. The assays were performed in three independent studies.

2.4 Experimental design

Twenty-four 4-week-old pigs (Large White/Landrace x Duroc) seronegative to PRRSV and *Mycoplasma hyopneumoniae* as determined by commercial ELISA test kits (IDEXX Laboratories, Westbrook, ME) were randomly divided into 4 groups of 6 pigs (3 castrated male and 3 female). The animals were housed at the swine research extension facility (Chiang Mai, Thailand). Group 1 pigs received i.m. injection with 2 ml of Amervac® PRRS MLV (PRRSV-1 VP046 Bis strain; Batch: 97PT-6; Hipra, Spain) at 0 dpv (day post vaccination). Group 2 pigs received i.m. injection with 2 ml of Amervac® PRRS MLV at 0 dpv and 1 mL of quil A (300 µg) at approximately 1 inch apart from the vaccination site at -1, 0, 1 dpv. The 300 µg dose of quil A was according to the recommendation by the manufacturer. Group 3 pigs received i.m. injection with 2 ml of vaccine solvent (Batch: 4C79-1; Hipra, Spain) used for resuspension of lyophilized Amervac® PRRS MLV at 0 dpv and served as challenge control. Group 4 pigs received no treatment and served as a strict control. Pigs of all groups except group 4 were challenged i.n. with 1 ml of 10^5 $TCID_{50}$ of

PRRSV-2 isolate 01NP1 at 28 dpv. The ORF5 nucleotide sequence of PRRSV-2 isolate 01NP1 (NCBI accession #AY297112) shared only 68% homology to the ORF5 of the vaccine strain (NCBI accession #DQ324668). Blood was collected weekly from jugular vein of all pigs at 0 until 49 dpv. Experimental schedule is summarized in Table 1. All serum samples were aliquoted and stored at -20^{o}C until assayed for PRRSV-specific antibodies or at -70^{o}C until assayed for the presence of PRRSV.

All animals were monitored daily and were fed *ad libitum* with commercial feed. The animals were sacrificed by an intravenous injection of sodium pentobarbital at 49 dpv. All experiments were carried out according to internal guidelines for care and use of experimental animals, which include the criteria regarding replacement, reduction, and refinement of experimental animals. The experimental procedure was approved by the animal care and use committee of Maejo University (Approval number MACUC 004S/2561).

2.5 Clinical evaluation and growth performance

Rectal temperature and clinical signs were recorded daily following PRRSV-2 isolate 01NP1 challenge. Clinical scores were calculated according to the criteria adjusted from Roca et al. (2012) (Table 2). Average daily weight gain (ADWG) was calculated from 28 to 49 dpv (corresponding to 0 to 21 days post inoculation [dpi]).

2.6 Antibody response

The presence of PRRSV-specific antibodies was determined by commercial ELISA test kit (IDEXX PRRS X3 Ab Test, IDEXX Laboratories, Westbrook, ME) and serum virus neutralization (SVN) test. For ELISA test, the antibody response was presented as the sample to positive (s/p) ratio, where s/p ratio equal to and greater than 0.4 was considered positive. SVN test was conducted as described previously (Yoon et al., 1995). Briefly,

250 serum samples were heat inactivated at 56^{o}C for 40 min, then serially diluted 2-fold in
251 MEM^{++}. One hundred microliters of diluted serum were mixed with an equal volume of
252 PRRSV-2 isolate 01NP1 containing 10^2 $TCID_{50}$. The mixtures were incubated at 37^{o}C for 1
253 h, then 200 µl of which was added to a 96-well flat-bottom plate containing 16-h-old
254 confluent MARC-145 cells. The cultures were incubated at 37^{o}C for 5 days and observed for
255 CPE. Each serum sample was run in triplicate. The Nab titers were expressed as the
256 reciprocal of the highest serum dilution in which no CPE was observed. The Nab titer equal
257 to or greater than 2^2 was considered positive.
258
259 2.7 Immune parameter gene expression
260 2.7.1 PBMC isolation and culture
261 PBMC were isolated from EDTA-anticoagulated blood by Ficoll-Hypaque gradient
262 centrifugation (Histopaque®-1077, Sigma, St. Louis, MO) as described previously
263 (Charerntantanakul et al., 2006b). Contaminating erythrocytes were lysed with 6 ml of cold
264 hypotonic solution comprising of 0.156 M ammonium chloride, 10 mM sodium bicarbonate,
265 and 1 mM EDTA for 90 seconds. The isotonicity was restored with 3 ml of 3x PBS. PBMC
266 were centrifuged at 1,250 rpm at 4^{o}C for 15 min and washed once with PBS prior to
267 resuspension in RPMI^{++} (RPMI-1640 with L-glutamine (Caisson Laboratories, Smithfield,
268 UT), 10% heat-inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml) and
269 amphotericin B (250 ng/ml)) to $5x10^5$ cells/ml. Cell viability was determined by trypan blue
270 dye exclusion assay. Greater than 95% viable PBMC were obtained.
271 Two hundred microliters of PBMC suspension (10^5 cells) was pipetted into 96-well
272 flat-bottom plates in duplicate setting. The wells then received 100 µl of PRRSV-2 isolate
273 01NP1 (10^4 $TCID_{50}$; approximately 1 m.o.i.), mock antigen (mock control), or were left
274 untreated (to be served as positive control). Plates were incubated in a humidified 5% CO_2

atmosphere at 37°C for 72 h. Eighteen hours prior to the conclusion of incubation period, wells to be served as positive control received 100 µl of poly IC (5 µg/ml final; Sigma, St. Louis, MO) or LPS from *E. coli* O111:B4 (5 µg/ml final; Sigma, St. Louis, MO). Wells receiving poly IC served as positive control for Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNα, IFNβ, IL-10, and TGFβ, and wells receiving LPS served as positive control for IFNγ, IL-2, IL-13, and TNFα.

2.7.2 Real-time PCR

Isolation of total RNA from cultured PBMC and elimination of contaminating DNA were performed using NucleoSpin® Blood kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instructions. Complete elimination of genomic DNA was confirmed by real-time PCR using total RNA preparation as template and primers for RPL32 (ribosomal protein L32) (Charerntantanakul et al., 2013). Reverse transcription was carried out, using RevertAid™ First Strand cDNA synthesis kit (Thermo Fisher Scientific Baltics, Lithuania). The concentration and purity of RNA and the concentration of cDNA were determined by spectrophotometry (Nanodrop ND-1000; NanoDrop Technologies). All RNA samples had absorbance values at 260 and 280 nm (A260/280) and 260 and 230 nm (A260/230) between 2.0-2.2 and 1.8-2.2, respectively.

Real-time PCR was performed on the thermal cycler (Applied Biosystems 7500 Fast Real-Time PCR system) in a total reaction volume of 20 µl, consisting of 2 µl of cDNA template, optimal concentration of each primer (Charerntantanakul and Fabros, 2018), and 10 µl SYBR® Green real time PCR master mix (Toyobo, Japan). All reactions were set up in duplicate. The PCR condition was 95°C (15 min); and 40 cycles of 94°C (15s), optimal annealing temperature (30s) (Charerntantanakul and Fabros, 2018), and 72°C (30s). The threshold cycles (C_T) of all genes were collected and used for calculation of immune parameter gene expression by $2^{-\Delta\Delta C_T}$ method (Pfaffl, 2001). The RPL32 and tyrosine 3-

monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) mRNA of the same animal were used for normalization of immune parameter gene expression. The expression values were transformed into $\log_2$ scale in order that the expression was relative to 0. Melting curve analysis was conducted following the completion of PCR cycles for product verification. Agarose gel electrophoresis was performed to confirm a single product of the expected size. In each run, nuclease-free water was included as no template control.

2.8 Viremia

2.8.1 Generation of recombinant PRRSV ORF7 plasmids

The RNA of PRRSV-2 isolate 01NP1 was extracted from virus aliquot using Nucleospin® RNA virus kit (Macherey-Nagel, Bethlehem, PA) according to the manufacturer's instructions. Elimination of contaminating DNA was performed using rDNase (Macherey-Nagel, Bethlehem, PA). Reverse transcription was carried out using RevertAid™ First Strand cDNA synthesis kit with 2 µl of total RNA. Amplification of ORF7 cDNA was performed using Quick Taq HS DyeMix (Toyobo, Japan) and 400 nM each of primer ORF7 149F (5'CGGAGAAGCCCCATTTTCCT3') and ORF7 346R (5'GGATCAGGCGCACAGTATGA3') designed according to NCBI accession #DQ056373. Agarose gel electrophoresis was conducted to confirm a single product of 198 bp. The PCR products were purified using PureLink™ PCR purification kit (Invitrogen, Carlbads, CA) and were sequenced. The PCR amplicons with 100% nucleotide homology to published PRRSV ORF7 sequence (NCBI accession #DQ056373) were cloned into pGEM-T easy vector (Promega, Madison, WI) according to manufacturer's instructions**.** The resulting recombinant plasmids were amplified in competent *E. coli* DH5α in LB medium, and subsequently the culture was spread onto LB agar supplemented with 40 µg/ml Xgal, 0.1mM IPTG, and 100 µg/ml ampicillin. The plasmids from white colonies were isolated using

Tianprep rapid mini plasmid kit (Tiangen, China), and were verified by restriction analysis with EcoRI (Thermo Fisher Scientific Baltics, Lithuania) and nucleotide sequencing.

2.8.2 Real-time PCR

Real-time PCR was performed with 150 µl of sera samples collected at 0, 28, 35, 42 and 49 dpv. The extraction of viral RNA, elimination of contaminating DNA, and reverse transcription were conducted as described above. All RNA samples had absorbance values at A260/280 and A260/230 between 2.0-2.2 and 1.8-2.2, respectively. Real-time PCR was performed in a total reaction volume of 20 µl, consisting of 2 µl of cDNA, 400 nM each of primer ORF7 149F and ORF7 346R, and 10 µl SYBR® Green real time PCR master mix (Toyobo, Japan). All reactions were set up in duplicate. The PCR condition was 95°C (15 min); and 35 cycles of 95°C (15s), 53°C (30s), and 72°C (30s). The C_T were collected and compared with C_T standard curve generated from 10^1-10^8 copy numbers of recombinant PRRSV ORF7 plasmids. Melting curve analysis was conducted following the completion of PCR cycles for product verification. Agarose gel electrophoresis was performed to confirm a single product of 198 bp. In each run, nuclease-free water was included as no template control.

2.8.3 Virus isolation

Virus isolation was conducted to confirm the presence of PRRSV. Serum samples were diluted 1:5 in MEM^{++}, and 200 µl of diluted serum samples were inoculated onto 16-h-old MARC-145 cells in 96-well flat-bottom plate. The inoculated cells were incubated at 37°C in humidified 5% CO_2 incubator for 7 days prior to observation of CPE and IPMA test.

2.9 Data analysis

Statistical analyses were performed using the SPSS software version 17 (IBM, Armonk, NY). Mean fold differences of ELISA s/p ratio, immune parameter gene

expressions, PRRSV copy numbers, rectal temperature, clinical scores, and ADWG were tested by one-way analysis of variance (ANOVA), followed by Dunnett's test. $P<0.05$ was set as a statistically significant level throughout this study.

3. Results

3.1 Optimal concentration of quil A for MARC-145 cells

Quil A was determined for its non-cytotoxic concentration to MARC-145 cells. As shown in Fig. 1, quil A at a final concentration of 15.6 µg/ml or higher was toxic to MARC-145 cells, yielding %viable cells of 25.40±3.03% to 92.72±2.51%. Quil A at a final concentration of 7.8 µg/ml was the highest concentration that was not toxic to MARC-145 cells, yielding %viable cells of 100.04±6.91%. Thus this concentration was used for subsequent studies.

3.2 Quil A did not interfere with PRRSV-1 infectivity and replication in MARC-145 cells

Quil A was evaluated for its anti-PRRSV activity prior to subsequent studies in vivo. The presence of anti-PRRSV activity of quil A, if any, may adversely affect PRRSV MLV vaccine immunogenicity and protective efficacy. Incubation of PRRSV-1 with quil A prior to subsequent inoculation to MARC-145 cells did not reduce PRRSV infectivity as there was no significant difference between PRRSV titers of quil A-pre-treated cells (3.96±0.43 $\log_{10}$ $TCID_{50}$/ml) and PRRSV control (4.04±0.19 $\log_{10}$ $TCID_{50}$/ml) (Fig. 2a). Moreover, addition of quil A into PRRSV-1-infected MARC-145 cells did not interfere with PRRSV-1 replication, as there was no significant difference of PRRSV titers between quil A-treated cells (4.04±0.33 $\log_{10}$ $TCID_{50}$/ml) and PRRSV control (4.08±0.26 $\log_{10}$ $TCID_{50}$/ml) (Fig. 2b). No unspecific binding of mAbs used in IPMA test to MARC-145 cells was detected.

3.3 Co-administrations of quil A did not improve antibody response compared to MLV vaccination alone

Both group 1 and group 2 pigs became seropositive for PRRSV at 14 dpv, and their s/p ratios peaked at 28 dpv. Upon PRRSV-2 challenge, both groups of pigs did not develop anamnestic antibody response (Fig. 3). No significant difference of s/p ratio between group 1 and group 2 pigs prior to and after PRRSV-2 challenge was detected (Fig. 3). Group 3 pigs became seropositive for PRRSV at 42 dpv (14 dpi). Group 4 pigs remained seronegative for PRRSV throughout the experiment. None of the groups had detectable Nab either before or after challenge (data not shown).

3.4 Immune parameter gene expression

3.4.1 Co-administrations of quil A significantly improved gene expressions of type I IFN-regulated gene, type I and II IFN, and inflammatory cytokine compared to MLV vaccination alone

Group 1 pigs demonstrated significantly increased gene expressions of Mx1, IRF7, OAS1, IFNγ, IL-10, and IL-13 at 7 to 28 dpv, and of IRF3, STING, OPN, IFNα, IFNβ, IL-2, and TNFα at 7 to 21 dpv, compared to group 3 pigs (Fig. 4). No significant difference of TGFβ gene expression was observed during 7 to 28 dpv.

When compared to group 1 pigs, group 2 pigs demonstrated significantly increased gene expressions of IRF3 and IFNα at 7 to 28 dpv, OPN at 7 to 21 dpv, IL-2 and IL-13 at 14 and 21 dpv, IFNβ and IFNγ at 7 dpv, IRF7 at 14 dpv, and Mx1 at 21 dpv (Fig. 4). However, they demonstrated significantly decreased gene expression of IL-10 and IL-13 at 28 dpv. No significant difference of OAS1, STING, TNFα, and TGFβ gene expressions was observed during 7 to 28 dpv.

3.4.2 Co-administrations of quil A significantly improved gene expressions of type I IFN-regulated gene, type I and II IFN, and inflammatory cytokine in MLV-vaccinated pigs after PRRSV-2 challenge compared to MLV vaccination alone

Pigs were challenged with PRRSV-2 at 28 dpv (0 dpi). Group 3 pigs demonstrated significantly increased gene expressions of Mx1, IRF7, OAS1, STING, OPN, IFNα, IFNβ, IL-10, IL-13, and TGFβ at 35 to 49 dpv, IRF3 and TNFα at 42 to 49 dpv, and IFNγ and IL-2 at 42 dpv, compared to group 4 pigs (Fig. 4). The animals, however, demonstrated significantly decreased TNFα gene expression at 35 dpv.

When compared to group 3 pigs, group 1 pigs showed significantly increased gene expressions of IL-2 at 35 to 42 dpv, IFNα at 42 to 49 dpv, IRF7 and STING at 35 dpv, IRF3 at 42 dpv, and IL-13 and IFNγ at 49 dpv (Fig. 4). On the other hand, the animals demonstrated significantly decreased gene expressions of TGFβ at 35 to 42 dpv, IFNβ at 42 to 49 dpv, Mx1, OAS1, and OPN at 35 dpv, and IRF3 and STING at 49 dpv. No significantly different gene expressions of IL-10 and TNFα were observed.

When compared to group 1 pigs, group 2 pigs demonstrated significantly increased gene expressions of IFNβ at 42 to 49 dpv, OAS1, OPN, IFNα, IFNγ, IL-2, and TNFα at 35 dpv, IL-13 at 42 dpv, and IRF3 at 49 dpv (Fig. 4). However, they demonstrated significantly decreased TGFβ gene expression at 42 dpv. No significant difference of Mx1, IRF7, STING, and IL-10 gene expressions was observed during 35 to 49 dpv.

3.5 Co-administrations of quil A significantly reduced viremia compared to MLV vaccination alone

After PRRSV-2 challenge at 28 dpv (0 dpi), all groups except group 4 pigs became viremic. The presence of PRRSV ORF7 gene and PRRSV was detected in 6/6, 4/6, and 2/6 of group 3 pigs at 7, 14, and 21 dpi, respectively (Fig. 5). The number of viremic pigs was

424 decreased to 5/6, 3/6, and 2/6, respectively, in group 1 pigs, and 4/6, 2/6, and 2/6,
425 respectively, in group 2 pigs (Fig. 5).
426 The number of PRRSV ORF7 copies was significantly reduced in group 1 and group
427 2 pigs as compared to that of group 3 pigs at all time points (Fig. 5). The number of PRRSV
428 ORF7 copies was significantly reduced, by approximately 1 $\log_{10}$, in group 2 pigs when
429 compared to that of group 1 pigs at 7 dpi (Fig. 5).
430
431 3.6 Co-administrations of quil A did not confer clinical protection nor improve ADWG
432 compared to MLV vaccination alone
433 All groups except group 4 pigs developed fever (rectal temperature higher than 40^{o}C)
434 and clinical signs from 2 dpi onwards (Fig. 6). The fever persisted until 12 dpi in group 3
435 pigs, but diminished earlier at 10 dpi in group 1 and group 2 pigs (Fig. 6). There was no
436 significant difference in mean rectal temperature among group 1, 2, and 3 pigs throughout the
437 experiment except at 11 and 12 dpi.
438 Group 3 pigs showed clinical signs of difficult breathing, weakness, and reduced
439 appetite as well as coughing more obvious than group 1 and group 2 pigs (Fig. 6). They had
440 significantly higher clinical scores from 5 to 13 dpi, compared to group 1 and group 2 pigs.
441 There was no significant difference between clinical scores of group 1 and group 2 pigs
442 throughout the experiment. Group 4 pigs remained clinically normal throughout the study.
443 In addition to clinical signs, group 3 pigs had significantly slower growth rate than the
444 other three groups. The animals had ADWG from 0 to 21 dpi of 0.32$\pm$0.04 kg/day, while
445 group 1, 2, and 4 pigs had ADWG of 0.46$\pm$0.03, 0.47$\pm$0.04, and 0.53$\pm$0.03, respectively
446 (Fig. 6).
447
448 4. Discussion

449 The present study evaluated the potential of saponin quil A on improving immune
450 response and protective efficacy of PRRSV-1 MLV vaccine against PRRSV-2 challenge in
451 growing pigs. Robust immune response to PRRSV MLV vaccine is an essential factor that
452 contributes to increasing levels of cross protection against heterologous PRRSV challenge.
453 Cross protective immunity is an important clinical issue, particularly in countries where co-
454 infection of PRRSV-1 and PRRSV-2 exists.
455 Prior to the injections of quil A in vivo, the anti-PRRSV activity of quil A was
456 evaluated in MARC-145 cells in vitro. This was to determine the potential that quil A
457 interfered with PRRSV-1 MLV replication, which may reduce vaccine immunogenicity and
458 protective efficacy. Results showed that there was no significant difference in PRRSV-1 titer
459 between quil A-treated and untreated cells, either in the assay that quil A was incubated with
460 PRRSV-1 prior to subsequent inoculation to the cells or in the assay that quil A was added to
461 MARC-145 cells that were already infected with PRRSV-1. These results indicated that quil
462 A neither directly interfered with PRRSV-1 infection nor inhibited PRRSV-1 replication.
463 Thus injections of quil A should not have adverse effect on PRRSV-1 MLV infection and
464 replication, and thereby should not have negative effect on PRRSV-1 MLV vaccine
465 immunogenicity and protective efficacy.
466 Injections of quil A did not improve antibody response to PRRSV-1 MLV vaccine.
467 The s/p ratio was not increased in group 2 pigs either before or after PRRSV-2 challenge as
468 compared to group 1 pigs. In either group, the s/p ratio was detected as early as 14 dpv and
469 peaked at 28 dpv. Group 3 pigs, on the other hand, had higher s/p ratio at 14 dpi as compared
470 to 14 dpv of group 1 and group 2 pigs, and their s/p ratio peaked at 21 dpi. Higher total
471 antibody response in group 3 pigs might not attribute to the vaccine solvent given to the pigs
472 at 0 dpv, but rather to the virulence of the challenge PRRSV-2 strain. It has been evident that
473 kinetics and magnitude of total antibody response to PRRSV correlate directly with PRRSV

virulence, in which more virulent PRRSV strains elicit stronger antibody response than less virulent PRRSV strains (Johnson et al., 2004). Similar to the s/p ratio, the Nab titers were not increased in group 2 pigs either before or after PRRSV-2 challenge as compared to group 1 pigs. The absence or a slight increase, but still below assay cut-off, of Nab titer of group 1 and group 2 pigs has often been reported in several heterologous PRRSV challenge studies (Roca et al., 2012; Park et al., 2015; Ko et al., 2016). This was proposedly attributed, at least in part, to low amino acid sequence identity (approximately 51-55%) of neutralizing epitopes between PRRSV-1 and PRRSV-2, glycan shielding of broadly neutralizing epitopes shared between PRRSV-1 and PRRSV-2, and the existence of decoy neutralizing epitopes (Murtaugh et al., 1995; Ostrowski et al., 2002; Ansari et al., 2006; Vu et al., 2011). Yet, the role of Nab in cross protection is not well evident, although some researchers propose its contribution to PRRSV cross protection (Robinson et al., 2015; Rahe and Murtaugh, 2017). On the other hand, levels of total antibody response have been reported to correlate positively with levels of cross protection, though the cross protective role of total antibodies is not well identified (Charerntantanakul et al., 2006a; Roca et al., 2012; Ko et al., 2016). Majority of PRRSV-specific antibodies elicited by MLV vaccine is against PRRSV N and matrix (M) proteins as well as nsp1, nsp2, and nsp7, which contain no neutralizing epitope (Molina et al., 2008; Brown et al., 2009). The amino acid sequence identity of these proteins between PRRSV-1 and PRRSV-2 varies greatly, ranging from 32% in nsp2 to 79% in N protein (Meng et al., 1995; Murtaugh et al., 1995; Allende et al., 1999). Although exact mechanisms of PRRSV MLV-elicited antibody-mediated cross protection is not known, possible mechanisms may include antibody-dependent cell-mediated cytotoxicity, antibody-dependent cellular phagocytosis, antibody-dependent complement-mediated cytotoxicity, and antibody-dependent complement-mediated virolysis (Rahe and Murtaugh, 2017).

Immunization with PRRSV-1 MLV vaccine significantly increased gene expressions of type I IFN-regulated genes, i.e. Mx1, IRF3, IRF7, OAS1, STING, and OPN; type I and II IFNs, i.e. IFNα, IFNβ, and IFNγ; pro-inflammatory cytokines, i.e. IL-2 and TNFα; and anti-inflammatory cytokines, i.e. IL-10 and IL-13 from 7 dpv to at least 21 dpv, compared with unvaccinated control pigs (Fig. 4). Injections of quil A further enhanced immune gene expressions induced by PRRSV-1 MLV vaccine, i.e. Mx1, IRF3, IRF7, OPN, IFNα, IFNβ, IFNγ, and IL-2, at least one time point from 7 to 28 dpv, and significantly decreased gene expressions of IL-10 and IL-13 at 28 dpv, compared with immunization with PRRSV-1 MLV vaccine alone (Fig. 4). Our previous study in vitro have demonstrated that quil A potentially enhanced gene expressions of type I IFN-regulated genes, i.e. Mx1, IRF3, IRF7, OAS1, and STING; type I and II IFNs, i.e. IFNβ, and IFNγ, and significantly decreased gene expression of anti-inflammatory cytokine, i.e. TGFβ in PRRSV-inoculated PBMC (Charerntantanakul and Fabros, 2018). Those in vitro results as well as our present findings together indicated that injections of quil A significantly improve mRNA expressions of type I IFN-regulated genes, type I and II IFNs, and inflammatory cytokines in response to PRRSV MLV vaccine. It is noteworthy that injections of quil A did not enhance IL-10 gene expression which was induced by PRRSV MLV vaccination. PRRSV reportedly elicited weak and delayed CMI response partly by up-regulation of IL-10 expression in infected myeloid antigen-presenting cells (APCs) and PBMC (Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003; Charerntantanakul et al., 2006b). Such up-regulation of IL-10 expression contributed significantly to suppressing gene expressions of pro-inflammatory cytokines, e.g. IL-1β, IL-12p40, and TNFα; co-stimulatory molecules, i.e. CD80 and CD86, and IFNγ in infected APCs (Charerntantanakul and Kasinrerk, 2010, 2012). Reduced expressions of these immune parameters contributed to reduced activity of infected myeloid APCs in activation of PRRSV-

522 specific T cells, particularly CD8β$^+$ T cells (Charerntantanakul et al., 2006b;
523 Charerntantanakul and Kasinrerk, 2010).

524 Following PRRSV-2 challenge, challenge control pigs demonstrated significantly
525 increased gene expressions of Mx1, IRF3, IRF7, OAS1, STING, OPN, IFNα, IFNβ, IFNγ,
526 IL-2, IL-10, IL-13, TNFα, and TGFβ, at least at one time point from 35 to 49 dpv (7 to 21
527 dpi), compared with strict control pigs (Fig. 4). PRRSV-1 MLV-vaccinated pigs
528 demonstrated significantly higher gene expressions of IRF3, IRF7, STING, IFNα, IFNγ, IL-2,
529 and IL-13, and significantly lower gene expressions of Mx1, IRF3, OAS1, STING, OPN,
530 IFNβ, and TGFβ, at least at one time point from 35 to 49 dpv, than challenge control pigs
531 (Fig. 4). These findings suggest that priming with PRRSV-1 MLV vaccine helps enhance
532 gene expressions of some type I IFN-regulated genes and type I and II IFNs following
533 PRRSV-2 challenge, although the expression levels of some genes, i.e. IRF3 and STING
534 were fluctuated from time to time. Among these up-regulated genes, increased IFNγ gene
535 expression was confirmed by previous reports that PRRSV-1 MLV-vaccinated pigs
536 demonstrated significantly increased numbers of IFNγ-secreting cells and levels of serum
537 IFNγ following PRRSV-2 challenge as compared to unvaccinated challenge control pigs
538 (Kim et al., 2015; Choi et al., 2016; Ko et al., 2016; Park et al., 2017). Pigs injected with quil
539 A demonstrated significantly increased immune gene expressions which were induced by
540 PRRSV-1 MLV vaccine, i.e. IRF3, OAS1, OPN, IFNα, IFNβ, IFNγ, IL-2, IL-13, and TNFα,
541 at least at one time point from 35 to 49 dpv, and further decreased TGFβ gene expression at
542 35 to 42 dpv as compared to pigs vaccinated with PRRSV-1 MLV vaccine alone (Fig. 4).
543 PRRSV-1 MLV-vaccinated pigs either with or without quil A demonstrated lower, but not
544 significantly, IL-10 gene expression at 35 dpv compared with challenge control pigs. The
545 lower IL-10 gene expression correlated with higher expressions of some immune genes, e.g.
546 IRF7, STING, IFNα, IFNγ, IL-2, and TNFα in pigs of MLV-vaccinated groups. These

finding suggests that priming with PRRSV-1 MLV vaccine helps reduce IL-10 gene expression following PRRSV-2 challenge, and the reduced IL-10 may attribute to increased expressions of some type I IFN-regulated genes and type I and II IFNs. In contrast to IL-10, exact role of TGFβ on PRRSV infection has not yet been elucidated. Its increased expression has been reported in monocyte-derived macrophages (MDMs) infected with PRRSV in vitro, and in tonsils, mediastinal lymph nodes and lungs of PRRSV-infected pigs (Gomez-Laguna et al., 2012; Charerntantanakul et al., 2013). Its decreased expression, on the other hand, has been reported in PRRSV-infected MDMs that highly expressed IFNγ after the cells were transfected with plasmids expressing porcine IFNγ (Charerntantanakul et al., 2013). The in situ expression of TGFβ has been reported to correlate positively with PRRSV antigen loads in lymphoid tissues and lungs of PRRSV-infected pigs (Gomez-Laguna et al., 2012). The increased or decreased expression of TGFβ may be relevant to the activity of PRRSV-infected MDMs and PRRSV-specific regulatory T cells which, the latter, have been shown to play a crucial role on reducing CMI response to PRRSV (Wongyanin et al., 2010; Cecere et al., 2012; Silva-Campa et al., 2012). Further studies are required to investigate precise role of TGFβ on PRRSV immunology and pathogenicity. These findings altogether suggest that quil A significantly contributed to enhancing mRNA expressions of type I IFN-regulated genes, type I and II IFNs, and inflammatory cytokines in pigs vaccinated with PRRSV-1 MLV vaccine and challenged with PRRSV-2.

Immunization with PRRSV-1 MLV vaccine reduced number of viremic pigs and significantly reduced PRRSV viremia, as compared to challenge control pigs (Fig. 5). Decreased number of viremic pigs was seen at 7 and 14 dpi, while significant reduction of viremic PRRSV can be detected as early as 7 dpi and persisted until 21 dpi. Injections of quil A further decreased number of viremic pigs at 7 and 14 dpi and significantly further reduced viremic PRRSV at 7 dpi, and reduced but not significantly at 14 and 21 dpi, as compared

572 with vaccination with PRRSV-1 MLV vaccine alone (Fig. 5). Significantly reduced viremic
573 PRRSV copies at 7 dpi, and reduced viremic PRRSV at 14 and 21 dpi in quil A-injected pigs
574 correlated well with significantly increased gene expressions of IFNα, IFNγ, IL-2, and TNFα,
575 and significantly decreased gene expression of TGFβ on those days. These findings suggest
576 that appropriate alteration of mRNA expressions of these immune parameters may contribute
577 to reducing PRRSV-2 viremia. The inhibitory roles of IFNα, IFNγ, and TNFα against
578 PRRSV replication have been reported (Albina et al., 1998; Bautista and Molitor, 1999;
579 Lopez-Fuertes et al., 2000; Rowland et al., 2001). Increased expressions of IFNα, IFNγ, and
580 TNFα also have been shown to correlate well with clinical and pathological protection in
581 homologous and heterologous PRRSV challenge studies (Lowe et al., 2005;
582 Charerntantanakul et al., 2006a; Zuckermann et al., 2007; Lunney et al., 2010b). In contrast
583 to viremia, benefits of quil A injections were not detected in reducing fever days and clinical
584 scores, and increasing ADWG, as compared to pigs vaccinated with PRRSV-1 MLV vaccine
585 alone. Nonetheless, pigs vaccinated with PRRSV-1 MLV either with or without quil A
586 showed significantly less fever days and clinical scores, and significantly higher ADWG than
587 challenge control pigs. Benefits of PRRSV-1 MLV vaccine on improving these parameters
588 against PRRSV-2 challenge have been reported (Roca et al., 2012; Ko et al., 2016).
589 In conclusion, injections of quil A significantly enhanced mRNA expressions of type
590 I IFN-regulated genes, type I and II IFNs, and inflammatory cytokines, but did not enhance
591 antibody response to PRRSV-1 MLV vaccine and PRRSV-2 challenge. Increased
592 expressions of these immune genes may potentially contribute to reducing viremia upon
593 PRRSV-2 challenge. Our findings suggest that effective cross protection may be achieved by
594 robust expression of type I IFN-regulated genes, type I and II IFNs, and pro-inflammatory
595 cytokines. Our findings also suggest that quil A has a potential to serve as immunostimulator
596 for enhancing CMI defense to PRRSV-1 MLV vaccine against PRRSV-2 challenge.

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Table 1 Summary of experimental schedule

Group	N	Treatment	Vaccine	Quil A	Challenge	Antibody detection/ Immune gene expression	Viremia	Rectal temperature/ Clinical score	Body weight
1	6	MLV	0 dpv	-	28 dpv	0, 7, 14, 21, 28, 35, 42, 49 dpv	0, 28, 35, 42, 49 dpv	28-49 dpv	0, 28, 49 dpv
2	6	MLV+Quil A	0 dpv	-1, 0, 1 dpv	28 dpv	0, 7, 14, 21, 28, 35, 42, 49 dpv	0, 28, 35, 42, 49 dpv	28-49 dpv	0, 28, 49 dpv
3	6	Vaccine solvent	0 dpv	-	28 dpv	0, 7, 14, 21, 28, 35, 42, 49 dpv	0, 28, 35, 42, 49 dpv	28-49 dpv	0, 28, 49 dpv
4	6	None	-	-	-	0, 7, 14, 21, 28, 35, 42, 49 dpv	0, 28, 35, 42, 49 dpv	28-49 dpv	0, 28, 49 dpv

dpv - Day post vaccination

Table 2 Criteria for evaluation of clinical signs following PRRSV-2 challenge

Parameters	Score			
	0	1	2	3
Breathing	None	Dyspnea	Dyspnea+ tachypnea	Dyspnea+ tachypnea+cyanosis
Behavior	Active	Less active but responding to stimulation	Less active even when stimulated	Inactive
Appetite	Normal	Reduced	No	-
Other (conjunctivitis, coughing, sneezing, vomiting, diarrhea)	None	One sign presented	Two signs presented	Three or more signs presented