PBMC transcriptomic responses to primary and secondary vaccination differ due to divergent lean growth and antibody titers in a pig model

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Volume 47: 470–478, 2015. First published July 14, 2015; doi:10.1152/physiolgenomics.00015.2015.—The genetic relationship between immune responsiveness and performance is not well understood, but a major topic of the evolution of resource allocation and of relevance in human medicine and livestock breeding, for instance. This study aims to show differences of transcript abundance changes during the time intervals before and after two tetanus toxoid (TT) vaccinations in domestic pigs differing in lean growth (LG) and anti-TT-antibody titers (AB) parameters of performance and immunocompetence. During response to the first vaccination all animals had a general decrease in transcript abundances related to various functional pathways as measured by comparative Affymetrix microarray hybridization and Ingenuity Pathway analyses. Low-AB phenotypes had predominantly decreased immune response transcripts. Combined phenotypes high-AB/high-LG had decreased transcripts related to growth factor signaling pathways. However, during the interval after the booster vaccination, high-LG and high-AB animals responded exclusively with increased immune transcripts, such as B-cell receptor signaling and cellular immune response pathways. In addition, high-LG and low-AB animals had predominantly increased transcripts of several cellular immune response pathways. Conversely, low-LG and high-AB animals had few elevated immune transcripts and decreased transcripts related to only two nonimmune-specific pathways. In response to booster vaccination high-LG phenotypes revealed enriched transcripts related to several different immune response pathways, regardless of AB phenotype. Different from the expected impact of AB titers, divergent AB phenotypes did not reflect considerable differences between immune transcripts. However, highly significant differences observed among divergent LG phenotypes suggest the compatibility of high performance and high vaccine responses.

leukocytes; pathway analysis; immune response; performance; microarray;
erated antigen-naive and therefore provides a suitable model antigen for immune stimulation. Using this model antigen we have previously analyzed the transcript abundance in PBMCs depending on lean growth (LG) performance and anti-TT antibody (AB) titers (36). However, this analysis does not allow any information about temporal dynamics of transcript abundances during the response to two vaccination events. In fact, major changes of transcript abundance due to primary and secondary vaccination can be expected that are obligatory and underlie only subtle biological variation. The suspected interrelation of performance and immune traits can be addressed as the differences of the vaccination-induced changes of transcript abundance between well-defined groups of probands of divergent combinations. Here, to reveal more insights into the time course of transcript abundance changes, we made these comparisons in the intervals from pre- to postvaccination among animals divergent for LG and AB titers (Fig. 1). Our interest was to identify and characterize the time interval that shows clear differences between phenotypes during the switch from innate to adaptive immune response. Based on significantly different transcript abundances, canonical signaling pathways and biofunctions were addressed to four groups of combined phenotypes of divergent performance and humoral immune response.

MATERIALS AND METHODS

Animals and vaccination. Animal care, vaccination, and blood collection were performed according to the guidelines of the German Law of Animal Protection. The experimental protocol was approved by the Animal Care Committee of the Leibniz Institute of Farm Animal Biology and the State Mecklenburg-Vorpommern (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei; LALLF M-V/TS/1221.3-2.1-020/09).

The experimental design is outlined in Fig. 1. Animals, vaccination, and blood sampling were described previously (3). In brief, a total of 160 5 wk old male and female piglets of a German Landrace outbred herd with known pedigree were initially vaccinated (day 0), and a booster vaccination was given at day 14. Vaccination was performed by 1 ml (30 IU) of TT vaccine (Equilis Tetanus-Vaccine, Intervet, Unterschleißheim, Germany) composed of TT and aluminum hydroxide as adjuvant. Of each animal 6 ml of venous blood was taken at the vena cava craniales into EDTA-coated tubes at days 0, 14, and 28. Juvenile animals (average age of 10 wk) were performance-tested during fattening and at termination (final average weight of 110 kg) according to the guidelines of the German performance test (50). Resulting from a principal component analysis of 32 traits related to performance, growth, body composition, and meat properties, the first factor was taken as a basis for the identification of LG performance phenotypes (3). Most significant parameters for LG were lean meat content, loin eye area, meat-to-fat ratio, and (negatively signed) fat area and backfat. Animals from the respective terciles of highest and lowest factor one values were categorized as high (hiLG) and low (loLG) LG performance, respectively.

Blood and plasma samples. Blood sampling was performed between 8:00 and 9:00 AM. EDTA blood samples were collected on ice until PBMC preparation. Plasma samples obtained during PBMC isolation were stored at −80°C until further analysis. Time until freezing at −80°C was about 30 min for plasma and 90 min for PBMC.

Plasma AB titers (all isotypes of anti-TT) at day 14 and day 28 were determined in triplicate using a commercially available ELISA...
PBMC EXPRESSION AT DIVERGENT LEANNESS AND ANTIBODY TITERS

(RE57441; IBL International, Hamburg, Germany) according to manufacturer’s directions. According to LG phenotype rating, animals assigned to the first and third terciles of day 28 AB titers were rated as high (hiAB) and low (loAB) humoral immune response phenotypes, respectively. By combination of LG and AB phenotypes four groups of differentiated phenotypes were set up: hiLG+hiAB, hiLG+loAB, loLG+hiAB, and loLG+loAB. In turn, from each of these groups 10 animals were randomly selected for microarray analyses.

**RNA preparation and microarray hybridization.** PBMC isolation, RNA isolation, and target preparation were performed as described (3). In brief, PBMCs were isolated from 6 ml blood samples by centrifugation on Histopaque density gradients (Sigma-Aldrich, Taukirchen, Germany). Total RNA was isolated using Qiazol reagent (Qiagen, Hilden, Germany), treated with DNase, and column-purified using the RNeasy Mini Kit (Qiagen). Absence of DNA contamination was assessed by PCR amplification of porcine GAPDH (forward primer: 5’-AACGAGGGATGATTCTGG-3’; reverse primer: 5’-ATGCCCCGGTGACACCAAC-3’). Each RNA sample was transcribed to DNA using the Ambion WT Expression Kit (Ambion, Austin, TX). DNA preparations were fragmented and labeled with a WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA). Labeled cDNA was hybridized on Affymetrix snowball arrays (16, 19). Microarray data are MIAME-compliant and were deposited in the Gene Expression Omnibus (GEO) repository (see below).

**Data processing and functional analyses.** The dataset supporting the results of this article is available in the GEO repository of the National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/geo (GEO: GSE47845).

To normalize quality-controlled raw data, the PLIER algorithm was applied using Expression Console 1.1 software (Affymetrix). Expression data were filtered by standard deviation (s ≤ 0.2). Changes of relative transcript abundance were determined by mixed model analysis, which is a well-established approach for dissection of variant data (34, 37). The model includes effects of sire, AB phenotype (hi or lo), LG phenotype (hi or lo), time (day 0, 14, or 28), and interactions between AB, LG, and time. Accordingly, the model \[ y = \mu + s + T + AB + LG + (AB \times LG) + e \] was fitted using the JMP Genomics 5.0 software (SAS Institute, Cary, NC). The model was combined with a repeated statement for the time component to take into account correlations among measurements made on the same subject by specifying a heterogeneous covariance structure. The three-way interaction between LG phenotype, AB phenotype, and time refers to the longitudinal experimental design and represents the 12 experimental units [2 (LG) \times 2 (AB) \times 3 (T)] that were defined. Comparisons of (day 0 vs. day 14) and (day 14 vs. day 28) were made within each of four groups of differentiated phenotypes hiLG+hiAB, hiLG+loAB, loLG+hiAB, and loLG+loAB based on the least square means of the three-way interaction. Subsequently, we compared the eight lists (2 comparisons \times 4 phenotype groups) of transcripts with significant different abundance to discriminate temporal changes of transcription that are common among phenotype groups and that are unique for a particular phenotype group (Fig. 2). Annotation data for the snowball arrays were obtained from the developers (19). Significantly altered transcripts (P < 0.05) were assigned to annotated genes and bioinformatically analyzed by Ingenuity Pathways Analysis (IPA) software (49). Affected canonical pathways and downstream biofunctions were subjected to IPA’s Benjamini-Hochberg multiple testing correction P value procedure (5). Cut-off criteria were set for canonical pathways to false discovery rate (FDR)-corrected P values < 0.05; for predictions of altered biofunctions, cut-off values were FDR-corrected P values < 0.05 and absolute values of activation z-scores > 2.0. We used IPA’s compare data tool to create Venn diagrams and calculate unique and common differentially expressed (DE) genes among phenotypes.

**RESULTS**

Blood samples for comparative microarray analyses and determination of AB titers were taken at day 0 (prevaccination) and 14 (before booster vaccination) and at day 28, i.e., 14 days after second vaccination. (Fig. 1). AB titers at day 14 were generally below the lower assay detection limit (<0.08 IU/ml). AB titers at day 28 ranged from <0.1 IU/ml to >1.0 IU/ml (mean = 0.33 IU/ml; standard deviation = 0.23 IU/ml) and were used as a basis for the identification of divergent phenotypes of high humoral immune response (hiAB, mean = 0.57 IU/ml, standard deviation = 0.13 IU/ml) and low humoral immune response (loAB, mean = 0.23 IU/mL, standard deviation = 0.04 IU/ml; P < 0.001).

Within each phenotype group, hiLG+hiAB, hiLG+loAB, loLG+hiAB, or loLG+loAB comparisons for the intervals from day 0 to day 14 (primary immune response, interval 1) and day 14 to day 28 (secondary immune response, interval 2) revealed changes of abundance from 433 to 1,770 transcripts; the numbers of genes with significantly different transcript abundances from each group and overlapping gene numbers among comparisons are illustrated by Venn diagrams in Fig. 2. The common and phenotype group-specific DE-specific temporal changes of transcript abundances among groups were considered in subsequent IPAs (49).

IPA genes with significantly different transcript abundances (hereafter referred to as DE genes) were assigned to canonical pathways (Fig. 3) and biofunctions, as defined in the Ingenuity Knowledge Base (Supplemental Table S1). Comparisons between day 0 and day 14 identified between 571 and 1,770 DE genes and revealed different proportions between increased and decreased transcript abundances among the four phenotypes at day 14 (Table 1). In response to the first vaccination for all groups significant canonical pathways presented generally decreased transcript abundances (Fig. 3). Accordingly, all affected biofunctions were predicted for decreased activation (Supplemental Table S1).

**Response of high LG phenotypes to first vaccination.** Comparison of hiLG animals between day 0 and day 14 revealed 1,201 and 1,770 DE genes for high and low AB responders, respectively (Table 1). DE genes with decreased transcript abundances from the hiLG+hiAB group were related to canonical pathways of several growth factors (ErbB signaling, GDNF family ligand-receptor interactions, PDGF signaling) and cellular immune response (LPS-stimulated MAPK signaling, IL-6 signaling, IL-3 signaling) (Fig. 3). Additional pathway analyses of DE genes unique (see Venn diagrams in Fig. 2) to hiLG+hiAB in contrast to hiLG+loAB and loLG+hiAB phenotypes, respectively, revealed that certain pathways are exclusively affected in the hiLG+hiAB group (ErbB Signaling, GDNF family ligand-receptor interactions, LPS-stimulated MAPK signaling; Supplemental Table S2). The most significant biofunctions predicted to decrease were related to cellular proliferation, leukocyte function, inflammatory response, and apoptosis of antigen-presenting cells (Supplemental Table S1).

The hiLG+loAB group had decreased transcripts related to canonical pathways predominated by cellular immune re-

\[ ^{1} \] The online version of this article contains supplemental material.
responses, protein ubiquitination, and cellular or oxidative stress (NRF2-mediated oxidative stress response, mitochondrial dysfunction) (Fig. 3). Moreover, protein ubiquitination, mitochondrial dysfunction, and the NFAT pathway were characterized as unique for this group (see Supplemental Table S2). Affected biofunctions were predicted to decrease quantities of blood cells, particularly mononuclear leukocytes and hematopoietic cells, and hematopoietic progenitor cells (Supplemental Table S1).

Response of low LG phenotypes to first vaccination. The \textit{loLG}+hiAB group had fewer significantly different transcript abundances with only 572 DE genes for \textit{interval 1}, whereas the \textit{loLG}+loAB group had 1,032 DE genes (Table 1). Accordingly, those transcripts were only related to pathways of protein ubiquitination and CTLA4 signaling in cytotoxic T lymphocytes (Fig. 3). Affected biofunctions were predicted to decrease quantity of blood cells and expansion of leukocytes (Supplemental Table S1).

The \textit{loLG}+loAB group presented DE genes with mainly immune response-related pathways, similar to \textit{hiLG}+loAB animals. Although these two groups shared five affected pathways, examination revealed unique DE genes constitute these pathways (CD28 signaling in T helper cells, calcium-induced T lymphocyte apoptosis, and NF-κB signaling) (Fig. 3). Altered

Fig. 2. Venn diagrams of unique and common differentially expressed (DE) genes in \textit{interval 1} and \textit{2} among the 4 phenotypes. Comparative microarray analyses revealed different transcript abundances (DE genes), as indicated by numbers, among groups between day 0 and day 14 (\textit{interval 1}) and day 14 and day 28 (\textit{interval 2}), respectively.
biofunctions were predicted to decrease quantity of leukocytes including hematopoietic stem cells and to decrease expression or transcription of RNA.

Response of high LG phenotypes to second vaccination. Comparison of hiLG animals between day 14 and day 28 revealed 442 and 994 DE genes for high and low AB responders, respectively (Table 1). Significant canonical pathways generally exhibit elevated transcript abundances. Accordingly, affected biofunctions were predicted for increased activation.

![Pathway diagram](image_url)
Table 1. Numbers of DE genes in the intervals between days 0 and day 14 as well as day 14 and day 28, i.e., primary and secondary response to vaccination, for divergent phenotypes of lean growth performance and antibody titers

<table>
<thead>
<tr>
<th>Interval:</th>
<th>day 0–day 14</th>
<th>day 14–day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>DE genes, n</td>
<td>hiLG+hiAB: 1,201</td>
<td>hiLG+loAB: 1,770</td>
</tr>
<tr>
<td>Transcript abundance ↑</td>
<td>292 (24%)</td>
<td>491 (28%)</td>
</tr>
<tr>
<td>Transcript abundance ↓</td>
<td>909 (76%)</td>
<td>1279 (72%)</td>
</tr>
</tbody>
</table>

For the hiLG+hiAB group, transcripts were predominantly related to canonical pathways of humoral and cellular immune responses, particularly B cell receptor (BCR) signaling (Fig. 3). BCR signaling is the crucial pathway for B cell activation in the humoral immune response and comprises antigen recognition by BCRs and intracellular signal transduction to B cell proliferation, AB production and secretion, memory B cell differentiation, cell survival, and apoptosis (10). Enrichment of BCR signaling-related transcripts was found uniquely in the hiLG+hiAB phenotype (see Supplemental Table S2). Other significant pathways were related to T-cell-mediated immune response, including NFAT regulation of immune response, Nur77 signaling, and PKCδ signaling in T lymphocytes. The most significant predicted biofunctions were increased cell proliferation, particularly of lymphocytes (Supplemental Table S1).

The hiLG+loAB group also showed a predominance of unique signaling pathways related to the immune response, such as B cell development, antigen presentation, and dendritic cell maturation (Fig. 3). Significant predicted biofunctions at day 28 were related to increased quantity, differentiation, and development of leukocytes (Supplemental Table S1).

Response of low LG phenotypes to second vaccination. In response to the booster vaccination given at day 14, loLG+hiAB animals presented 533 DE genes and loLG+loAB animals presented 1,239 DE genes (Table 1). For the loLG+hiAB group, IPAs revealed decreased transcripts related to mitochondrial dysfunction and protein ubiquitination pathways and increased transcripts related to immune response signaling (B cell development, antigen presentation, and Nur77 signaling in T lymphocytes) (Fig. 3). However, no corresponding biofunctions were identified (Supplemental Table S1).

Additional pathway analyses of unique genes (see Venn diagrams in Fig. 2 and Supplemental Table S2) in contrast to loLG+loAB or hiLG+hiAB failed to clearly characterize this phenotype. B cell development and Nur77 signaling appeared as unique by comparison to loLG+loAB, and mitochondrial dysfunction, protein ubiquitination, and antigen presentation were found among unique DE genes in contrast to hiLG+hiAB.

The loLG+loAB group showed a balance between increased and decreased transcript abundances within significant pathways related to intracellular and second messenger signaling, cellular and organismal growth and development, and cellular immune responses (Fig. 3). Only tight junction signaling transcripts decreased, while only B cell development transcripts increased. Pathway analyses of unique DE genes revealed phospholipase C, tight junction, gap junction, and ephrin B signaling as affected only in the loLG+loAB phenotype. Affected biofunctions were related to increased quantity of mononuclear leukocytes or B lymphocytes and hematopoietic progenitor cells and to decreased activation, aggregation, and engulfment of blood cells (Supplemental Table S1). The observed clear contrasts between the high and low LG groups in terms of canonical pathways were poorly reflected by proportions of shared and unique DE genes, respectively (Fig. 2). While hiLG+hiAB compared with loLG+hiAB revealed only few shared DE genes (~8%) the comparison between hiLG+loAB and loLG+loAB showed a proportion of about 50% shared DE genes.

DISCUSSION

The present study explores time courses effects of transcriptional responses and response differences between divergent LG and AB phenotypes. Comparison of day 0 transcriptome profiles were performed among all groups but did not reveal significant differences in terms of affected canonical pathways or biofunctions (data not shown). This indicates that the phenotype groups did not differ before vaccination and the animals were in a naïve state before the antigenic challenge. It is to be assumed that the first vaccination induces an innate immune reaction, whereas the booster induces the adaptive immune system. AB titers at day 14 were consistently low, approximately at the assay detection limit. It was not until the second vaccination that considerable AB titers were detected and thus high and low responders could be identified. Comparing day 0 and day 14, all four phenotypes had a general decrease in transcripts of signaling pathways and predicted biofunctions. Significant pathways in loAB phenotypes were predominantly related to immune response, whereas hiAB and hiLG groups correlated with several growth factor signaling processes. No influence of hiLG or loLG phenotypes was observed. Decreased transcripts within significant pathways at day 14 correlates with our previous study of multiple time points after an initial and booster vaccination (2). Within 24 h after the initial vaccination, transcripts related to immune responses and other biofunctions increased. However, at day 14 both studies show more decreased transcripts, possibly suggesting tissue reorganization and cell population modification occur 2 wk after initial challenge.

After the second immune stimulation (i.e., day 28), hiLG animals respond exclusively with elevated transcripts of hu-
moral and cellular immune processes. Since BCR signaling is significant only in hiAB animals, the humoral response seems to be more pronounced in hiAB animals, whereas loAB animals may have a bias to the cellular branch.

The loLG phenotype revealed few significant pathways and biofunctions in the context of hiAB. A small number of gene transcripts assigned to immune functions were elevated, and two nonimmune-specific transcript pathways were decreased at day 28. LoLG+loAB animals had transcripts correlated with pathways assigned to multiple functions, but directionality could not be predicted because gene transcripts were not consistently increased or decreased.

Only hiLG phenotypes showed considerable activation of immune responses that can be expected after immune stimulation in the transition from day 14 to 28. Elevated cellular immune response transcripts were found in hiLG animals regardless of AB titer; however, humoral immune response—crucial BCR signaling was significant only in the hiAB group. BCR signaling is crucial for activation of B cells and comprises BCR antigen recognition (membrane-bound immunoglobulins and associated molecules CD79a and CD79b), signal transduction, and processing leading to gene transcription and altered cell metabolism and cytoskeletal organization (10). For the hiLG+hiAB phenotype, we found increased transcript abundances for the adapter proteins GAB, BLNK, and BAM32, which are involved in initial signal generation. In addition, Btk, p38, and JNK1/2 kinases and Oct-2, a B cell-specific transcription factor (26), transcripts were elevated. p38 and JNK1/2 enter the nucleus and activate several transcription factors. Although these results indicate the activation of B cell-mediated immune responses, it should be taken into account that the lymphocyte fraction of peripheral blood is composed of a smaller fraction of B lymphocytes than T cells, and moreover, within the T helper population, Th2 lymphocytes are less abundant than Th1 cells. Both may impair, to some extent, the detection of shifted transcript quantities in B cells and T helper cells indicative for the humoral immune response.

In summary, consistent with our previous studies (2, 3), hiLG phenotypes have enriched transcripts of immune response pathways in the context of both hiAB and loAB. In addition, our results reveal that only after booster vaccination can ongoing immune response activation be observed at the transcriptional level. To our surprise, AB phenotype differences did not considerably impact immune response pathways. Thus, under certain circumstances results from immune assays may not reflect the actual immunocompetence as previously reported (1).

However, differences between hiLG and loLG phenotypes became clearly visible in response to booster vaccination, as hiLG animals had numerous enriched immune response transcripts and loLG animals had few. Studies on the relationship between production performance and immune response have revealed similar results. Yorkshire pigs selected for high cellular and humoral immune responses also show increased weight gain (31, 46, 47). In particular, the authors state that “the reason for advantages in growth rate are not known but may reflect efficient response to clinical and subclinical infection with reduced duration of illness and reduced muscle growth” (46).

In general, it seems plausible to assume that animals with higher and therefore effective immune responses benefit from decreased incidence of infection and therefore unimpaired growth performance. A further explanation may be given by the fact that in pigs, exposure to stressors promotes catabolic processes. Stressed pigs therefore show reduced growth and decreased lean-to-fat ratios (25, 45). Hence, in addition to immune activation, the correlating stress induction may play a crucial role in metabolic effects. It is conceivable that hiLG animals either possess highly effective immune responses or are better able to handle the stressors caused by vaccination.

Further understanding of the interaction between the immune system and the metabolic processes important for the expression of production traits is needed. Several studies report that certain factors, such as infection, poor hygiene conditions, or physical and psychological stressors, lead to metabolic impairment and, thus, lower production performance. Furthermore, immune responses require physiological costs (9), and resource allocation between immune function and growth performance can be assumed (38). However, such immune system activation and resulting adverse effects on metabolism should be differentiated from genetically determined differences in immunocompetence and performance potential in terms of weight gain, LG, and other production traits. As already indicated, immune traits are heritable, and considerable individual variation of immune responses can be observed. Today’s breeding programs are faced with demanding requirements (33), and the aim of integration of immune traits leading to disease resistance reconcilable with animal welfare underlines the necessity of a comprehensive understanding of the genetic control between them.

In conclusion, in response to booster vaccination, hiAB animals did not have pronounced activation of immune responses at the transcriptional level compared with loAB animals. However, LG phenotype animals revealed predominantly enriched immune response transcripts. For hiAB animals, BCR signaling was the most significant function, whereas loAB animals had enriched gene transcripts assigned mainly to cellular immunity. Hence, both hiLG phenotypes were characterized by several favorable production traits and a combined humoral and cellular immune response in hiAB animals, as well as a predominance of cellular immune response in loAB animals. The highly significant differences observed among divergent LG phenotypes on the one hand suggest the compatibility of LG and immunocompetence and, on the other hand, point to an important role of LG traits within the genetic interaction of metabolism and immunocompetence.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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