Immune biomarkers associated with infection or protection against *Ehrlichia ruminantium*.
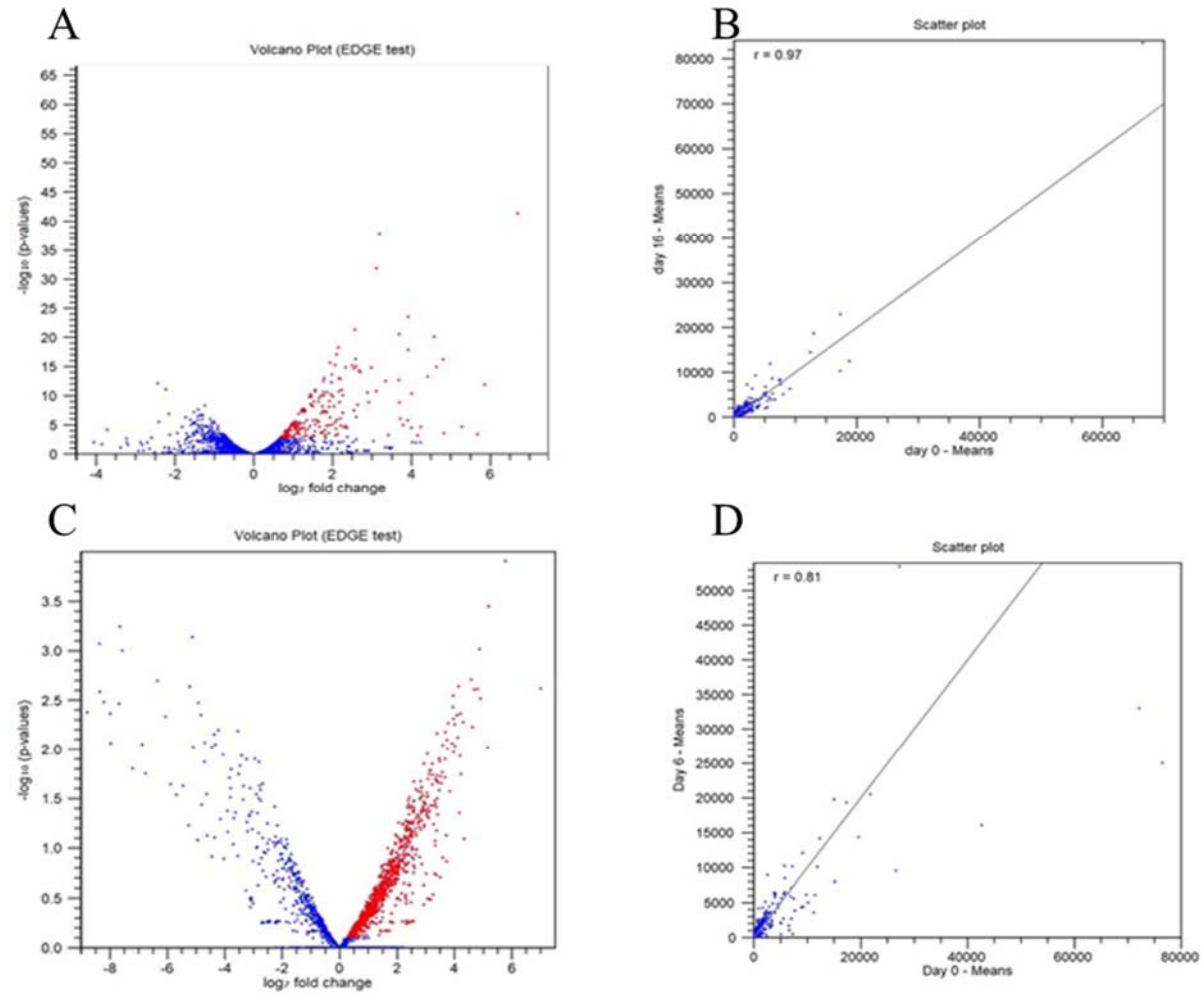
<u>**Supplementary Figures and Tables:**</u>

Figure S1: A-D) Representative volcano and scatter plots using the EDGE statistics, day 16 primary infection (A&B) and day 6 challenge infection (C&D), on proportions with the log2 of the fold change on the axis and the –log10 (p-value) y- axis, red dots representing upregulated genes and scatter plot; x and y axis with normalised means of all sheep.

A — 31B D11 00005566 238 [Ungated] SS / FS

Gate	Number	%Total	%Gated
All	10,000	100.00	100.00
Lymphocytes	4,868	48.68	48.68

B — 31B D11 00005566 238 [AU] FL4 / SS — AV: 34.92%

Gate	Number	%Total	%Gated	X-Med
All	4,556	45.56	100.00	0.46
AV	1,591	15.91	34.92	8.53

C — 31S D12 00005554 226 [A] FL2 / FL4 — V1: 22.37%, V2: 0.15%, V3: 69.94%, V4: 7.54%

Gate	Number	%Total	%Gated	X-Med	Y-Med
All	4,657	46.57	100.00	0.30	0.28
V1	1,042	10.42	22.37	0.23	6.60
V2	7	0.07	0.15	13.47	7.78
V3	3,257	32.57	69.94	0.30	0.23
V4	351	3.51	7.54	21.44	0.26

D — 31S D12 000055554 222 — Live: 93.76%, Dead: 6.24%

Gate	Number	%Total	%Gated
All	2,582	28.61	100.00
Dead	161	1.78	6.24
Live	2,421	26.83	93.76

E — 31 B NEG D3 00005337 052 [Lymphocytes] FL4 / SS — BY: 0.16%

Gate	Number	%Total	%Gated	X-Med
All	5,685	56.85	100.00	0.30
BY	9	0.09	0.16	0.88

F — 30 CD8 00005096 032 — V1: 0.00%, V2: 0.00%, V3: 88.70%, V4: 11.30%

Gate	Number	%Total	%Gated	X-Med	Y-Med
All	7,196	71.96	100.00	0.27	0.18
V1	0	0.00	0.00	N/A	N/A
V2	0	0.00	0.00	N/A	N/A
V3	6,383	63.83	88.70	0.26	0.18
V4	813	8.13	11.30	13.68	0.17

G — 31S D0 00006271 356 [A] FL2 / FL4 — V1: 0.86%, V2: 0.12%, V3: 91.06%, V4: 7.96%

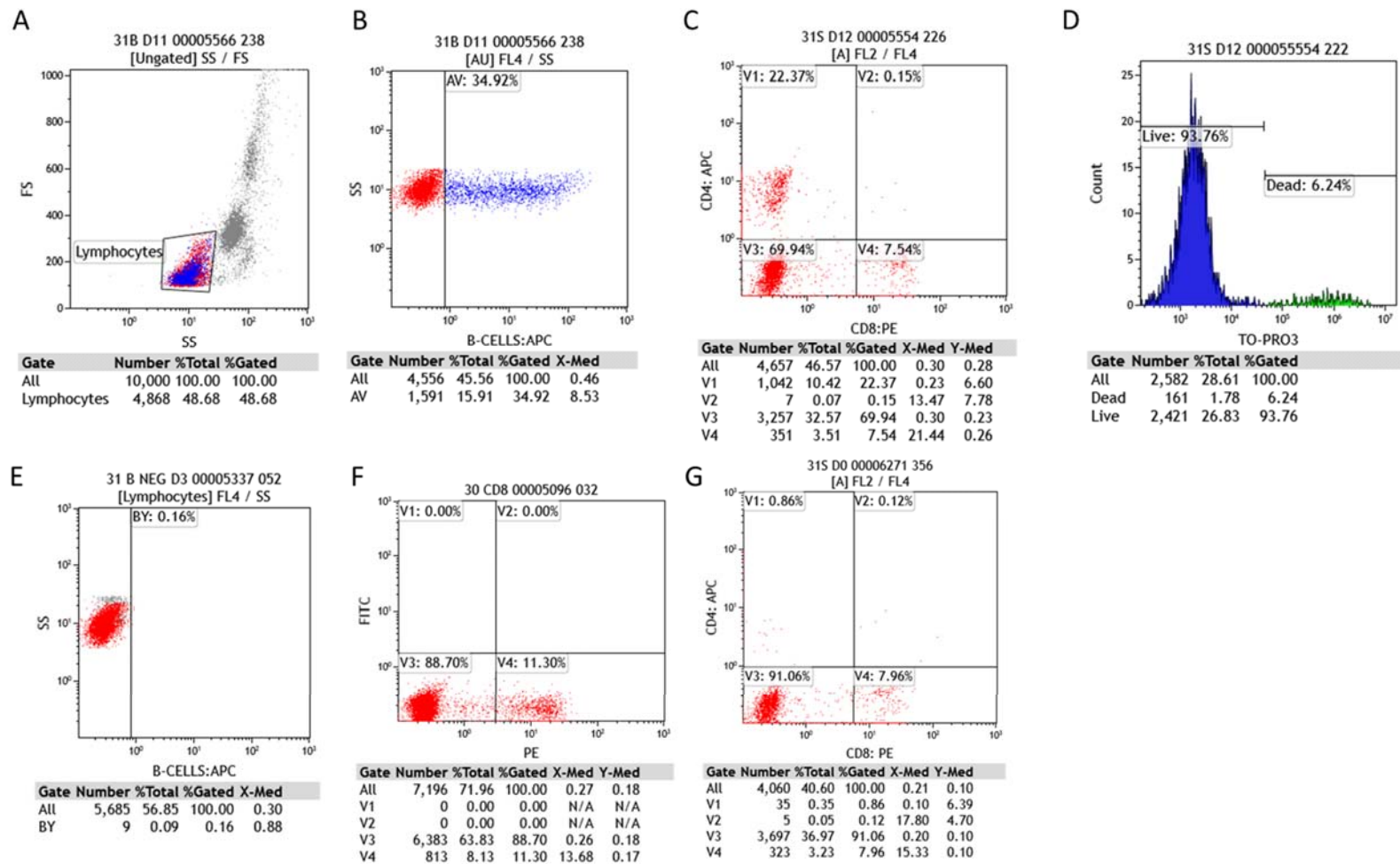| Gate	Number	%Total	%Gated	X-Med	Y-Med
All	4,060	40.60	100.00	0.21	0.10
V1	35	0.35	0.86	0.10	6.39
V2	5	0.05	0.12	17.80	4.70
V3	3,697	36.97	91.06	0.20	0.10
V4	323	3.23	7.96	15.33	0.10

Figure S2: Gating strategy for PBMC phenotyping through flow cytometry using S30 and S31 as representative data. The gating strategy is indicated by gating from Lymphocytes in the FS vs SS density plot (A). Density plot SS vs B cell marker is shown in (B) with the unstained control in (E). Histogram for CD4: APC vs CD8: PE is shown in (C) with the FMO controls in (F and G). A representative histogram for live/dead staining of whole blood processed within 4 h of isolation and RBC removed is shown in (D).

Table S1. *E. ruminantium* copy numbers detected in sheep (s30-s34) blood collected daily after tick infection (D=day, during/after tick feeding) and daily temperatures recorded. (Red text = Febrile reaction; T = Treated; neg = Negative; pos = Positive).

| Days	Sheep 30			Sheep 31			Sheep 32			Sheep 33			Sheep 34		
	Temp	Copy no/µl	PCR +/-	Temp	Copy no/µl	PCR +/-	Temp	Copy no/µl	PCR +/-	Temp	Copy no/µl	PCR +/-	Temp	Copy no/µl	PCR +/-
0	38	neg	neg	39.4	neg	neg	39.1	neg	neg	39.9	neg	neg	39.6	neg	neg
1	39.3	neg	neg	39.5	neg	neg	39.5	neg	neg	39.1	neg	neg	39.3	neg	neg
2	39.1	neg	neg	39.2	neg	neg	38.9	neg	neg	39.3	neg	neg	39	neg	neg
3	39.4	neg	neg	39.4	neg	neg	39.2	neg	neg	39.1	neg	neg	39	neg	neg
4	39.5	neg	neg	39.5	neg	neg	39.3	neg	neg	39.5	neg	neg	39	neg	neg
5	39.5	neg	neg	39.5	neg	neg	39	neg	neg	39.2	neg	neg	38.9	neg	neg
6	39.4	neg	neg	39.4	neg	neg	38.8	neg	neg	39.5	neg	neg	39	neg	neg
7	39.2	neg	neg	39.5	neg	neg	39.1	neg	neg	39.5	neg	neg	39.4	neg	neg
8	39.4	neg	neg	39.6	neg	neg	39.2	neg	neg	39.4	neg	neg	39.5	neg	neg
9	39.8	neg	neg	39.6	neg	neg	39.2	neg	neg	39.3	neg	neg	39.4	neg	neg
10	39.6	neg	neg	39.7	neg	neg	39.4	neg	neg	39.5	neg	neg	39.3	neg	neg
11	39.8	neg	neg	39.4	neg	neg	39.4	neg	neg	39.2	neg	neg	39.4	neg	neg
12	39.5	neg	neg	39.4	neg	neg	39.3	neg	neg	39	neg	neg	39	neg	neg
13	39.1	neg	neg	39.9	1×10^5	**pos**	39.1	neg	neg	39.3	neg	neg	39.6	neg	neg
14	39.5	neg	neg	41.2	4×10^6	**pos**	39.4	neg	neg	39.3	neg	neg	38.2	neg	neg
15	39.1	neg	neg	41.6	1×10^7	**pos**	40	2×10^6	**pos**	40.1	5×10^6	**pos**	39.9	neg	neg
16	39.2	neg	neg	41.9[T]	5×10^6	**pos**	40.3	4×10^6	**pos**	40.6	2×10^6	**pos**	40.7	**pos**	4×10^6
17	39.1	neg	neg				41.7	2×10^7	**pos**	41.4	1×10^6	**pos**	41.2	**pos**	9×10^6
18	39.6	neg	neg				42[T]	1×10^7	**pos**	42[T]	1×10^5	**pos**	41.8[T]	**pos**	3×10^5
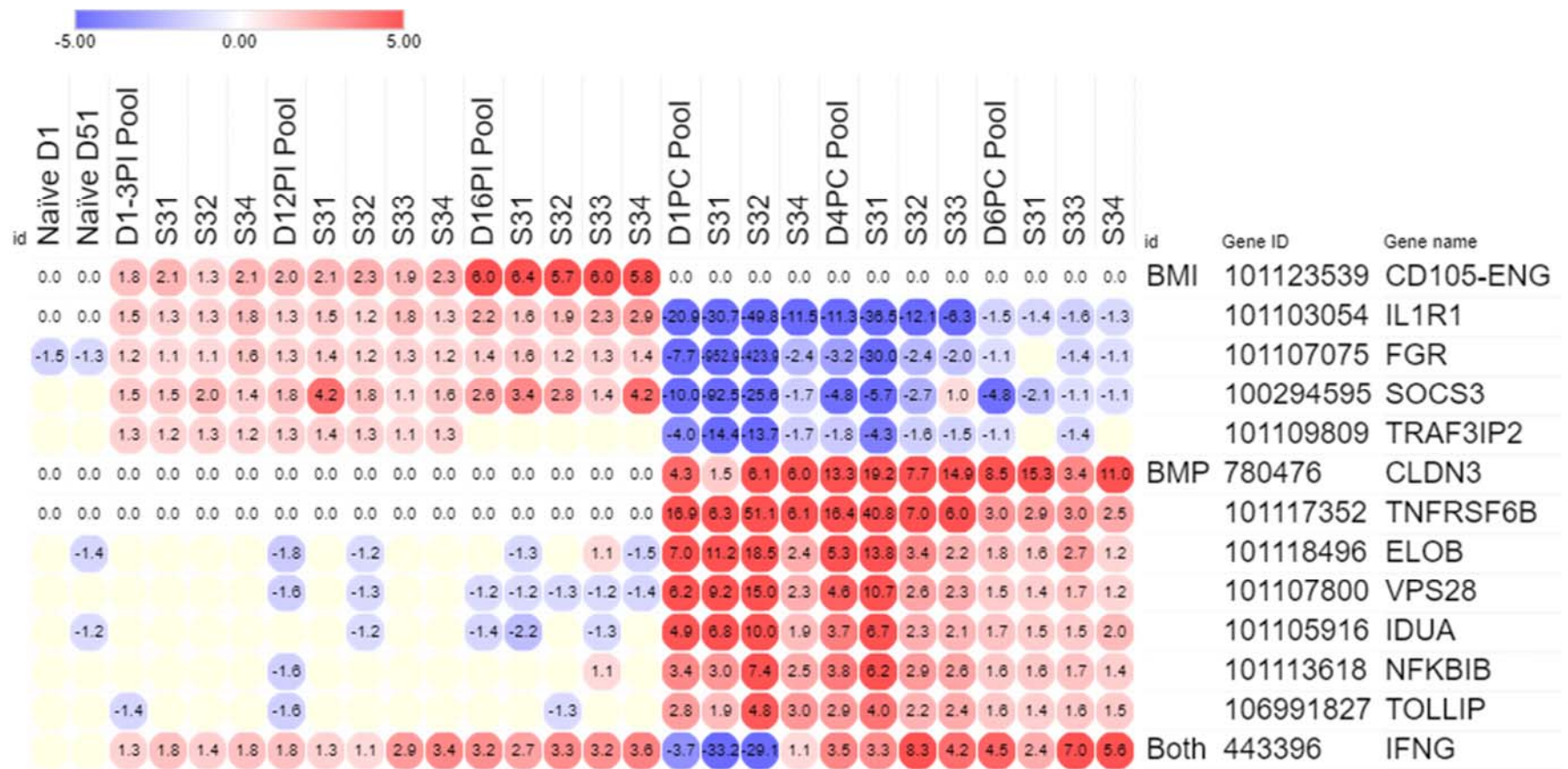
Figure S3: Heat map showing representative data for DEGs selected to be either BMP or BMI. Values indicated as "0.0" shows DEGs with zero RNA transcripts (RPKM < 10), spots without values are FC between 1.1 and -1.1 or not significant, red indicates upregulated while blue represents downregulated DEGs. Darkest colours include those DEGs with FC< -3 and >3 so that lower expression values are also visible on the map. Maps were created with https://software.broadinstitute.org/morpheus/.
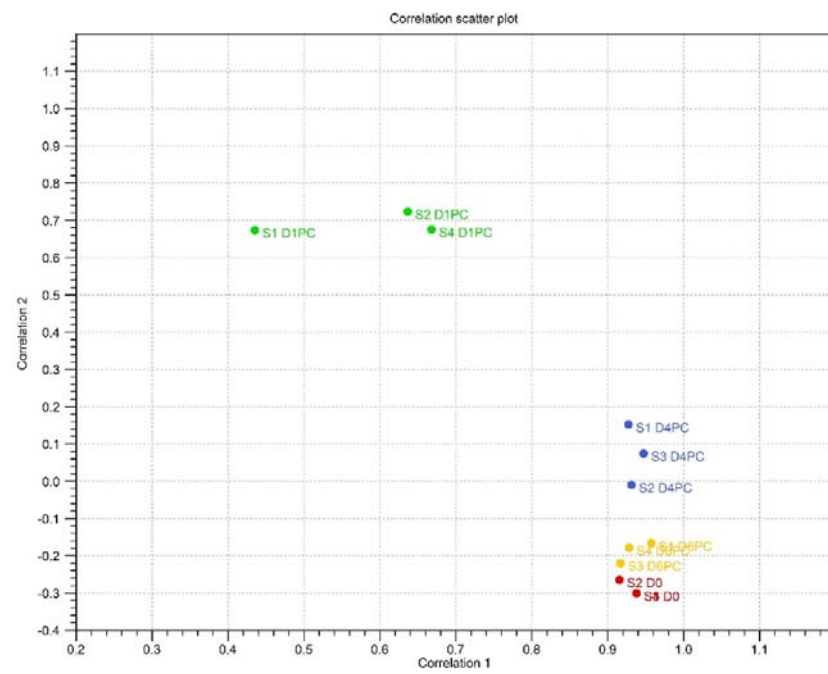
A
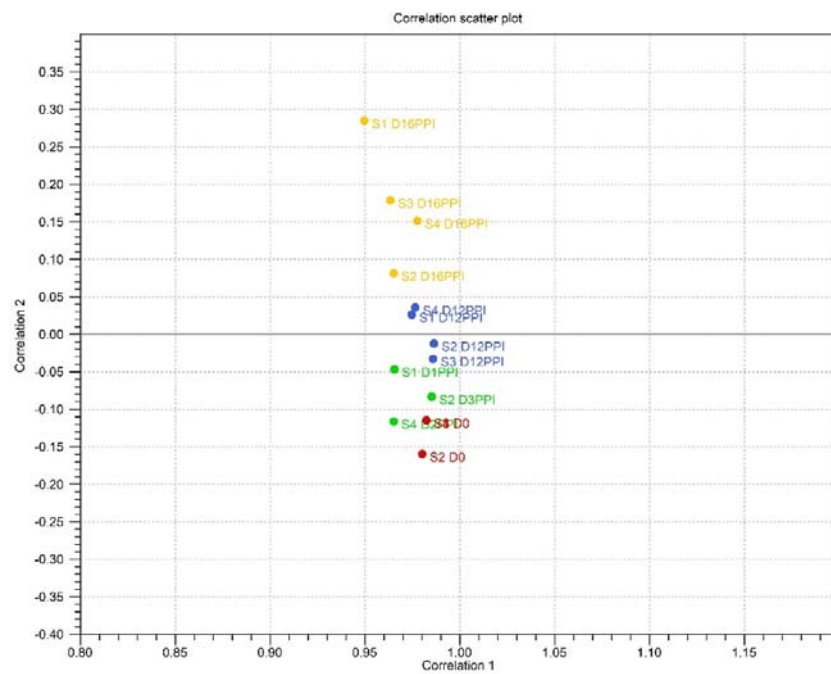
B

Figure S4: Principal component analysis correlation scatter plots of A) the post infection samples and B) the post challenge samples.
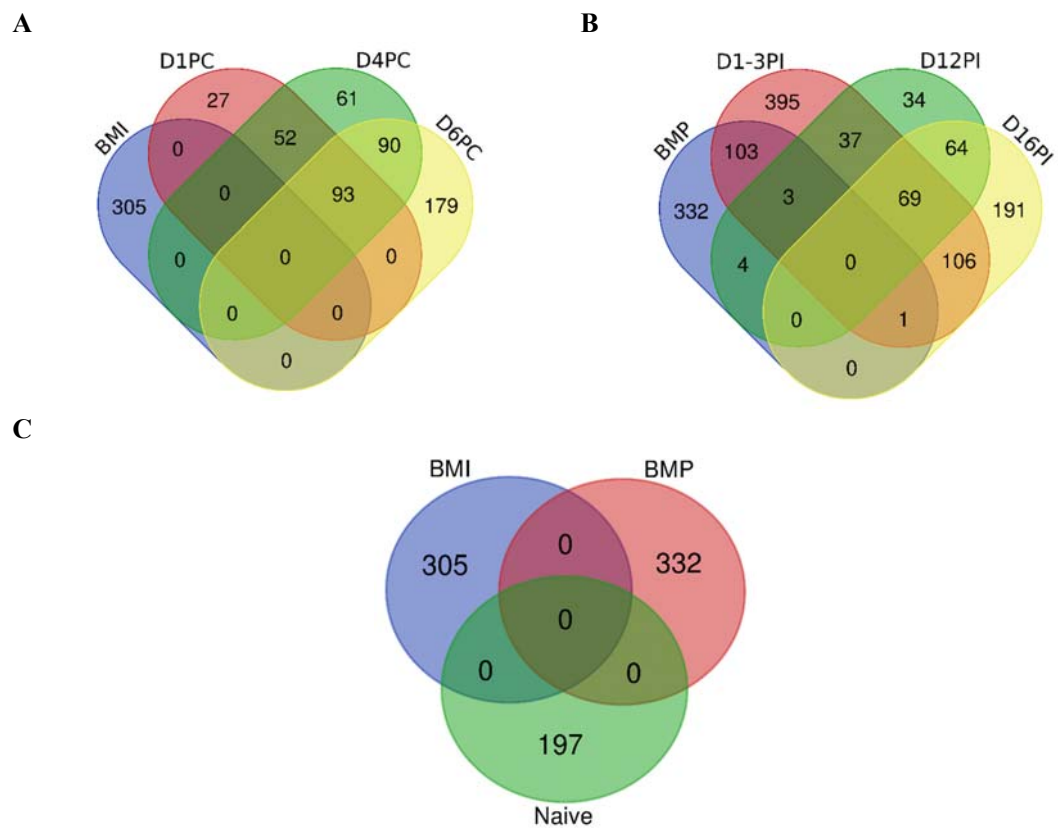
A

B

C

Figure S5: Venn analysis strategy used for the identification of BMI and BMP. Comparison of the absolutely expressed (A) and the upregulated (B) BMP DEGs compared with the upregulated DEGs in the opposite data set (these included DEGs that was upregulated in the pooled gene set and in at least 2 individual animals per time point). The selected BMI and BMP DEGs were then compared with all upregulated DEGs at any one of the two naïve time points selected in (C).
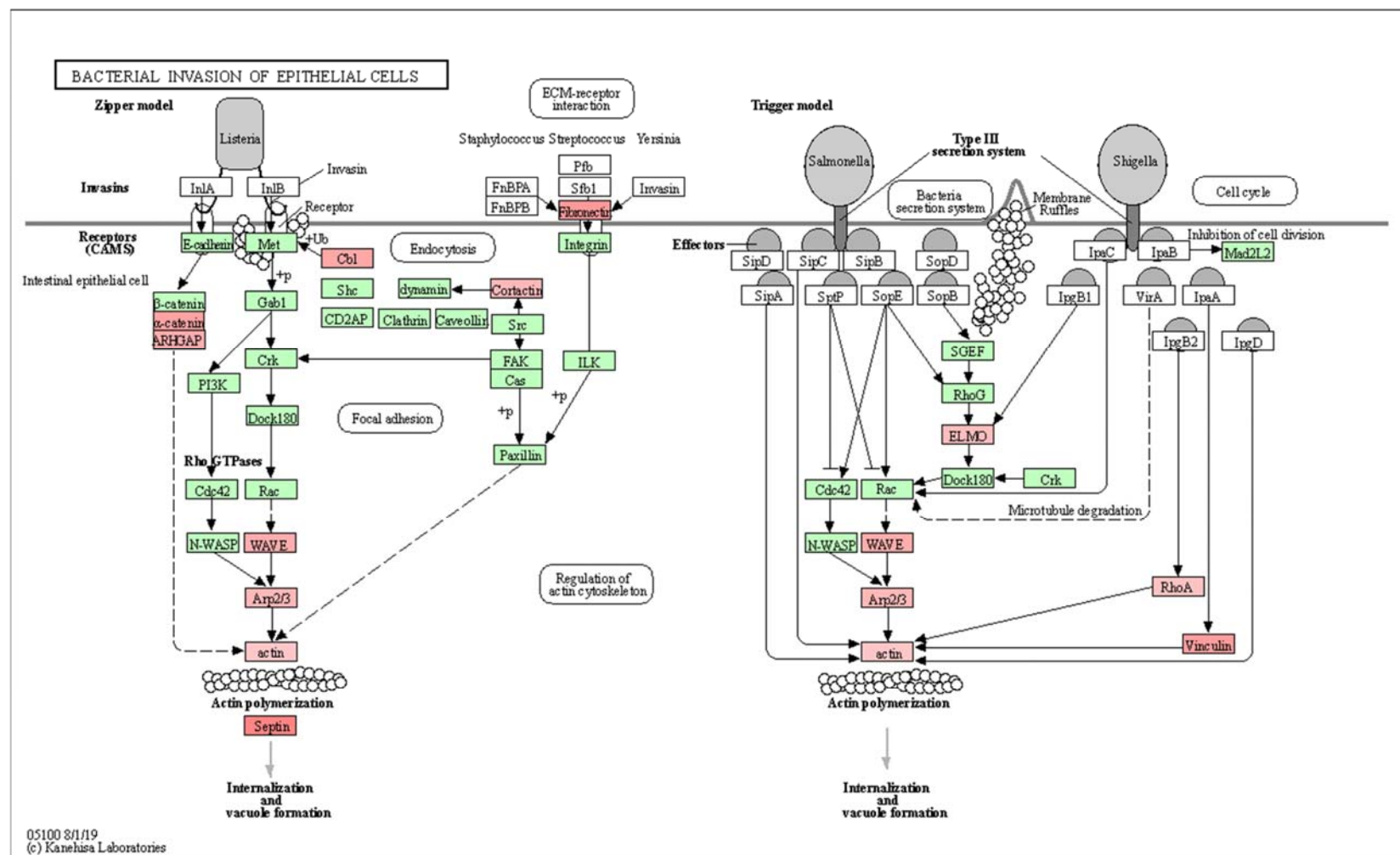
Figure S6: Heat maps of DEGs identified as BMI and BMP expressed at one or two time points. DEGs considered being BMI are shown in (A and B) and BMP are shown in (C and D). Exclusively downregulated DEGs both during primary infection and post challenge are indicated in (D). Yellow blocks indicate DEGs that was not significantly expressed and/or with RPKM values of between -1.1 and 1.1, white blocks indicate those that were not expressed (RPKM values less than 10), blue represent downregulated and red blocks upregulated DEGs. Darkest colours include those DEGs with FC< -3 and >3 so that lower expression values are also visible on the map. Maps were created with https://software.broadinstitute.org/morpheus/.

Figure S7: KEGG Bacterial invasion of host cell pathway analysis (https://www.genome.jp/pathway/oas05100) of the 305 DEGs considered BMI identified at the three PI time-points. This gene map uses epithelial cells as an example but according to the KEGG pathway description may also refer to any host cells infected, such as endothelial cells in the case for *E. ruminantium*. Red boxes indicate identified DEGs upregulated in at least one time point during infection and the expression levels are indicated from minimum (light red) to maximum (dark red).

Figure S8: KEGG Apoptosis pathway analysis (https://www.genome.jp/pathway/oas04210) of the 332 DEGs considered BMP identified at the three PI time-points. DEGs in violet were upregulated in at least one time point PC and the expression levels are indicated from minimum (light violet) to maximum (dark violet).
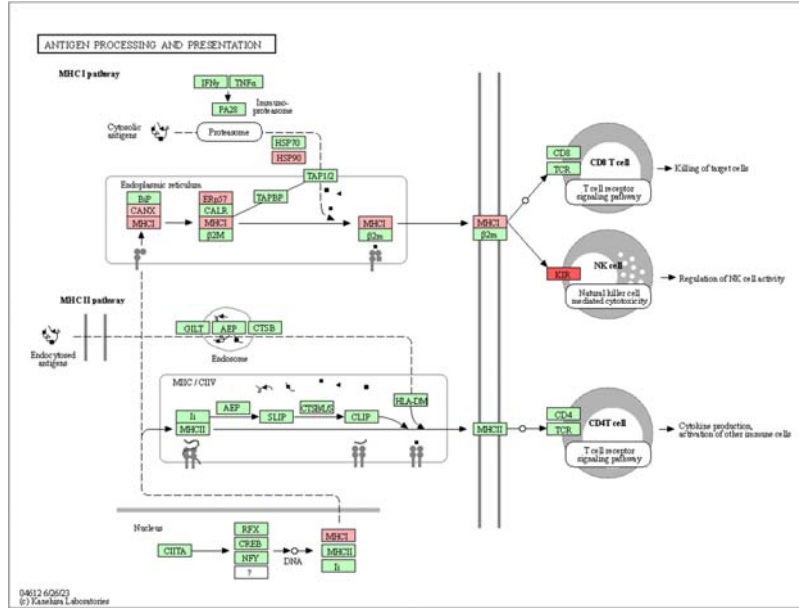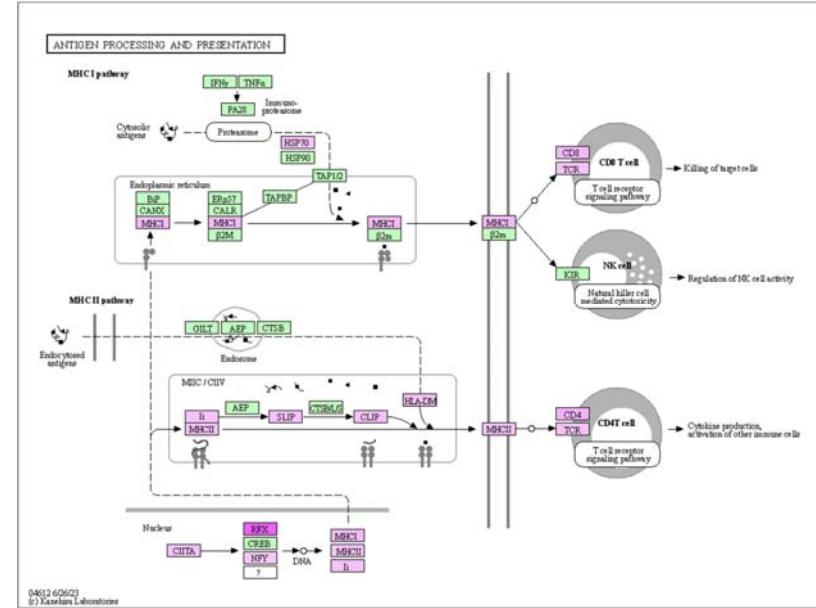
Figure S9: KEGG antigen processing and presentation pathway analysis (https://www.genome.jp/pathway/oas04612) of the 305 DEGs considered BMI (A, red) and the 332 DEGs considered and BMP (B, violet) identified at the three PI or PC time-points. It is clear that an innate NK-cell specific response was activated PI while memory response including CD8 and CD4 T cells were activated and identified as BMP, although these were only detected from D6PC. DEGs in red or violet were upregulated in at least one time point during infection. The expression levels are indicated from minimum (light) to maximum (dark).

Figure S10: Reactome analysis (https://reactome.org/PathwayBrowser/#TOOL=AT) comparing pathways identified using upregulated DEGs with the three PC time-points compared in a Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/). In addition to the unique pathways (underlined) at each time point indicated, D16PI activated many pathways in common with the PC time points (indicated with stars). There were 25 pathways in common between D1PC and D4PC and 42 pathways in common at all three the PC time-points indicated.

11