System immunology-based identification of blood transcriptional modules correlating to antibody responses in sheep

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Lacking immunogenicity, inactivated vaccines require potent adjuvants. To understand their effects, we used a system immunology-based analysis of ovine blood transcriptional modules (BTMs) to dissect innate immune responses relating to either antibody or haptoglobin levels. Using inactivated foot-and-mouth disease virus as an antigen, we compared non-adjuvanted to liposomal-formulated vaccines complemented or not with TLR4 and TLR7 ligands. Early after vaccination, BTM relating to myeloid cells, innate immune responses, dendritic cells, and antigen presentation correlated positively, whereas BTM relating to T and natural killer cells, as well as cell cycle correlated negatively with antibody responses. Interestingly, BTM relating to myeloid cells, inflammation and antigen presentation also correlated with haptoglobin, but in a reversed manner, indicating that acute systemic inflammation is not beneficial for early antibody responses. Analysis of vaccine-dependent BTM modulation showed that liposomal formulations induced similar responses to those correlating to antibody levels, while addition of TLR ligands reduced myeloid cells, inflammation and antigen presentation BTM expression despite promoting antibody responses. Furthermore, this vaccine was more potent at downregulating T and natural killer cell BTM. When pre-vaccination BTM were analyzed, we found that high vaccine responders expressed higher levels of cell cycle and myeloid cell BTMs as compared with low responders. In conclusion, we have transferred human BTM to sheep and identified early vaccine-induced responses associated with antibody levels or unwanted inflammation. Such readouts are applicable to other veterinary species and very useful to identify efficient vaccine adjuvants, their mechanism of action, and factors related to low responders.

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INTRODUCTION

In the past, vaccines were developed empirically requiring many animal studies with a long-term follow-up to assess vaccine efficacy. Furthermore, although the mechanisms of many vaccine adjuvants at the molecular and cellular level are clear, in vivo protection is much more complex and still difficult to understand and predict. Systems immunology offers powerful approaches towards dissecting the early innate immune response relating to potent adaptive immune responses in human.1-3 This enabled to identify genes expressed in peripheral blood leukocytes within the first days after vaccination, which correlated to CD8 T-cell or antibody responses.2,3 Nevertheless, a main problem with this relatively simple approach was the high heterogeneity of individual responders and the difficulty to understand immunological processes from individual gene information. For these reasons, a much more powerful analysis of transcriptomic data was developed by identifying gene sets such as blood transcriptional modules (BTMs) through large-scale data integration.1,4 To this end, Li et al.1 established human blood transcriptomes from 540 data series containing over 32,000 samples obtained from many disease-related studies to identify master networks of genes co-regulated and interacting in peripheral blood immune system during many physiological and pathological conditions.1 Certain BTM were demonstrated to correlate with adaptive immune responses in a vaccine type-specific manner enabling conclusions on immunological processes associated with desired vaccine responses.1

For foot-and-mouth disease (FMD), a devastating viral infection affecting cloven-hooved animals, traditional vaccine approaches are based on the formulation of inactivated antigen with conventional adjuvants such as oil-in-water emulsions.5 In countries free of FMD, besides culling infected herds, vaccines can have an important role in the control of outbreaks. Nevertheless, the ability to induce early protection is an essential element of such vaccines. A problem associated with the elimination of FMD from endemic areas is the fact that current vaccines offer protection with a very limited duration of immunity.6 A potential to improve such vaccines would be to enhance their ability to activate the innate immune system by targeting Toll-like receptors (TLRs), which can promote T-cell help and differentiation of B lymphocytes into memory or long-lived plasma cells.7 TLRs are a family of pathogen recognition receptors

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individual transcripts correlating with neutralizing antibody titers

Individual changes in gene expression were calculated and correlated with the SNT on day 7, 14, and 28 p.v. (Supplementary Fig. 3). For the D0 vs. D3 differentially expressed genes, 26 transcripts (ZNF646, STIM2, C9orf3, NPEPPS, SDR39U1, HEXDC, NUCLB2, GHDC, LSR, MYO1E, TRAPPC6A, DCAF12, ABCC2, ZDHHC24, TRIM41, GZMA, NAGK, GMNN, OLFML3, MYO7A, TNFSF13B, GGA3, ASB8, ARNLT, GNG3, PLA2G12A) were identified to correlate with the SNT of all three time points (7, 14, and 28 days). For the D0 vs. D7 differentially expressed genes, six transcripts were found to correlate with SNT of all three time points (CHPT1, RPL12, RNASEL, CDCDC93, MFAP2, MYO1E). Only MYO1E was correlating at D3 as well as D7 with the SNT at all time points.

Translation of human BTM to the sheep

While some of the genes identified above are clearly linked to antibody responses such as TNFSF13B (B-cell-activating factor), the potential involvement of most genes in promoting antibody responses remained elusive. We therefore employed gene set enrichment (GSEA) using BTM developed in the human system. These have been demonstrated to provide a comprehensive picture of human immune responses correlating with adaptive immune responses induced by different classes of vaccines. To this end, the gene composition of the BTM was adapted to sheep by replacing genes known to differ in name for human and sheep such as those belonging to the MHc, CD1, TCR, Ig and IFN families with sheep homologues. In addition, we manually annotated many sheep genes focusing on those represented in many BTM. This resulted in a median coverage of 88.2% (Supplementary Fig. 4). The list of all BTM with their gene compositions can be downloaded from the Supplementary Data.

BTM correlating with antibody responses

The correlation of BTM with antibody responses measured at 7, 14, and 28 days p.v. were determined for the D3 and D7 transcriptome (Fig. 2). Although some BTM correlated only with one of the time points p.v., other correlated with two or all of the time points analyzed. This was expected as some animals with low early antibody levels caught up at day 28 p.v. (Fig. 1b). Nevertheless, for D3 the BTM M209 (lysosome) and M23 (RA, WNT, CSF receptor network) positively correlated with the SNT at all time points. Furthermore, despite the variation in antibody responses over time, a number of BTM were found that negatively correlated to antibody responses measured at all three time points. These were mainly BTM related to cell proliferation (M4.10, M37.3, M10.0, M182) and T/natural killer (NK) cells (M7.2, M61.2, M7.3).

Antibody-correlating D7 BTM were reduced in numbers (Fig. 2). Nevertheless, some BTM were found again, including M146 (MHC-TLR7-TLR8 cluster, positive correlation), M40 (complement and other receptors, positive correlation), M35.0/M35.1 (signaling in T cells, negative correlation) and M230 (cell cycle, mitotic phase, negative correlation), S1 (NK cell surface signature, negative correlation), S3 (plasma cell surface signature, negative correlation) and a number of unnamed BTM (M102, M116, M177 and M249). BTM 7.4 (T cell activation, positive correlation) was the only BTM found with inverse correlation.

To enable an easier interpretation of the BTM results, we classified them into six BTM families (Table 1 and Supplementary Excel file “BTM composition and families”). To this end, BTM were attributed to immune cell types or immunological processes, for which we decided to make large families to obtain a simplified synopsis of early immune responses correlating to antibody titers. As an example, the BTM family “myeloid cells/inflammation” was composed of all BTM’s relating to monocytic cells, neutrophils, and...
**a Study design**

| Groups and adjuvants | Antigen | Route | Sheep per group | Age | Gender | PBMC Sampling | Serum sampling |
|----------------------|---------|-------|-----------------|-----|--------|---------------|---------------|
| 1. None (PBS)        | 40μg inactivated FMDV particles | One injection i.m. | six 5-6 month old and nine 12-20 month old, equally distributed | Five female and one castrated male in each group | 0, 3, 7 days p.v. | 0, 3, 7, 14, 28 days p.v. |
| 2. Liposomes         |         |       |                 |     |        |               |               |
| 3. Liposomes with MPLA & guardiquimod |         |       |                 |     |        |               |               |

**b Antibodies**

![Antibody titers following vaccination of sheep with antigen in phosphate buffered saline (PBS), antigen formulated in liposomes (L) and antigen formulated in liposomes with TLRL (L(TLRL)).](image)

**c Haptoglobin**

![Haptoglobin responses.](image)

**d Differentially expressed genes**

![Vulcano plots showing differentially expressed genes which were up- or downregulation on day 3 (D3) and day 7 (D7) compared with before immunization, respectively. Green dots represent significant genes (p adjusted < 0.05). Digits in the plots indicate the number of significantly down or upregulated genes. In b and c, statistical significant differences between two groups were calculated using unpaired two-way ANOVA with repeated measures followed by Tukey’s multiple comparisons test.](image)

Fig. 1 Neutralizing antibodies, haptoglobin and differentially expressed genes in PBMC induced by vaccination. **a** Study design. **b** Serum neutralizing antibody titers following vaccination of sheep with antigen in phosphate buffered saline (PBS), antigen formulated in liposomes (L) and antigen formulated in liposomes with TLRL (L(TLRL)). **c** Vaccine-induced haptoglobin responses. **d** Vulcano plots showing differentially expressed genes which were up- or downregulation on day 3 (D3) and day 7 (D7) compared with before immunization, respectively. Green dots represent significant genes (p adjusted < 0.05). Digits in the plots indicate the number of significantly down or upregulated genes. In **b** and **c**, statistical significant differences between two groups were calculated using unpaired two-way ANOVA with repeated measures followed by Tukey’s multiple comparisons test (**p < 0.001; **p < 0.002; *p < 0.033)
antibody levels in analogy to

Fig. 2

| Correlating BTM D0 vs D3 | Correlating BTM D0 vs D7 |
|--------------------------|--------------------------|
| day 7 14 28              | day 7 14 28              |

other elements of inflammatory reactions. We also included BTM related to coagulation and complement activation, as these are processes induced during inflammation (Table 1). The result of such an analysis for the correlating BTM is shown in Fig. 3 and demonstrate that the D3 dominant BTM families positively

Fig. 2  BTM correlating to serum neutralizing antibody titers. a Heatmap showing the correlation of D3 BTM with antibody levels on day 7, 14, or 28 after immunization. Animals from all groups were included (n = 18, cutoff p < 0.05). b Heatmap showing the correlation of D7 BTM with antibody levels in analogy to a

with antibody responses were “myeloid cells/inflammation” and “DC/antigen presentation,” whereas the BTM families “T/NK cells” and “cell cycle” were the most prominent to negatively correlate. This dominance was seen in terms of the number of correlating BTM’s attributed to the family for each of
Vaccine-induced BTM's

We next calculated BTM's significantly induced by the different vaccines with the aim to compare these responses with the antibody-correlating BTM, and to determine the effects of formulation and TLRL. The results are shown as BTM families in Fig. 3b and as heatmap for individual BTM in Supplementary Fig. 5. In the PBS group, only few BTM were significantly modulated at both D3 and D7. On D3 the liposomal formulations strongly promoted "myeloid cells/inflammation" and "DC/antigen presentation" but downregulated "T/NK cells" and "cell cycle" BTM families, creating a picture resembling the correlation data shown in Fig. 3a. Interestingly, the addition of the TLRL abrogated the induction of "myeloid cells/inflammation" and "DC/antigen presentation" BTM. In contrast, on D7 we only found many significantly modulated BTM in the L(TLRL) group. While "myeloid cells/inflammation" and "DC/antigen presentation" were downregulated, the "B cell" and "cell cycle" family was found to be upregulated only in the TLRL group.

As a main aim of the present study was to identify modules induced by our vaccine formulation and correlating with antibody response we combined the data of Fig. 3 to search for BTM which would correlate to the antibody response and also be induced by a particular vaccine. This approach was also used to identify BTM correlating independent from the vaccine-induced responses. Figure 4 shows average correlations coefficients for BTM which significantly correlated with antibody responses found at least for two different time points. The BTM were grouped in BTM which were not found to be induced by a vaccine ("only corr."), which were induced by the PBS-formulated vaccine (corr & PBS), by the liposomal formulation (corr. & L) or by the TLRL-adjuvanted liposomal vaccine (corr. & L(TLRL)). On D3, for many BTM such as those belonging to the "DC/antigen presentation" and "B cells" families, correlating BTM were actually not identified to be significantly induced by the vaccines, indicating that these could more reflect differences in the host response to the vaccination. The L vaccine group induced many correlating "myeloid cells/inflammation" BTM, while the L(TLRL) group was potent at downregulating "T/NK cell" BTM (Fig. 4a). Overall, this demonstrated a strong immunomodulatory effect of the TLRL. On D7, none of the correlating BTM were not found to be induced by the vaccines (Fig. 4b), again indicating that these BTM reflect differences in the host responses to the vaccines.

Impact of vaccines on peripheral blood mononuclear cell populations

Considering that changes in many BTM could have been the result of a modulation of relative frequencies of cell populations we quantified CD14+ and CD16+ monocytes as well as CD4+ and CD8+ T cells in D0 and D3 PBMC. Only a significant reduction of CD8+ T cells was found in the L and L(TLRL) groups (Supplementary Fig. 6), indicating that the vaccine-induced BTM modulation is not solely reflecting changes in the cellular composition of the PBMC.

BTM correlating to the acute phase response

To differentiate innate immune responses associated with antibody responses or unwanted inflammation, we calculated the correlation of BTM with the haptoglobin responses (Fig. 5 and Supplementary Fig. 7). We excluded the PBS group as no haptoglobin responses were found in these animals (Fig. 1c). For the D3 transcriptome, many negative correlations were found for the "myeloid cells/inflammation" and "DC/antigen presentation" but positive correlation for the "T/NK cells" and "cell cycle" BTM. In addition, a few "B cells", "cell cycle" and "others" BTM families were negatively correlated. For the haptoglobin responses on day 7 p.v., the dominant modulation was again in the "myeloid cells/inflammation" BTMs. For the D7 transcriptome the number of correlations was markedly reduced. If BTM families are compared with those correlating with antibody levels measured at D3, we found that "myeloid cells/inflammation" and "DC/antigen presentation" BTM families were regulated in an opposite manner. When looking at individual BTM (D0 vs D3), only seven out of 93 correlated in the same direction to the antibody responses (M130, M61.2, M210, M65, M39, M14, M0, Supplementary Fig. 7 and Fig. 2). Five correlated in an opposite manner (M11.1, M220.0, M181, M23, M111.0). These results indicate that the host responses associated with the acute phase response are largely not linked to those resulting in potent antibody responses.

| Table 1. Definition and composition of BTM families |
|-----------------------------------------------|
| **BTM families** | **Immune cells and processes** | **BTM included** |
| Myeloid cells/inflammation | Myeloid cells, innate/inflammatory response (inflammatory cytokines and chemokines), leukocyte migration, coagulation and platelet activation, complement activation | M0, M4.3, M4.15, M11.0, M11.1, M11.2, M16, M21, M23, M24, M27.0, M27.1, M29, M33, M37.1, M38, M39, M45, M53, M59, M73, M78, M81, M85, M86.0, M86.1, M88.0, M91, M112.0, M112.1, M115, M118.0, M118.1, M132, M163, M199, S4, M30, M32.0, M32.1, M37.0, M42 |
| DC/antigen presentation | DC surface (resting and activated), antigen processing and presentation, DC activation | M5.0, M25, M28, M40, M43.0, M43.1, M50, M64, M67, M71, M87, M95.0, M95.1, M119, M138, M139, M146, M165, M168, M200, M209, S5, S10, S11 |
| T/NK cells | NK and T-cell surface, differentiation, activation, signaling, costimulation, and proliferation | M4.5, M4.6, M4.9, M4.11, M5.1, M7.0, M7.1, M7.2, M7.3, M7.4, M12, M14, M18, M19, M35.0, M35.1, M36, M44, M46, M52, M61.0, M61.1, M61.2, M65, M126, M157, M223, S0, S1, S6, S7 |
| B cells | B cells, BCR signaling, B-cell development and differentiation, plasma cells, immunoglobulins | M9, M47.0, M47.1, M47.2, M47.3, M47.4, M58, M69, M83, M123, M156.0, M156.1, M217, S2, S3, S8, S9, M152.0, M152.1, M152.2, M182 |
| Cell cycle | Cell cycle, proliferation, DNA repair, nucleotide and amino acid metabolism, splicing | M76, M103, M4.10, M4.12, M4.0, M4.1, M4.2, M4.4, M4.7, M4.8, M6, M8, M10.0, M10.1, M20, M22.0, M22.1, M31, M32.2, M32.4, M37.3, M49, M144, M167, M175, M181, M230, M250, M137, M152.1, M158.3, M158.4, M158.5, M158.6, M158.7 |
| IFN type-I | Nucleic acid sensing, IFN type-I induction, and IFN response signature | M13, M68, M75, M111.0, M111.1, M127, M150, M158.0, M158.1 |

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Pre-vaccination BTM associated with antibody levels
Considering the heterogeneity of vaccine-induced antibody responses (Fig. 1b), we tested if certain BTM measured before vaccination would be associated with higher levels of antibody response. To this end, in each vaccine group animals having an SNT at day 28 of > 50% above the median were defined as “high responders” (n = 6) and those with < 50% of the median as “low responders” (n = 6). A comparison of high and low responders

Fig. 3  BTM families correlating with antibody responses and induced by the vaccines. BTM families were created as described in Table 1. a The polar plots show the correlation coefficients for BTM of the module families with significant positive or negative correlation to neutralizing antibody levels in serum (p < 0.05). BTM for the D3 (upper plots) and D7 (lower plots) transcriptome are shown. b Vaccine-dependent BTM modulations were calculated as normalized enrichment scores (NES) using GSEA. The significantly (p < 0.05) modulated BTM were grouped as BTM families as in a. The data are shown was calculated for each vaccine group with the D3 (upper plots) and D7 (lower plots) transcriptome
using GSEA demonstrated that 10 BTM related to cell cycle and DNA repair, four BTM related to myeloid cells, two BTM related to inflammation and innate immune responses, one to NK cells were expressed at relatively higher levels in the “high responders” (Fig. 6). No BTM was higher in the “low responder” animals.

### DISCUSSION

The present study adapted BTM originally developed for human blood to sheep PBMC to understand vaccine-induced responses correlating with antibody and acute-phase responses. For efficient delivery to antigen-presenting cells, we employed synthetic...
liposomes as vaccine platform and tested the effect of a combination of TLR4 and TLR7 ligands on antibody and acute-phase protein response. These ligands were selected based on their synergistic potentiation of antibody responses in a mouse model.\(^7\) Specifically, the TLR7 ligand Gardiquimod was selected based on its ability to induce proliferation and induce IFN-\(\alpha\) in PBMC,\(^13,14\) and the TLR4 ligand monophosphoryl lipid A (MPLA) based on its potent triggering of nuclear factor-\(\kappa B\) signaling, which promotes pro-inflammatory responses in mononuclear phagocytes.\(^15,16\) Furthermore, MPLA is clinically approved and was also recently reported to induce a similar response in sheep compared with human PBMC.\(^17\) The vaccination of sheep was successful in terms of induction of neutralizing antibody titers to levels likely associated with protective immunity.\(^18-20\) As anticipated, the addition of the TLRL significantly enhanced the responses. Nevertheless, it is clear that the present vaccine still requires significant improvements, and thus future studies focussing on the effects of formulation, TLR ligand selection and dose in sheep are needed.

Interestingly, although the increase in antibody responses induced by the TLRL was weak compared to previous studies in mice,\(^7\) it had a clear effect on the expression of individual genes. Strikingly, the overall perturbation of gene expression was reduced at D3 in terms of differentially up- or downregulated genes when compared with the L group. Furthermore, on day three p.v., the PBS group shared many upregulated genes with the L, but not with the L(TLRL) group. At day 7, this was even more evident in that differentially regulated genes were found almost exclusively in the TLRL group. Also, comparing the vaccine groups using BTM confirmed clear effects of the TLRL. The reduction of upregulated “myeloid cells/inflammation” and DC/antigen presentation” BTM at D3 together with the above reduced perturbation of individual genes and the reduced induction of haptoglobin indicates an anti-inflammatory effect of the TLRL. Future studies are required to understand how this is induced and if it is an effect of one of the TLRL or their combinations. The fact that at D7 the L (TLRL) group showed the strongest perturbations, both at the individual gene and BTM level, demonstrates that this is not a simple suppressive effect but that rather long-lasting immunomodulatory circuits are induced.

The analysis of genes correlating positively or negatively with antibody titers revealed a relatively large number of genes. We focused mainly on those found to correlate at least for two different time points to increase the chance to have truly relevant genes. Nevertheless, for most of the correlating genes, their role in immune responses is unclear, with the exception of TNFSF13B representing BAFF. Furthermore, only few of the correlating genes matched those identified in PBMC from humans vaccinated with an inactivated viral vaccine.\(^2\) For these reasons, we employed the BTM as a much more powerful bioinformatics analysis of transcriptomic data. BTM represent master networks of

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**Fig. 5** BTM families correlating to vaccine-induced haptoglobin levels. BTM families were created as described in Table 1. The polar plots show correlation coefficients for module families with significant \(p < 0.05\) positive or negative correlation to serum haptoglobin levels. BTM families for the D3 (upper plots) and D7 (lower plots) transcriptome are shown.

**Fig. 6** Pre-vaccination BTM associated with antibody levels. a GSEA was used to determine the BTM enriched in “high responders” \((n = 6,\) red\) and “low responders” \((n = 6,\) blue\). The colors indicates the NES. b Heatmap for the BTM “monocyte surface signature” \((S4)\) as an example of the analysis in a. Each square represents the indicated gene expression for individual animals (same color code as in a).
...immunological genes strongly co-regulated and interacting during many physiological and pathological conditions. This approach permitted to identify many BTM expressed on D3 and D7 correlating with antibody responses on day 7, 14, and 28 p.v. Remarkably, many of the correlating BTMs were also found in human vaccination studies using protein-based or inactivated vaccines. Prominent amongst these are positively correlating BTM relating to monocytes such as M11.0, M23, and M118,21,23 to complement activation such as M112,23 to TLR4/D7 correlating with antibody responses on day 7, 14, and 28 p.v. In sheep, many T-cell modules including CD4 T-cell BTM (M46, S6) as well as general T-cell activation modules (M7.X) were negatively correlated with antibodies. Nevertheless, the various reports on human studies indicate that this can strongly vary depending on the time point being analyzed, the vaccine employed, the number of vaccinations, and the age.1,2,21,24 Although M7.1 and M7.2 were often negatively correlated with antibody response also in human studies,1,21,23,24 our phenotypic data confirmed that at least for the CD8 T cells the liposomal vaccine formulations induced a relative reduction of circulating T cells. For the cell cycle modules which were also among the BTM with a negative correlation in sheep, this was rarely found in the human studies using protein-based vaccines, indicating possible species differences or alternatively a specific effect of the liposomal vaccine employed in the present study. Clearly, the correlating BTM response strongly depends on the type of vaccine. Altogether, we propose that the common correlating BTM found for several different inactivated/protein-based vaccines in both species could be particular robust measurements. From our data, we also conclude that measuring the transcriptome early after vaccines provides more correlating BTM and, therefore, more information regarding the effects of the vaccine on the immune system.

For the immunological interpretation of these results, it must be considered that blood represents only a transit compartment for the immune cells. As a result of vaccine-induced innate immune responses, haematopoiesis of myeloid cells and DC is likely to be enhanced explaining the increase of many modules relating to these cell types. Furthermore, it is obvious that despite the intramuscular injection, the vaccines cause systemic inflammatory effects, some of which obviously correlate with antibody responses. These are not only a number of modules relating the DC and antigen presentation but also to complement activation and blood coagulation. With these reactions, it is also possible to explain the negative correlation of T/NK cell modules, as these cells are very quickly recruited to lymphoid tissues following acute innate immune response such as virus infections. The negative correlation of cell cycle BTM could be related to the same mechanisms as T cell represent the main cell fraction in PBMC and many such BTM were actually related to T cells, in particular CD4 T cells.

An important question arising from such studies is whether the above BTM represent either mechanistically relevant modules, non-functional correlates or even modules that also reflect unwanted side effects of strong adjuvants. To address this question, we performed two additional analyses, which were to compare the correlating BTM with the vaccine-induced BTM, as well as to identify BTM correlating with an acute-phase protein. Our results demonstrate that many of the correlating BTM were actually not identified to be significantly induced by the vaccines, indicating that these could more reflect differences in the host response of individual animals to vaccination. Some BTM relating to "DC/antigen presentation" and "cell cycle" appeared to be particular interesting to identify individual variability due to their strong correlation with antibody levels without apparently being influenced by the vaccine. These analyses also permitted to identify potent effects of the TLRL potentially involved in their beneficial action. While BTM related to "cell cycle" had a strong negative correlation to antibodies they were strongly induced by the L vaccine but not by L(TLRL) formulation. Similar observations but as positive correlation/upregulation were made for some "myeloid cells/inflammation" and "DC/antigen presentation" BTM. In contrast, some "T/NK cells" BTM correlated negatively with antibodies and were also downregulated by the L(TLRL) vaccine. It will thus be interesting to address which of the above effect of the TLRL, in particular the prominent effects on the T/NK cell modules, are mechanistically relevant to better understand their mode of action in promoting immune responses.

The main difference with the PBS group was that it did not impact the "T/NK cell" BTM and had an opposite effect (upregulation) on the "cell cycle" BTM. We speculate that in the absence of liposomal formulation the inflammatory responses and the presence of antigen-loaded activated DC in draining lymph nodes could be reduced explaining a reduced recruitment of these cells to lymphoid tissue.

The comparison of the BTM correlation analyses to antibodies and haptoglobin levels indicated that the host responses associate with the acute phase response are largely not linked to those resulting in potent antibody responses. In particular, the BTM families "myeloid cells/inflammation" and "DC/antigen presentation" were found to correlate in a opposite manner at 3 days p.v. Finally, we also tested whether different pre-vaccination expression levels of BTM can be identified in low or high antibody-producing sheep. Our data indicate that this seems possible even with relatively small group sizes, although additional work is required for confirmation. The relative upregulation of cell cycle, monocyte, DC and innate/inflammatory response BTM in the "high responders" would indicate that the immune system of those animals is in a kind of pre-activated status. A possible explanation for the combination of increased levels of myeloid cell/DC and cell cycle BTM could be increased hematopoiesis and egress of cells from the bone marrow and thymus. Future studies could aim at inducing such a status to enhance vaccine-induced responses.

Obviously, there many other possibilities to analyze gene sets and a choice from a multitude of possible gene sets, including pathways and molecular network-based gene sets, must be made. An example for a different approach selected in cattle is the study by Laughlin et al. using established canonical pathways and gene ontology networks analyzed using a method called Dynamic Bayesian Gene Group Activation. Although many of the gene sets identified to correlate are related to those found using the BTM method, a direct comparison is difficult. To enable a comparison of our data with a relatively large set of human vaccination data, we selected the BTM method for the present study.

In conclusion, the present work has developed a system immunology readout in the ovine model composed of many immunologically relevant gene expression networks, which correlate with antibody responses. We anticipate that this is applicable to other species and will be very useful to both the identification of efficient vaccine adjuvants causing little unwanted side effects and to find factors related to low vaccine responders.

**METHODS**

**Vaccine preparation**

The TL84 ligand MPLA was from Avanti Polar Lipids (USA) and the TL7 ligand guardiquimod from Chemdea (USA). DPPC, DCChol, and PHAD were purchased from Avanti Polar Lipids; Hapes from Applichem; sucrose,
chloroform, and ethanol from Sigma-Aldrich (Switzerland). Extraducer membranes of 400 and 200 nm pore size, as well as draining disks, were purchased from WVR International (Switzerland). Small-scale liposome formulations for evaluation were prepared as follows. DPPC and DCChol stocks were prepared at 50 mg/ml in methanol/methylene-chloride 1:1 (Sigma-Aldrich). Sixteen milligrams of total lipid mix was prepared and evaporated on an Eppendorf Vacufuge. Lipids were resuspended in 1 ml 20 mM HEPES, 10% sucrose buffer on a shaker for 60 min. Liposomes were serially extruded using an Avanti Mini Extruider (Avanti, USA) with 400, 200, and 100 nm membranes (Avanti). For each filtration step, liposomal solutions were passed 11 times. Large-scale liposomal formulation for the in vivo vaccine trial were made as follows. A solution of DPPC (2.6 ml) at 30 mg/ml in chloroform (78.5 mg) and 1.9 ml of a solution of DCChol at 30 mg/ml in chloroform (57.3 mg) were mixed in a 50 ml round bottom flask. The solvent was evaporated with rotary evaporator (Laborota 4001, Heidolph) at 40 °C. The lipid film was rehydrated with 8.5 ml of pre-warmed (40 °C) 20 mM HEPES buffer containing 10% sucrose, pH 7.4, to obtain a lipid concentration (DPPC + DCChol) of 16 mg/ml, a DPPC concentration of 9.2 mg/ml, and DCChol concentration of 6.7 mg/ml. The mixture was magnetically stirred for 25 min and incubated for 5 min at room temperature (RT). The mixture was inserted into an extruder (T001, Northern Lipids, Inc.) equipped with a thermostable of 10 ml and equilibrated at 50 °C. After five extrusion cycles with a filter of 0.22 μm filter to recover 7.5 ml of the sterile product. The filtered product was flushed with a nitrogen stream and stored at 4 °C. Cationic liposomes containing Gardiquimod were prepared with the same procedure by rehydrating the lipid film with 8.5 ml of pre-warmed (40 °C) 20 mM HEPES buffer containing 10% sucrose and gardiquimod at 1 mg/ml, final pH 7.4. Cationic liposomes containing MPA were prepared with the same procedure, except that 1.7 ml of a solution of MPA in chloroform at 1 mg/ml was added to the chloroform solutions of DPPC and DCChol before evaporation and formation of the lipid film. Liposomes were formulated with antigen to final concentration of 8 mg/ml IgG and 20 μg/ml FMDV 146S antigen using a HEPES/Sucrose buffer under stirring. The 146S antigen represents purified inactivated FMDV particles (sedimentation coefficient of 146) prepared from FMDV A Iran96 and was kindly provided by Merial Ltd, Pirbright, UK. The antigen dose was selected as the highest possible dose not strongly altering the physical characteristics of the liposomal vaccine formulations (Supplementary Fig. 1). Vaccine characterization The liposome products were characterized immediately after preparation by measuring pH, average particle size, polydispersion, zeta potential (light scattering), and 20 mM HEPES, 10% sucrose buffer on a shaker for 60 min. Liposomes were serially extruded using an Avanti Mini Extruider (Avanti, USA) with 400, 200, and 100 nm membranes (Avanti). For each filtration step, liposomal solutions were passed 11 times. Large-scale liposomal formulation for the in vivo vaccine trial were made as follows. A solution of DPPC (2.6 ml) at 30 mg/ml in chloroform (78.5 mg) and 1.9 ml of a solution of DCChol at 30 mg/ml in chloroform (57.3 mg) were mixed in a 50 ml round bottom flask. The solvent was evaporated with rotary evaporator (Laborota 4001, Heidolph) at 40 °C. The lipid film was rehydrated with 8.5 ml of pre-warmed (40 °C) 20 mM HEPES buffer containing 10% sucrose, pH 7.4, to obtain a lipid concentration (DPPC + DCChol) of 16 mg/ml, a DPPC concentration of 9.2 mg/ml, and DCChol concentration of 6.7 mg/ml. The mixture was magnetically stirred for 25 min and incubated for 5 min at room temperature (RT). 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Cationic liposomes containing Gardiquimod were prepared with the same procedure by rehydrating the lipid film with 8.5 ml of pre-warmed (40 °C) 20 mM HEPES buffer containing 10% sucrose and gardiquimod at 1 mg/ml, final pH 7.4. Cationic liposomes containing MPA were prepared with the same procedure, except that 1.7 ml of a solution of MPA in chloroform at 1 mg/ml was added to the chloroform solutions of DPPC and DCChol before evaporation and formation of the lipid film. Liposomes were formulated with antigen to final concentration of 8 mg/ml IgG and 20 μg/ml FMDV 146S antigen using a HEPES/Sucrose buffer under stirring. The 146S antigen represents purified inactivated FMDV particles (sedimentation coefficient of 146) prepared from FMDV A Iran96 and was kindly provided by Merial Ltd, Pirbright, UK. The antigen dose was selected as the highest possible dose not strongly altering the physical characteristics of the liposomal vaccine formulations (Supplementary Fig. 1).
reads was assessed using fastqc v.0.11.2 (http://www.bioinformatics. babraham.ac.uk/projects/fastqc/). Ribosomal RNA (rRNA) was removed by mapping the reads to a collection of rRNA sequences (ensembl release 3.1) with Bowtie2 v. 2.2.1.20 The remaining reads were mapped to the Ovis aries reference genome (Oar_v3.1) with Tophat v.2.0.13.31 We used htseq-count v.0.6.1 to count the number of reads overlapping with each gene, as specified in the ensembl annotation (Oar_v3.1.86). The Bioconductor package DESeq2 v. 1.10.133 was used to test for differential gene expression between the different time points and vaccines. As cutoff for significance we employed an adjusted p value of 0.05 based on the Benjamini–Hochberg procedure. Detailed information about the genes including the Entrez Gene ID, the description of the gene, the human homolog ensembl gene ID and the human homolog gene name was obtained using the Bioconductor package BioMart v. 2.26.1.34 The raw data was uploaded to the European Nucleotide Archive accession number PRJEB26387.

BTM analyses
To further analyze the molecular signatures of the response to different vaccines, we performed a GSEA using blood transcription modules defined by Li et al. These modules are composed of 9 up to 347 different genes. Modules were translated to Ensembl annotation and some genes lacking human homolog gene name were manually added to allow higher coverage. To this end we employed Biomart (www.ensembl.org/info/data/ biomart) to create a list of putative sheep homologue Ensembl numbers. All genes known to differ in name for human and sheep were replaced by the sheep homologues if sufficient information was found. This included MHC, CD1, TCR, Ig and IFN genes. We also completed the list with many genes not annotated, focussing on those represented in many BTM. Ovine genes with high homology (>80) to another mammal were manually annotated and included in the BTM. Human genes without HGNC symbol in the original BTM list published by Li et al. were removed as most of them were found to be either withdrawn, represent non-coding RNAs or pseudogenes. The composition of sheep BTM can be downloaded from the Supplementary Material. The GSEA tool (software.broadinstitute.org) was used to calculate enrichment scores for BTMs with each individual animal. Significant modules were defined using p < 0.05 corrected for multiple testing by false discovery rate q < 0.05. Pearson’s correlation of enrichment scores with antibody or haptoglobin levels was calculated in R. GSEA was also used to compare to compare the different vaccines. To this end, a grouped GSEA was performed. P < 0.05 was considered significant. To analyze difference in high and low antibody responders, animals in each vaccine group having an SNT at day 28 of >50% above the median were arbitrarily defined as “high responders” (n = 6) and those with < 50% of the median as “low responders” (n = 6). Two animals per vaccine group fitted into each these criteria. Then GSEA was used to compare BTM levels found in high and low responders. Heatmaps, scatter plots, and polar plots were created using the ggplot 2 package or Graphpad Prism 7.0.

DATA AVAILABILITY
The raw sequence data was uploaded to the European Nucleotide Archive accession number PRJEB26387. Other data sets are available in the Supplementary Data. Raw flow cytometry data are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS
R.O.B.: design of the study, data acquisition and analysis, and drafting the manuscript. L.B.: vaccine preparation, data acquisition, and analysis. K.W.: design of the study and bioinformatic analysis. G.A., O.G.N., S.P., B.Z., and V.G.: data acquisition and data analysis. M. S.: data analysis and critically reviewing the manuscript. N.C., C.B., and R.B.: design of the study and data analysis. A.S.: design of the study, data analysis, and finalizing manuscript.

ADDITIONAL INFORMATION
Supplementary information accompanies the paper on the npj Vaccines website (https://doi.org/10.1038/s41541-018-0078-0).

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REFERENCES
1. Li, S. et al. Molecular signatures of antibody responses derived from a systems biology study of five human vaccines. Nat. Immunol. 15, 195–204 (2014).
2. Nakaya, H. I. et al. Systems biology of vaccination for seasonal influenza in humans. Nat. Immunol. 12, 786–795 (2011).
3. Querec, T. D. et al. Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. Nat. Immunol. 10, 116–125 (2009).
4. Obermoser, G. et al. Systems scale interactive exploration reveals quantitative and qualitative differences in response to influenza and pneumococcal vaccines. Immunity 38, 831–844 (2013).
5. Patil, P. K. et al. Early antibody responses of cattle for foot-and-mouth disease quadrivalent double oil emulsion vaccine. Vet. Microbiol. 87, 103–109 (2002).
6. Doel, T. R. Natural and vaccine induced immunity to FMD. Curr. Top. Microbiol. Immunol. 288, 103–131 (2005).
7. Kasturi, S. P. et al. Programming the magnitude and persistence of antibody responses with innate immunity. Nature 470, 543–547 (2011).
8. Ferguson, N. M., Donnelly, C. A. & Anderson, R. M. The foot-and-mouth epidemic in Great Britain: pattern of spread and impact of interventions. Science (New York) 292, 1155–1160 (2001).
9. McFadden, A. M. et al. Epidemiology of the 2010 outbreak of foot-and-mouth disease in Mongolia. Transbound. Emerg. Dis. 62, e45–51 (2015).
10. Ramanoon, S. Z., Robertson, I. D., Edwards, J., Hassan, L. & Isa, K. M. Outbreaks of foot-and-mouth disease in Peninsular Malaysia from 2001 to 2007. Trop. Anim. Health Prod. 45, 373–377 (2013).
11. Quaye, I. R. Haptoglobin inflammation and disease. Trans. R. Soc. Trop. Med. Hyg. 102, 735–742 (2008).
12. Eckersall, P. D. et al. Maternal undernutrition and the ovine acute phase response to vaccination. BMC Vet. Res. 4, 1 (2008).
13. Auray, G. et al. Characterization and transcriptomic analysis of porcine blood conventional and plasmacytoid dendritic cells reveals striking species-specific differences. J. Immunol. 197, 4791–4806 (2016).
14. Braun, R. O., Python, S. & Summerfield, A. P. Porcine B cell switch responses to Toll-like receptor ligands. Front Immunol. 8, 1044 (2017).
15. Raetz, C. R. & Whitfield, C. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. 73, 635–700 (2002).
16. Takeda, K. & Akira, S. TLR signaling pathways. Semin Immunol. 16, 3–9 (2004).
17. Enkhaabtar, P. et al. Comparison of gene expression by sheep and human blood stimulated with the TLR4 agonists lipopolysaccharide and monophosphoryl lipid A. PLoS ONE 10, e0144345 (2015).
18. Doel, T. R. Natural and vaccine induced immunity to FMD. Curr. Top. Microbiol. Immunol. 288, 103–131 (2005).
19. Parida, S. Vaccination against foot-and-mouth disease virus: strategies and effectiveness. Expert Rev. Vaccines 8, 347–365 (2009).
20. Pay, T. W. & Hingley, P. J. The use of serum neutralizing antibody assay for the determination of the potency of foot and mouth disease (FMD) vaccines in cattle. Dev. Biol. Stand. 64, 153–161 (1986).
21. Kazmin, D. et al. Systems analysis of protective immune responses to RTS,S malaria vaccination in early childhood. Proc. Natl Acad. Sci. USA 114, 2425–2430 (2017).
22. Nakaya, H. I. & Pullender, B. Vaccinology in the era of high-throughput biology. Philos. Trans. R. Soc. Lond. B Biol. Sci. 370, pii: 20140146 (2015). https://doi.org/10.1098/rstb.2014.0146.
23. Nakaya, H. I. et al. Systems biology of immunity to MFS9-adjuvanted versus nonadjuvanted trivalent seasonal influenza vaccines in early childhood. Proc. Natl Acad. Sci. USA 113, 1853–1858 (2016).
24. Nakaya, H. I. et al. Systems analysis of immunity to influenza vaccination across multiple years and in diverse populations reveals shared molecular signatures. Immunity 43, 1186–1198 (2015).
25. Summerfield, A. & Ruggli, N. Immune responses against classical swine fever virus: between ignorance and lunacy. Front Vet. Sci. 2, 10 (2015).
26. Zak, D. E., Tam, V. C. & Aderem, A. Systems-level analysis of innate immunity. Annu Rev. Immunol. 32, 547–577 (2014).
27. Chaussabel, D. & Baldwin, N. Democratizing systems immunology with modular transcriptional repertoire analyses. Nat. Rev. Immunol. **14**, 271–280 (2014).
28. Laughlin, R. C., Drake, K. L., Morrill, J. C. & Adams, L. G. Correlative gene expression to protective seroconversion in rift valley fever vaccinates. PLoS ONE **11**, e0147027 (2016).
29. Reed, L. J. & Muench, H. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol. **27**, 493–497 (1938).
30. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. Nat. Methods **9**, 357–359 (2012).
31. Kim, D. et al. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol. **14**, R36 (2013).
32. Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics **31**, 166–169 (2015).
33. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. **15**, 550 (2014).
34. Durinck, S. et al. BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinformatics **21**, 3439–3440 (2005).
35. Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA **102**, 15545–15550 (2005).