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Full Length Article

T Regulatory and T Helper 17 Populations with Transcription Factors in Pig Tissues in Response to Chronic Heat Stress

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Abstract

Both Treg cells (CD4+ CD25+ FOXP3+) and Th17 cells are pivotal in sustaining the immune balance in most experimental animal models. But this is not clear in heat stressed pigs. The present study was aimed at determining if T regulatory and T helper 17 populations along with transcription factors in animals such as pigs are affected by persistent heat stress. Changes in $CD4^+$ $CD25^+$ $FOXP3^+$ Treg cells of the peripheral blood mononuclear cells (PBMC), thymus, spleen, mesenteric lymph nodes, and colon lamina propria of heat-stressed pigs over 9 days were studied. Transcription and the levels of Interleukin 17 (IL-17) protein along with specific transcription factors including *retinoic acid receptor related orphan receptor gamma t (RORγT)* and *forkhead box P3 (FOXP3)* were also measured. Treg cell frequency increased in PBMC and spleen at days 1, 3 and 6, followed by a significant decrease on day 9 ($p < 0.05$). However, the Treg cell frequency in the thymus was seen to decrease at every measurement. ($p < 0.05$). A marked increase in plasma, spleen, and colon lamina propria IL-17 concentration was evident during early stages of the heat stress experiment. Transcription of *RORγT* and *IL-17* but not *FOXP3* was markedly upregulated in all the tissues examined. We concluded that heat stress affected the T regulatory and T helper 17 populations along with transcription factors in pig tissues, and that this is dependent on the duration of the thermal stress during chronic heat stress.

Keywords: T lymphocyte; Imbalance; Pig; Heat stress

Introduction

Heat stress represents a major cause of economic loss in the swine industry because it can cause immunosuppression which can lead to disease outbreaks (Morrow-Tesch *et al.*, 1994; St-Pierre *et al.*, 2003), and significant functional and structural damage to the gastrointestinal tract, such as shorter intestinal villi, shallower crypts, and severe diarrhoea syndrome (Sangild *et al.*, 2003; Liu *et al.*, 2009; Yu *et al.*, 2010). Furthermore, stressed animals are more prone to diseases including some of the emerging pig diseases including some which are zoonotic, and this can be a threat to public health (Salak-Johnson and McGlone, 2007).

Different types of immune cells, like the B cells, $CD8^+$ T cells, basophils, macrophages, mast cells, neutrophils, and eosinophils are involved in adaptive immune responses. The role of $CD4^+$ T helper (Th) cells is to recruit and activate these cells (Zhu and Paul, 2010). Th cells have been primarily distributed into four major genetic heredities, namely Th1, Th2, Th17 and T regulatory (Treg) based on their different functions and cytokine secretion patterns (Zhu and Paul, 2010). The lineages are derived from naïve CD4 T cells regulated by specific transcription factors including the T-box transcription factor (T-bet), retinoid receptor related orphan receptor gamma t (RORγT), forkhead box P3 (FOXP3) and GATA-binding protein 3 (GATA3), (Rissoan *et al.*, 1999). Which of these transcription factors are expressed is dependent on which cytokines are present and where the naïve CD4 T cells are located (Dong, 2008; Wing and Sakaguchi, 2009; Nurieva and Chung, 2010). T-bet and GATA3 get expressed due to facilitation by IL-12 and IL-4, respectively, and this causes naïve CD4 T cells to differentiate into Th1 and Th2 cells (Zhu and Paul, 2010). This differentiation is enhanced by adaptive concentrations of transforming growth factor-β (TGF- β) and IL-6/retinoic acid respectively (Ivanov *et al.*, 2009).

A major role of Treg cells ($CD4^+$ $CD25^+$ $FOXP3^+$) *in vivo* is to in maintain the immune balance by suppression of

$CD4^+$ $CD25^+$ T-cell functions (Neighbors *et al.*, 2006). Deficiency of Treg cells can lead to development of thyroiditis (Flynn *et al.*, 2007), diabetes (Salomon *et al.*, 2000), bowel disease (Maloy and Powrie, 2001), and systemic lupus erythematosus (Miyara *et al.*, 2005). Interleukins IL-17A and IL-17F, which are required for induction of maximal autoimmune inflammation in experimental animal models of allergic encephalomyelitis (Komiyama *et al.*, 2006), collagen-induced arthritis (Nakae *et al.*, 2003), and colitis (Lim *et al.*, 2008) are produced by Th17 cells. Favre *et al.* (2009) found that in pigtailed macaques infected with Simian immunodeficiency virus there was a marked and selective reduction in Th17 cells and also the Th17/Treg balance in the blood, lymphoid organs, and mucosal tissue was skewed (Favre *et al.*, 2009). Similar results were reported in patients with persistent hepatitis B virus infection (Zhang *et al.*, 2010) and atherosclerosis (Cheng *et al.*, 2008).

Porcine $CD4^+$ $CD25^+$ $FOXP3^+$ T cells affect Th cells, cytotoxic T lymphocytes, and T-cell receptor γδ T cells, and share a role in suppressing immunity in mice and humans (Kaser *et al.*, 2008a; Kaser *et al.*, 2008b; Kaser *et al.*, 2011). Heat stress induces a loss of balance between CD4 and CD8 T cells (Ju *et al.*, 2011a), and affects the inflammatory cytokines IFN-γ, IL-2 and IL-8 secretions (Ju *et al.*, 2014) and upstream molecules such as Toll-like receptors (Ju *et al.*, 2011b; Ju *et al.*, 2013). But it is unclear whether porcine T regulatory and T helper 17 populations with transcription factors are disturbed by heat stress, a key stress factor in South China pig production. In this study, the Treg cells/IL-17 expression cells ratio and changes in transcription factors including FOXP3 and RORγT were examined in pigs over nine consecutive days of heat stress.

Materials and Methods

Animals and Housing

Thirty crossbreed pigs (local breed × Landrace, castrated at 10–14 days, aged 3–4 months) were purchased from a commercial farm. Prior to the experiments, pigs were allowed to acclimatize for approximately 3 weeks. Three littermates were housed together in nursery pens with floor space of 1.5 m^2 for each pig. The pigs had ad libitum access to water and were fed thrice daily. Their diet was developed as per the recommended nutrient allowances for the breeds (http://yzxy.nxin.com/html/20130710/18316.html).

Experimental Design

A total of 30 pigs, 3 in each pen, were used. Fifteen pigs (in 5 pens) were allocated to the control group (CON). Air temperature in the housing was 28±3°C and the relative humidity ~90%. The rema 15 (also in 5 pens) underwent heat stress treatment (HEAT) and were fed in an artificial climate chamber at 35 ± 1°C and ~ 90% relative humidity, for nine consecutive days.

Ethics Approval and Consent to Participate

All animal experiments were conducted under the Centre for Disease Control and Prevention's Institutional Animal Care and Use Committee guidelines, in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Guangdong Ocean University (Permit No.: 201-1231). All surgeries were performed under sodium pentobarbital anaesthesia, and every effort was made to minimize suffering.

Blood and Tissue Sample Collection

Blood and tissue samples were collected from 3 pigs each from the CON and HEAT pigs on days 0, 1, 3, 6, and 9. Blood collection was done by holding the pigs in a supine position. Anterior vena cava puncture was used to collect 10 mL of blood into vacutainers containing 143 USP units of heparin. Blood was used for PBMC isolation and plasma collection. Three pigs each from the CON and HEAT groups were euthanized at each sampling day, and the thymus, spleen, mesenteric lymph nodes, and colon lamina propria were collected. Each tissue type was quickly cut into 3 portions, one part was frozen in liquid nitrogen for mRNA extraction, the second was minced and digested using 1 mL collagenase (0.6 mg/mL; Sigma-Aldrich, Beijing, China) and 3 mL pancreatin (0.25%; Invitrogen, Shanghai, China) for 30 min, filtered with a 40 μM cell strainer and used for subsequent multicolour staining. The remaining tissue was homogenized for IL-17 measurement. PBMC and tissue lymphocytes were isolated by density gradient centrifugation using LTS-1077 (density = 1.077 g/mL; TBD, Tianjin Haoyang Biological Manufacturing Co., Tianjin, China) as per manufacturer's protocols. Isolated cells were washed twice in Hank's medium, counted, and resuspended in the culture medium (RPMI 1640, Gibco, Carlsbad, CA, USA) at 7×10^6/mL for subsequent analyses.

Total RNA Extraction and Complementary DNA Synthesis

The extraction of total RNA and synthesis of complementary DNA (cDNA) were performed according to the manufacturer's instructions. RNA was extracted from nitrogen-frozen tissues using commercially available RNAiso Plus kits (TaKaRa Biotechnology, Dalian, China). The total RNA yield (10–20 μg) was resuspended in diethylpyrocarbonate-treated distilled water and stored at −70°C for future use. For cDNA synthesis, total RNA was reverse-transcribed with a PrimeScript RT Reagent Kit (TaKaRa). The reaction mixture (10 μL) contained 2 μL RNA (100 ng), 2 μL PrimeScript Buffer, 0.5 μL PrimeScript RT Enzyme Mix I, and 0.5 μL random primers (50 μM).

Table 1: Primer sequences and amplicon characteristics

Gene	Sequence	Reference	Amplicon	
			Length(bp)	Tm (°C)
ROR-γT	5′-CCTATCTATGACCTCACCTC-3′ 5′-GCTCCTCCATCGTGTATT-3′	XM_003480537	157	53.6
FOXP3	5′-CATTCGCCACAACTTGAG-3′ 5′-CCCTGTCCATCCTTCTTT-3′	AY669812.2	177	53.7
IL-17	5′-GCCCTCAGATTACTCCAA-3′ 5′-CCACTGTCACCATCACTT-3′	NM_001005729.1	242	53.6
TBP	5′-GTAGTTATGAGCCAGAGT-3′ 5′-CCTTTAGGATAGGGTAG-3′	DQ845178	159	48.0
B2M	5′-TATCTGGGTTCCATCCG-3′ 5′-AACTATCTTGGGCTTATCG-3′	NM_213978	197	51.0

bp base pairs, *Tm* melting temperature

qRT-PCR

FOXP3, RORγT, and IL-17 expressions were evaluated by real time qPCR (RT-qPCR), with TBP (TATA-box-binding protein) and B2M (β_2-microglobulin) as reference genes. All primers were synthesized by GeneCore (Shanghai GeneCore BioTechnologies, Shanghai, China) (Table 1). The iCycler IQ Real-Time PCR Detection System was used to perform RT-qPCR (Bio-Rad, Hercules, CA, USA). Each 25 μL reaction contained 12.5 μL 2× SYBR Premix Ex *Taq* (TaKaRa), 0.5 μL forward primer (20 μmol/L), 0.5 μL reverse primer, 2 μL RT product, and 9.5 μL distilled water. The amplification program consisted of 40 cycles of 95°C for 4 s, annealing at 55°C for 15 s, and extension at 72°C for 10 s, ended with a melting program ranging from 60°C to 95°C with a heating rate of 0.5°C per 5 secs. The PCR efficiency was determined and was >97% for each primer set, with the use of six serial dilutions of cloned products as templates. Relative gene expression value was calculated using the threshold cycle (CT) method. This was applied to each of the genes by calculating the expression $2^{-\Delta\Delta CT}$, where ΔΔCT is the sum of: $[CT_{gene} - CT_{reference}]_{(Heat\text{-}stressed)} - [CT_{gene} - CT_{reference}]_{(Control)}$.

Flow Cytometry Analysis

Treg cells were stained using mouse anti-porcine CD4-phycoerythrin antibody (clone 74-12-4, IgG2b; Southern Biotech, Birmingham, AL, USA) and mouse anti-porcine CD25 antibody (clone K231.3B2, IgG1; AbD Serotec, Raleigh, NC, USA) and incubated for 30 min. All appropriate isotype controls were obtained from eBioscience (San Diego, CA, USA). Then, goat anti-mouse immunoglobulin G-allophycocyanin (IgG1; SouthernBiotech) was added and incubated for 30 min and stained with eBioscience FOXP3 Staining Buffer (San Diego, CA) as per the manufacturer's protocols. The cells were washed and analysed on a 4-laser BD LSR-II flow cytometer using a high-throughput-system plate reader (BD Biosciences, Franklin Lakes, NJ, USA), and FlowJo software v6-8 (TreeStar, Ashland, OR, USA) was used to analyse the data.

Determination of IL-17 by ELISA (Enzyme-Linked Immunosorbent Assay)

Plasma and tissue homogenates of the thymus, spleen, mesenteric lymph nodes, and colon lamina propria were stored at −80°C prior to analyses. IL-17 was measured by enzyme-linked immunosorbent assay (ELISA) (Laville *et al.,* 2007) kits for porcine IL-17 (Abcam, Sha Tin, Hong Kong) following the manufacturer's instructions. Plates were read at 405 nm using a microplate reader (BioTek, Winooski, VT, USA).

Statistical Analyses

The SAS version 8 (SAS Institute, Cary, NC, USA) was used to perform statistical analyses. Data was analysed by one-way analysis of variance (ANOVA). RT-qPCR and cytokine concentration results are expressed as mean ± SEM. Differences between the CON and HEAT pigs in FOXP3 frequency, IL-17 concentration, Treg/Th17 ratio, *FOXP3*/*RORγT* mRNA and *FOXP3*/*IL-17* mRNA ratios were analyzed using the Student *t*-test for independent pairs at each sampling point. A *p*-value of < 0.05 meant it was of statistical significance.

Results

Effect of Heat Stress on PBMC

The dynamics of Treg cells/Th17 in PBMCs of the heat-stressed (HEAT) pigs are shown in Fig. 1. Treg cell frequency in the PBMC increased on day 1, 3 and 6 of heat stress ($p < 0.05$) but decreased substantially on day 9 ($p < 0.05$). Since it is difficult to purchase a commercial antibody against porcine IL-17, the IL-17 concentration in the plasma/tissue homogenates was measured to indirectly determine the Th17 cell number, because IL-17 is primarily produced by Th17 cells (Onishi and Gaffen, 2010). Plasma IL-17 was markedly upregulated on day 1 and 3 of heat stress ($p < 0.05$). The ratio of the Treg cells to IL-17 also increased markedly on day 1 of heat stress in PBMC ($p < 0.01$) contributing to an imbalance between Treg and IL-17 populations.

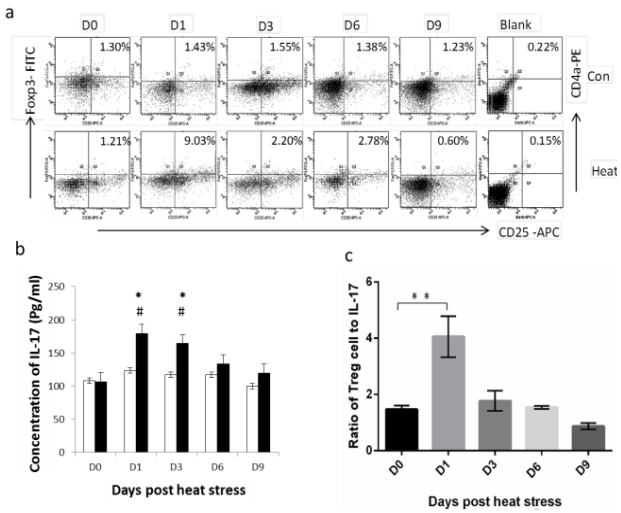

Fig. 1: Differential dynamic of Treg/Th17 cells in PBMC collected from heat-stressed pigs. Multi-parameter flow cytometric analysis of the frequency of CD25+ CD4+ Foxp3+ T cells amongst total CD4+ T cells in PBMC collected at the indicated times following exposure to heat stress **(a)**. IL-17 concentration in porcine plasma determined by ELISA **(b)** and the ratio of Treg cell frequency in PBMC to IL-17 concentration in plasma **(c)**, served as an indirect measure of the balance between Treg and Th17 cells. Data are presented as mean ± SEM. The heat-stress data were normalized to that of the controls. P-values were obtained on a per-group basis (#) using the Student t-test (when comparing heat stress and control at a given time) or over time (*) by ANOVA (linear scale) (comparing each sampling point to the day 0 value in heat-stressed pigs). * or # indicate that $p \leq 0.05$. PE, Phycoerythrin; FITC, fluorescein isothiocyanate; APC, allophycocyanin

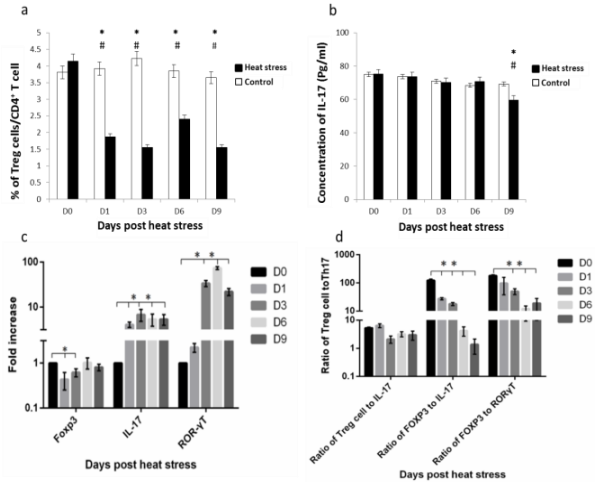

Fig. 2: Differential dynamic of Treg/Th17 cells in the thymus of heat-stressed pigs. Multi-parameter flow cytometric analysis of the frequency CD25+ CD4+ FOXP3+ T cells amongst total CD4+ T cells in thymus obtained at the indicated times following exposure to heat stress **(a)**. IL-17 concentration in the tissue fluid of porcine thymus was determined by ELISA **(b)**. The expression of FOXP3 mRNA, IL-17 mRNA and RORγT mRNA were detected by RT-qPCR **(c)**. The ratio of Treg cell frequency to IL-17 concentration in porcine plasma **(d)**, served as an indirect measure of the balance between Treg and Th17 cells. The ratios of FOXP3 mRNA to IL-17 mRNA and of FOXP3 mRNA to RORγT mRNA in the thymus were also analysed (d). Data are presented as mean ± SEM. The heat-stress data were normalized to that of the controls. P-values were obtained on a per-group basis (#) using the Student t-test (when comparing heat stress and control at a given time) or over time (*) by ANOVA (linear scale) (comparing each sampling point separately to the day 0 value in heat-stressed pigs). * or # indicate that $p \leq 0.05$

Effect of Heat Stress on the Thymus

The profiles of Treg cell/Th17 in the thymus are shown in Fig. 2. Treg cells in the thymus decreased on days 3, 6 and 9 ($p < 0.05$) (Fig. 2a). However, no significant differences in IL-17 concentration between the CON and HEAT pigs were observed until day 9 when it decreased significantly ($p < 0.05$) (Fig. 2b). The thymus *IL-17* and *RORγT* were significantly increased on all sampling days (1, 3, 6 and 9) with IL-17 upregulated approximately 7-fold on day 3 and RORγT 74-fold on day 6 in HEAT pigs ($p < 0.05$) (Fig. 2c). But *FOXP3* mRNA expression decreased ~2-fold on days 1 and 3, and ratios of *FOXP3/RORγT* mRNA and *FOXP3/IL-17* mRNA decreased significantly ($p < 0.01$) at different times over the 9 days (Fig. 2d).

Effect of Heat Stress on the Spleen

Heat stress increased both Treg cell number (Fig. 3a) and IL-17 (Fig. 3b) in the spleen on days 1 and 3 ($p < 0.05$). Although *FOXP3* mRNA expression decreased approximately 5-fold in HEAT pigs on day 9 ($p < 0.05$), the *IL-17* and *RORγT* increased significantly on all days, and were upregulated approximately 14-fold and 10-fold respectively on day 3 (Fig. 3c). The ratios of *FOXP3/RORγT* mRNA and *FOXP3/IL-17* mRNA decreased significantly on different days ($p < 0.01$). A decrease in the ratio of Treg cell number to IL-17 concentration was observed in the spleen on days 6 and day 9 ($p < 0.05$), indicating a disparity between Treg and IL-17 cells in the spleen after prolonged heat stress (Fig. 3d).

Effect of Heat Stress on the Intestinal Tract

In HEAT pigs, Treg cell numbers increased in the colon lamina propria (Fig. 4a) and mesenteric lymph nodes (Fig. 5a) on day 1 but decreased on day 6. IL-17 also increased in the colon lamina propria (Fig. 4b) and mesenteric lymph nodes (Fig. 5b) on day 3 ($p < 0.05$) but decreased on day 9 in the mesenteric lymph nodes (Fig. 5b). There was a decrease in the Treg/IL-17 ratio in the colon lamina propria on days 3, 6 and 9 ($p < 0.05$ (Fig. 4d). The transcription factor *FOXP3* decreased on days 1, 3 and 6 in the colon lamina propria (Fig. 4c), and on days 3, 6, and 9 in the mesenteric lymph nodes (Fig. 5c) ($p < 0.05$). Both *IL-17* and

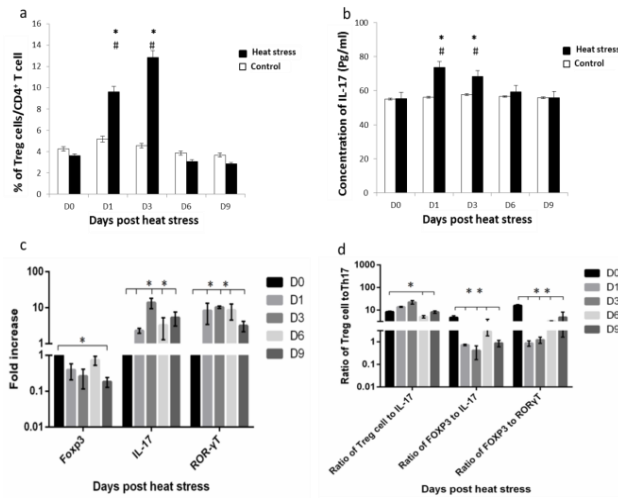

Fig. 3: Differential dynamic of Treg/Th17 cells in the spleen of heat-stressed pigs. Multi-parameter flow cytometric analysis of the frequency CD25+ CD4+ FOXP3+ T cells amongst total CD4+ T cells in porcine spleen obtained at the indicated times following exposure to heat stress **(a)**. IL-17 concentration in tissue fluid of porcine spleen determined by ELISA **(b)**. The expression of FOXP3 mRNA, IL-17 mRNA and RORγT mRNA detected by RT-qPCR **(c)**. The ratio of Treg cell frequency to IL-17 concentration in porcine plasma (d), serving as an indirect measure of the balance between Treg and Th17 cells. The ratios of FOXP3 mRNA to IL-17 mRNA and of FOXP3 mRNA to RORγT mRNA in the thymus were also analysed **(d)**. Data are presented as mean ± SEM. The heat-stress data were normalized to that of the controls. P-values were obtained on a per-group basis (#) using the Student t-test (when comparing heat stress and control at a given time) or over time (*) by ANOVA (linear scale) (comparing each sampling point separately to the day 0 value in heat-stressed pigs). * or # indicate that $p \leq 0.05$

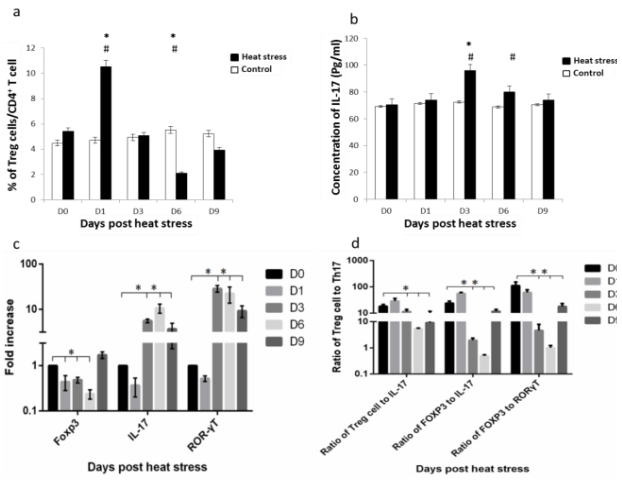

Fig. 4: Differential dynamic of Treg/Th17 cells in the colon lamina propria of heat-stressed pigs. Multi-parameter flow cytometric analysis of the frequency CD25+ CD4+ FOXP3+ T cells amongst total CD4+ T cells in porcine colon lamina propria obtained at the indicated times after heat stress was imposed **(a)**. IL-17 concentration in the tissue fluid of colon lamina propria determined by ELISA **(b)**. The expression of FOXP3 mRNA, IL-17 mRNA and RORγT mRNA were detected by RT-qPCR **(c)**. The ratio of Treg cell frequency to IL-17 concentration in porcine plasma **(d)**, served as an indirect measure of the balance between Treg and Th17 cells. The ratios of FOXP3 mRNA to IL-17 mRNA and of FOXP3 mRNA to RORγT mRNA in the thymus were also analysed (d). Data are presented as mean ± SEM. The heat-stress data were normalized to that of the controls. P-values were obtained on a per-group basis (#) using the Student t-test (when comparing heat stress and control at a given time) or over time (*) by ANOVA (linear scale) (comparing each sampling point separately to the day 0 value in heat-stressed pigs). * or # indicate that $p \leq 0.05$

RORγT transcription increased markedly from day 3 to day 9 ($p < 0.01$) in both these tissue types. With the exception of day 1, the *FOXP3/RORγT* mRNA and *FOXP3/IL-17* mRNA ratios decreased significantly in both tissue types but on different days ($p < 0.01$).

Discussion

Disorders in the secretion of inflammatory cytokines are common in stressed animals. Heat stress cause mature dendritic cells to stimulate naïve T-cells, with resultant ponding Th1 polarization (Hatzfeld-Charbonnier *et al.*, 2007), and upregulation of IL-15 expression *via* activation of the nuclear factor κB (Wang *et al.*, 2010). As has been previously reported by our group, heat stress increases IL-12 expression but downregulates interferon γ and IL-2 in the plasma of Bama miniature pigs (Ju *et al.*, 2014). Moreover, in pigs, IL-2, IL-6, and IL-10 increased to significantly high levels after 1 h of transportation stress, but IL-6 and IL-10 reduced at 4 h (Lv *et al.*, 2011). As suppressive cells, Treg are vital in reducing inflammation as they control the functioning of Th1, Th2 and Th17 cells (Chen *et al.*, 2007). With a few exceptions, *FOXP3* mRNA and Treg cell frequency decreased near the end of the present 9-day experiment. This may partially explain the disordered inflammatory cytokines observed in the heat-stressed pigs (Ju *et al.*, 2014).

In the early stages (on days 1 or 3) of heat stress, Treg cell numbers increased in the PBMCs, spleen, mesenteric lymph nodes, and colon lamina propria, but this was accompanied by a decline in *FOXP3* mRNA expression. This lack of agreement may be due to gene degradation or post-transcriptional modifications. Similar responses have been reported in genomic and proteomic responses in largemouth bass exposed to environmentally relevant concentrations of dieldrin, where 54 genes were upregulated and 220 downregulated but only 90 proteins showed significant changes (Martyniuk *et al.*, 2010). Another study reported only 57 or 14% directional agreement between differentially regulated mRNAs and proteins respectively in the hypothalamus of fish treated with several dopaminergic agonists (Popesku *et al.*, 2010).

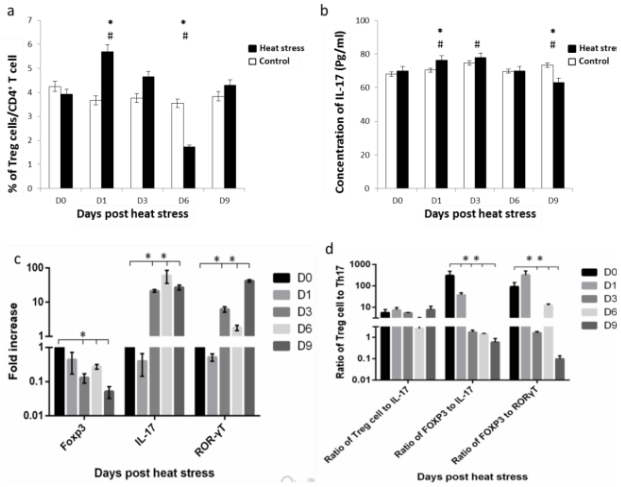

Fig. 5: Differential dynamic of Treg/Th17 cells in mesenteric lymph nodes of heat-stressed pigs. Multi-parameter flow cytometric analysis of the frequency CD25+ CD4+ FOXP3+ T cells amongst total CD4+ T cells in porcine mesenteric lymph nodes obtained at the indicated times following exposure to heat stress **(a)**. IL-17 concentration in the tissue fluid of mesenteric lymph nodes determined by ELISA **(b)**. The expression of FOXP3 mRNA, IL-17 mRNA and RORγT mRNA detected by RT-qPCR **(c)**. The ratio of Treg cell frequency to IL-17 concentration in porcine plasma **(d)**, served as an indirect measure of the balance between Treg and Th17 cells. The ratios of FOXP3 mRNA to IL-17 mRNA and FOXP3 mRNA to RORγT mRNA in the thymus were also analysed (d). Data are presented as the mean ± SEM. The heat-stress data were normalized to that of the controls. P-values were obtained on a per-group basis (#) using the Student t-test (when comparing heat stress and control at a given time) or over time (*) by ANOVA (linear scale) (comparing each sampling point separately to the day 0 value in heat-stressed pigs). * or # indicate that $p \leq 0.05$ for each test, respectively

Th17 cells are widely distributed in non-lymphoid and secondary lymphoid tissues (Ivanov *et al.*, 2006). They can increase host ability to resist bacterial infection in the gastrointestinal tract (Kolls and Linden, 2004) and also support the maintenance of mucosal barrier integrity (Higgins *et al.*, 2006; Khader *et al.*, 2007). The decrease in Th17 cells in those infected by human immunodeficiency virus is accompanied by an induction of many inflammatory cytokines, loss of mucosal integrity and increased microbial translocation (Brenchley *et al.*, 2006; Zheng *et al.*, 2008). Th17 cells have been observed in the PBMC, thymus, and lung lymphocytes of pigs (Kiros *et al.*, 2011). Although other cells, such as natural killer cells (NK cells), γδ T cells, and $CD8^+$ T cells can secrete IL-17, the main source is Th17 cells (Cupedo *et al.*, 2008; Cua and Tato, 2010). Upregulation of IL-17 in HEAT pigs along with increases in the specific transcription factor RORγT was observed in this present study. The ratio of Treg/Th17 decreased in many of the tissues analysed, both at cellular level and gene level, indicating that the balance of Treg/Th17 was affected in HEAT pigs.

Treg cells are widely distributed in the colon lamina propria and occur at a higher frequency than in other tissues (Honda and Littman, 2012). Indigenous *Clostridium* species can induce aggregation of Treg cells in the colon (Feuerer *et al.*, 2010; Atarashi *et al.*, 2011), and there is a special receptor in Treg cells that recognizes gut probiotics (Nagano *et al.*, 2012). Barnes and Powrie hypothesised that Treg cells, through probiotics recruitment and immunosuppression, assist in the maintenance of the balance of the intestinal tract (Barnes and Powrie, 2009). However, changes to the external environment such as heat stress, psychological stress, invasion of pathogenic bacteria, nutritional deficiency, overstrain, diet changes, and intestinal ischemia may lead to a shift of Treg cells to Th17 cells (Alison and Bested, 2013). In this study, the ratio of Treg/Th17 decreased after 9 days of heat stress not only in immune tissues such as the spleen, thymus and mesenteric lymph nodes, but also in the colon lamina propria on several days, mostly during late stages of the experiment. Treg cells are often immunosuppressive (Sakaguchi *et al.*, 2008). Treg and Th17 population turnover is under a tight control *in vivo* by apoptosis and/or the activation-induced cell death pathway (Meyaard *et al.*, 1992), indole-amine 2,3-dioxygenase-mediated tryptophan deprivation (Munn *et al.*, 2002), and galectin-1 signalling (Toscano *et al.*, 2007). A decrease in the ratios of FOXP3/IL-17, *FOXP3/RORγT* and *Treg/Th17* during heat stress was observed in the present study. However, at cellular level, the Treg/Th17 ratio (expressed as Treg/IL-17) decreased only in the colon lamina propria and spleen. We speculate that heat stress can stimulate naïve T cells with resultant Th17 polarization and that both these responses can alter the secretion of inflammatory cytokines in pigs experiencing heat stress.

Conclusion

Chronic heat stress affected the T regulatory and T helper 17 populations along with transcription factors in pig tissues in a time-dependent manner.

Acknowledgements

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