

Effects of Syndyphalin-33 on Immune Function During a *Salmonella* Challenge in Recently Weaned Pigs

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Abstract: The objective of this experiment was to characterize the effect of the synthetic opioid Syndyphalin-33 (SD-33) on immune cell populations with and without a concurrent inoculation with a common enteric pathogen, *Salmonella enterica* Typhimurium (SALM) in recently weaned pigs. On day 0, pigs (8 barrows and 6 gilts, 24±1 days of age, 8.43±0.82 kg) were weaned and fitted with jugular catheters. The following day, pigs were administered either SD-33 (0.5 µmol kg⁻¹, given i.m.) or saline (VEH; 0.5 mL, given i.m.) and SALM (oral gavage of 5×10⁹ CFU) or sterile broth (CON; 3 mL oral gavage) in a factorial arrangement with 4 treatment groups: VEH + CON (n = 4), SD-33 + CON (n = 3), VEH + SALM (n = 3) and SD-33 + SALM (n = 4). There were no differences in Feed Intake (FI) or Body Weight (BW) among the SALM treated animals over time (p>0.05). Cumulatively, FI among the SD-33 + CON pigs was greater (p<0.05) compared to the SD-33 + SALM pigs. White Blood Cell (WBC) populations increased (p<0.05) over the 4 days postinjection period. On day 2 postchallenge, circulating neutrophils and lymphocytes were lower (p<0.05) in VEH + SALM but not in SD-33 + SALM pigs relative to VEH + CON and SD-33 + CON pigs, demonstrating the ability of SD-33 to abrogate the affect of *Salmonella*. Also, on day 2 postchallenge, circulating monocyte populations were greater (p<0.05) in pigs receiving SD-33 relative to controls regardless of SALM treatment. The results of this preliminary study suggest that the opioid SD-33 may modulate the immune axis in recently weaned pigs.

Key words: Immune, opioid, pig, *Salmonella*, stress, weaning

INTRODUCTION

The process of weaning pigs involves many stressors and the proliferation and introduction of a pathogen occurs more readily in new arrivals at the nursery facility (Kahn and Line, 2005). Syndyphalin-33 (Tyr-D-Met (O)-Gly-N-methylphenethyl-amide; SD-33) is a synthetic enkephalin with prolonged analgesic activity (Kiso *et al.*, 1981) and has the ability to increase Feed Intake (FI) in adult wethers (Obese *et al.*, 2007). The effect on FI was observed in wethers 48 h after intravenous administration of SD-33, but was not observed in wethers subjected to both SD-33 and a lipopolysaccharide challenge. Recently, we have observed an increase in cumulative feed intake starting at 9 days postweaning in healthy pigs given a single intramuscular injection of SD-33 at weaning (Kojima *et al.*, 2009).

Inoculation of pigs with the bacteria *Salmonella enterica* simulates a common immune challenge pigs frequently encounter during weaning (Baliji *et al.*, 2000). The Typical Lipopolysaccharide (LPS) challenge initiates a well-characterized inflammatory response and fever (Johnson and von Borell, 1994; Parrott and Vellucci, 1998),

but the response occurs quickly and is short-lived (Parrott *et al.*, 1995; Parrott and Vellucci, 1998). The response to a *Salmonella enterica* challenge, however is gradual in onset and sustained for up to 4 days (Wilcock and Schwartz, 1992). Such enteric diseases rank the highest among diseases frequently observed in weaned pigs (Wells *et al.*, 2009).

As an opioid agonist capable of stimulating appetite and inducing analgesia, SD-33 may offer some protection during the weaning process by increasing the overall health and well-being of pigs during this critical period. Recent reports suggest that opioids may modulate immune function through altering chemotaxis and immune cell function. Therefore, the objective of this experiment was to characterize the effect of SD-33 on immune cell populations with and without a concurrent inoculation with a common enteric pathogen, *Salmonella enterica* Typhimurium.

MATERIALS AND METHODS

Animals and diets: All animal procedures were reviewed and approved by the University of Tennessee animal care

and use committee. Crossbred pigs (Landrace x Duroc x Hampshire) were farrowed in standard farrowing pens and processed according to usual University of Tennessee Experiment Station practice at 4-7 days of age. Procedures for processing included clipping needle teeth, docking tails, iron injection, tagging ears and castration of males. The pigs were kept in farrowing pens with their dams until weaning, with creep feed (Diet 554PE, Tennessee Farmers Cooperative, LaVergne, TN) available 10 days after birth.

Experimental design: Fourteen pigs (8 barrows and 6 gilts, 24±1 days of age, 8.43±0.82 kg) were removed from their sows, weighed and fitted nonsurgically with an indwelling jugular angiocatheter as described previously (Carroll *et al.*, 1999). Briefly, an angiocatheter was inserted into the jugular vein while the pigs were immobilized with isoflurane for approximately, 10 min. Immediately after catheter placement, pigs were placed in individual pens (1.3 m²) with nursery feed and water provided *ad libitum*.

Approximately, 24 h following cannulation, pigs were weighed and allocated by gender and weight to 4 treatment groups in a 2×2 factorial design: SD-33 + SALM (n = 4), VEH + SALM (n = 3), SD-33 + CON (n = 3) and VEH + CON (n = 4). Pigs in the SD-33 treatment groups received 0.5 µmol kg⁻¹ SD-33 (Bachem, Torrance, CA) in saline by a single i.m. injection of 0.5 mL or less and pigs in the VEH groups received a similar injection of 0.5 mL saline. Pigs in the SALM groups were inoculated with an oral dose of 5×10⁹ CFU of live *Salmonella enterica* serovar Typhimurium culture in a volume of 3 mL (Strain 798-4232, National Animal Disease Control, USDA, Ames, IA) as described by Ebner and Mathew (2000) and Mathew *et al.* (2005). Pigs in CON groups received a 3 mL oral gavage of sterile broth.

Serial blood sampling was performed such that 4 mL of blood was collected every 30 min for 6 h. Immediately after collection of the initial blood sample, pigs were administered their respective treatments. Body Weight (BW) and FI were measured and blood (1 mL) was collected daily at 0 (immediately prior to treatment), 24, 48, 72 and 96 h postinjection. Only the data from the daily blood samples (white blood cell differentials) are described herein; the data resulting from the 6 h serial blood collection is not included.

Blood collection and analysis: Blood (1 mL) was collected in tubes spray-coated with 5.4 mg of K₂ EDTA and immediately shipped on ice to a commercial clinical laboratory (Vet Path Labs, Tulsa, OK) for determining White Blood Cell (WBC) count and differentials. Reported are total WBC concentration (WBC/µL); the concentration of neutrophils, lymphocytes and

monocytes and the percentage of neutrophils, lymphocytes and monocytes relative to total WBC concentration.

Statistical analysis: Variables were analyzed with mixed model ANOVA, using a model for a factorial design. Pig was the experimental unit. For all variables, the model included treatment (±SD-33) and challenge (±SALM) as main effects, along with the interaction between main effects, with repeated measures when appropriate. Initial body weight (weaning weight prior to cannulation) was included as a covariate for all analyses. Square root transformation was performed as necessary to maintain homogeneity of variance. For variables presented as repeated measures, post-hoc analysis was conducted for each separate time-point (t-test). A significance level of p<0.05 was used for all testing; trends where p<0.10 were also reported. All graphical and textual descriptions of results are reported as raw means and standard errors.

RESULTS AND DISCUSSION

Feed intake and body weight: Daily FI increased (p<0.0001) in each of the 4 days measured in all treatment groups (Fig. 1a). Treatment with SALM resulted in an overall decrease (p<0.05) in FI, particularly on day 3 postchallenge (p<0.05). An interaction occurred such that at 2 days postchallenge, SD-33 + SALM pigs consumed very little feed whereas SD-33 + CON pigs consumed the greatest amount of feed (p<0.05). Cumulative FI over the 4 days trial was decreased (p<0.05) in SALM pigs relative to CON pigs (Fig. 1b). A trend (p = 0.065) was observed for a treatment interaction with the effect of *Salmonella* being evident in SD-33 (0.27±0.09 kg) pigs than in VEH (0.66±0.17 kg) pigs.

Cumulative weight gain was decreased (p<0.05) in SALM pigs relative to CON pigs (Fig. 1c). A trend (p = 0.067) was observed with SD-33 + SALM pigs showing greater gains compared to VEH + SALM pigs (0.05±0.27 versus -0.13±0.19 kg), respectively.

Circulating white blood cell populations: Total WBC numbers increased (p = 0.0001) through day 4 (Fig. 2a). Populations of WBC were increased (p = 0.0001) in SD-33 pigs relative to VEH pigs. On day 2, WBC concentrations were decreased (p<0.05) in VEH + SALM pigs, but not SD-33 + SALM pigs, relative to CON pigs.

Circulating neutrophil concentrations increased (p<0.0001) over time and were greater (p<0.05) in SD-33 pigs relative to VEH pigs (Fig. 2b). Post-hoc analysis revealed that at 24 h postchallenge, VEH + SALM pigs had fewer (p<0.05) circulating neutrophils than did

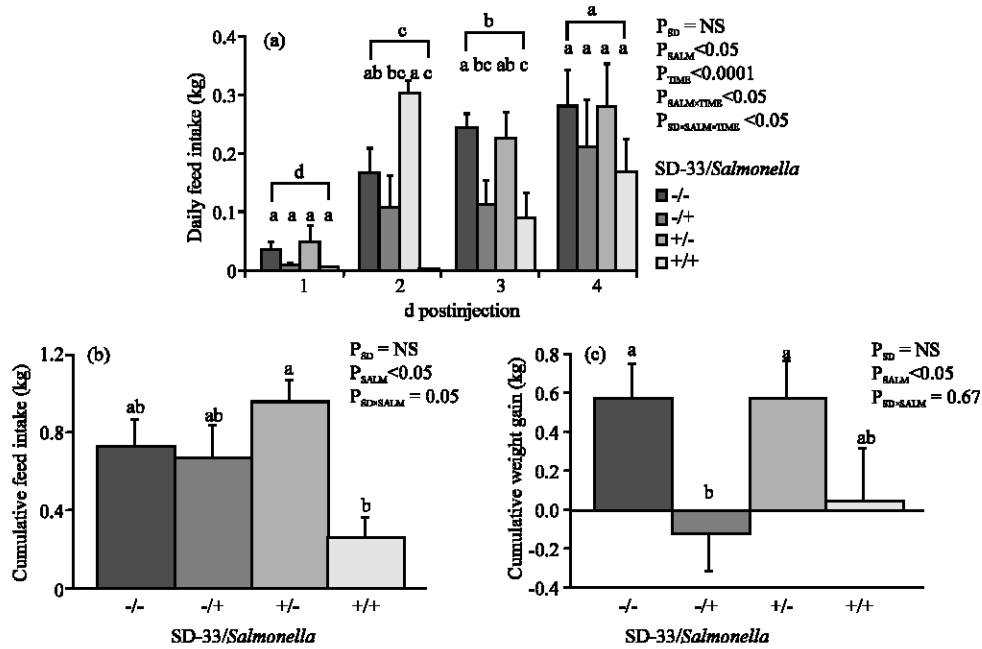


Fig. 1: Daily (a) and cumulative (b) feed intake and cumulative weight gain (c) of pigs injected with SD-33 ($0.05 \mu\text{mol kg}^{-1}$ syndyphalin-33) or saline, concurrent with an oral gavage of *Salmonella enterica* Typhimurium (5×10^9 CFU) or sterile broth. For all graphs, raw means and standard errors are shown. Above brackets, means of time points (across treatments) not sharing same letters differ ($p < 0.05$). Below brackets, means of treatments (within a time) not sharing same letters differ ($p < 0.05$). For cumulative data, means of treatments not sharing same letters differ ($p < 0.05$)

SD-33 + SALM pigs. This pattern was also evident (but was not statistically significant) at 48, 72 and 96 h. Although, treatment did not affect the percentage of WBC that were neutrophils (Fig. 2c), percentages tended ($p = 0.076$) to change over time, suggesting that neutrophil percentages across treatments were elevated at 24 h relative to the pre-challenge state.

Circulating lymphocyte concentrations increased ($p < 0.0001$) over time across treatment groups (Fig. 2d). An interaction was observed with VEH + SALM pigs having lower ($p < 0.05$) numbers of circulating lymphocytes compared to VEH + CON pigs at 48 h postchallenge. At 72 h, no effect of SALM was observed, but SD-33 + CON pigs had greater numbers of circulating lymphocytes than did VEH + CON pigs. The percentage of WBC that were lymphocytes was not affected by treatment with SD-33 or SALM (Fig. 2e). A trend was observed however, with SD-33 + CON pigs having a lower ($p = 0.087$) percentage of WBC that were lymphocytes relative to VEH + CON pigs at 48 h postchallenge.

Circulating monocytes increased ($p < 0.0001$) over time across all treatment groups and in contrast to lymphocytes, were elevated following SD-33 ($p < 0.001$) and SALM ($p < 0.05$) treatments, although the effect of SALM tended ($p = 0.082$) to be influenced by time

(Fig. 2f). It should be noted that circulating monocyte concentrations differed between treatment groups prior to treatment (0 h). Treatment with SD-33 tended ($p = 0.071$) to increase the percentage of WBC that were monocytes. The percentage of monocytes in SALM treated pigs increased ($p < 0.01$) as well (Fig. 2g). The percentage of WBC that was monocytes decreased ($p < 0.005$) at h 24 relative to pre-treatment levels. The percentage of circulating monocytes then gradually increased at 72 and 96 h to become greater ($p = 0.005$) than 0 h levels.

As expected, FI was adversely affected by weaning in all treatment groups as seen 1 day postchallenge (2 days postweaning). Pigs in all groups except SD-33 + SALM recovered by day 2 and all groups were eating by day 3. At 2 days postchallenge, SD-33 + CON pigs ate more than the other treatment groups, consistent with what we have observed previously in healthy pigs given SD-33 immediately prior to weaning (Kojima *et al.*, 2009). Inoculation with *Salmonella* markedly reduced FI, particularly at 3 days postchallenge. This reduction in FI is in agreement with previous studies which have shown decreased FI for up to 120 h postchallenge, depending somewhat on the dose given and the age of the pigs (Balaji *et al.*, 2000; Jenkins *et al.*, 2004). Simultaneous treatment with a

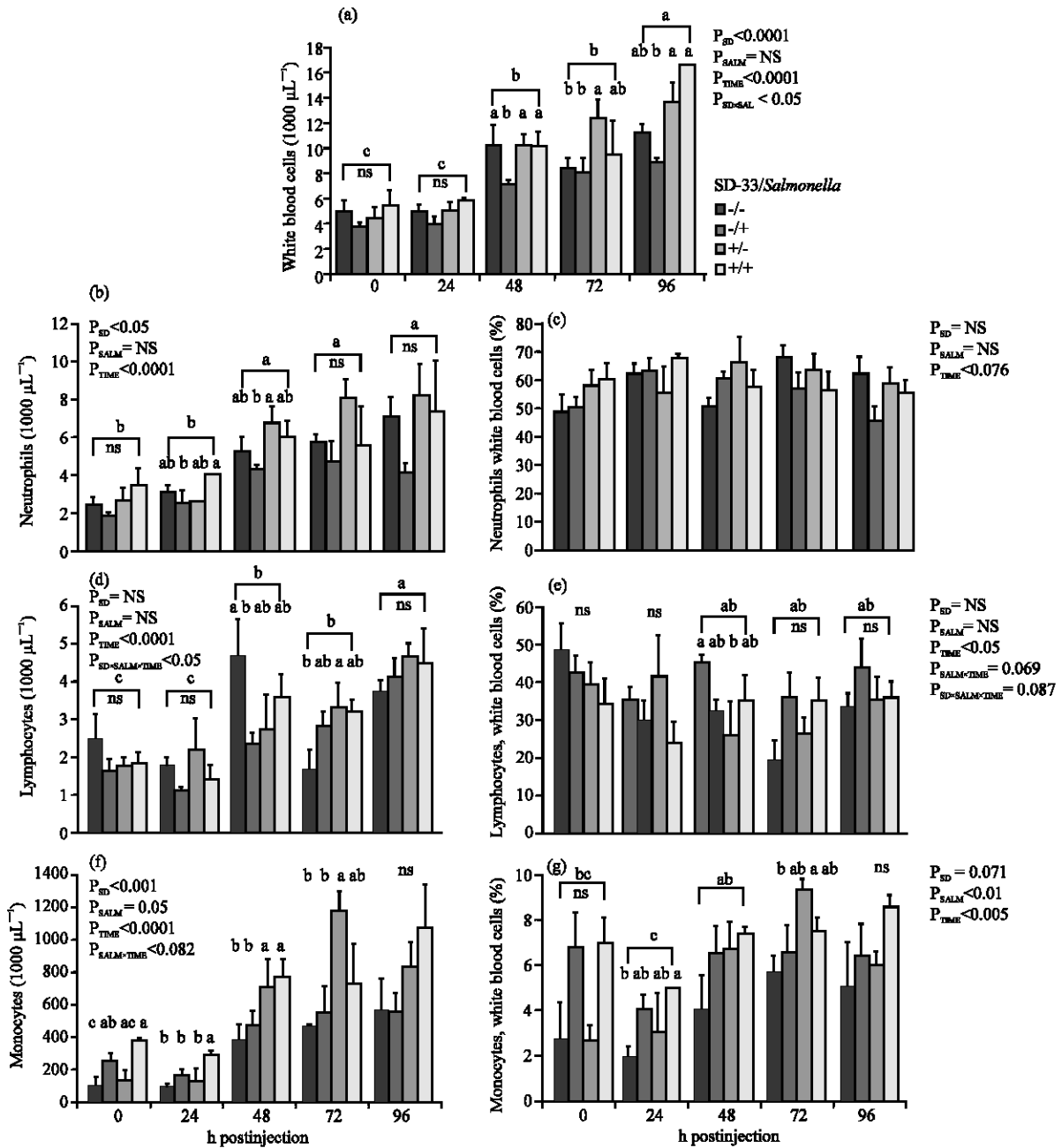


Fig. 2: Total white blood cell count (a), absolute counts (b, d, f) and percentage (c, e, g) of neutrophils, lymphocytes and monocytes (respectively) of pigs injected with SD-33 (0.05 $\mu\text{mol kg}^{-1}$ syndyphalin-33) or saline, concurrent with an oral gavage of *Salmonella enterica* Typhimurium (5×10^9 CFU) or sterile broth. Raw means and standard errors are shown. Above brackets, means of time points (across treatments) not sharing same letters differ ($p < 0.05$). Below brackets, means of treatments (within a time) not sharing same letters differ ($p < 0.05$)

single i.m. injection of 0.5 $\mu\text{mol kg}^{-1}$ SD-33 did not increase FI in immune-challenged pigs. Similarly, Obese *et al.* (2007) also found that SD-33 did not counteract the reduction in FI due to an LPS challenge in adult wethers. The effects of SD-33 may not be potent

enough to overcome the decreased appetite seen in an immune challenge; timing of injection or strength of dose may need to be altered.

Pigs challenged with *Salmonella* grew less over the 4 days trial period regardless of SD-33 treatment. Even

though, the interaction between SD-33 and SALM did not reach significance, VEH + SALM pigs lost weight relative to pre-trial BW, whereas the SD-33 + SALM pigs did not. In previous studies, Kojima *et al.* (2009) no effect on BW was observed in healthy, weaned pigs treated with SD-33, in spite of increased FI. As opioids exert their effects on many physiological processes, including appetite and immune function, SD-33 may be altering the partitioning of calories toward some other function than growth.

The primary function of WBC is to prevent and fight infections. In the young pig, its success in dealing with a pathogen may be dependent on the degree to which stress is affecting immune function. In the current study, the pigs were relatively stressed due to weaning and cannulation the day before. Neutrophil, lymphocyte and monocyte numbers increased over time across all treatment groups. We have previously reported increases in total WBC count, neutrophils, lymphocytes and monocytes over time in newly weaned pigs (Kojima *et al.*, 2008, 2009; Cooper *et al.*, 2009). Circulating immune cell populations increased at 1 day postweaning and returned to pre-weaning levels by 7 days postweaning (Kojima *et al.*, 2008; Cooper *et al.*, 2009). It would appear that immune cell populations continue to increase for several days postweaning. This may indicate prolonged inhibition of chemotaxis by cortisol and a progressive accumulation of cell numbers.

Treatment with SD-33 increased circulating monocyte concentrations, most noticeably at 2 days postchallenge. We have previously shown that SD-33 tended to selectively increase the number of circulating monocytes in healthy pigs given SD-33 immediately prior to weaning (Kojima *et al.*, 2009), although the response did not reach statistical significance.

Opioids are known to have immuno-modulatory functions and cells of the immune system (neutrophils, monocytes and lymphocytes) express opioid receptors, as reviewed by Finley *et al.* (2008). Grimm *et al.* (1998) reported that endogenous met-enkephalin and morphine induced monocyte chemotaxis, but inhibited chemokine-induced chemotaxis of human neutrophils and these responses were blocked by naloxone. Recent evidence (Finley *et al.*, 2008) suggests that activation of κ -opioid receptors may induce an anti-inflammatory response, while activation of μ -opioid receptors induces a pro-inflammatory response. Naloxone, although primarily known as a μ -opioid receptor agonist, is also an antagonist to κ - and δ -opioid receptors. While most of the published data suggests that SD-33 is a μ -opioid, the possibility that this molecule may also bind one of the other opioid receptor subtypes must be considered.

An oral challenge of *Salmonella* would typically recruit circulating WBC to the gut, thereby decreasing circulating concentrations of neutrophils and lymphocytes. While this decrease in circulating cell numbers was observed at 48 h postchallenge in VEH + SALM pigs, co-treatment with SD-33 appears to have blocked this effect. The exact mechanism for this is as yet undetermined, but may involve the inhibition of chemotaxis of neutrophils (and other WBC cell types) as described above.

In an intriguing examination of the effects of opioids, Stefano and Kream (2008) stated that opioids originated as immune signaling molecules for microbial invasions. Proenkephalins contain an antibacterial peptide called enkelytin (Goumon *et al.*, 1996) that is co-secreted with other immune signaling molecules to instantly attack pathogens while allowing time for immune recruitment (Stefano *et al.*, 1998). Syndyphalin-33 was originally designed as a synthetic met-enkephalin (Kiso *et al.*, 1981) and the results indicate that SD-33 has many effects, including those modulating immune function.

CONCLUSION

We have demonstrated that although, co-treatment with SD-33 during an immune challenge did not result in increased feed intake, SD-33 exerted effects relating to circulating populations of immune cells. Further research is needed to determine if these effects are beneficial or harmful to the immune-challenged newly weaned pig.

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