Comparison of the immune response between experimental bovine mastitis caused by different strains of *Escherichia coli*

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Supplementary Material

Supplementary Table S1: ANOVA results of parameters measured throughout the study, including significance (*P* values) for the strain and time effects and the strain × time interaction (S × T), number of measurements (n), R^2 and the percentage of variance in the trial attributed to variance between cows. In bold, statistically significant results ($P < 0.05$) between strains throughout time (S × T). Results refer only to glands challenged with one of the three mammary pathogenic E. coli (MPEC) strains studied, without control glands and glands challenged with the environmental strain K71.

	strain	time	S × T	n	r^2	Variance between cows (%)
Log_{10} SCC	0.4335	<0.0001	**0.0032**	103	0.923	NS
Leukocytes ($CD18^+$)	0.3008	<0.0001	0.9437	104	0.797	NS
$CD18^+G1^+$	0.9463	<0.0001	0.9347	104	0.689	NS
$CD4^+$	0.135	<0.0001	0.559	102	0.784	18.8
$CD8^+$	0.9161	<0.0001	0.9642	103	0.714	20.5
$CD14^+$ macrophages	0.8299	0.0015	0.9911	102	0.594	28.2
$CD14^+$ PMN	0.0344	<0.0001	0.9107	101	0.678	NS
IgG	0.4475	<0.0001	0.5566	96	0.737	24.6
$TLR4^+$ $CD18^+$	0.059	<0.0001	0.0731	104	0.779	NS
TNF^+ total	0.3392	<0.0001	0.3112	91	0.892	19.4
TNF^+ PMN	0.1041	<0.0001	**0.0224**	91	0.870	NS
TNF^+ macrophages	0.7127	<0.0001	0.6858	84	0.823	NS
TNF^+ lymphocytes	0.7787	<0.0001	0.9156	87	0.864	55.6
TNF blood	0.0142	0.3927	0.6602	44	0.880	42.4
TNF milk	0.8663	<0.0001	0.4542	99	0.799	16.3
IL-1β	0.6909	<.0001	0.6704	99	0.696	NS
IL-4	0.7665	0.4467	0.9805	99	0.370	NS
IL-6	0.1056	<0.0001	0.1185	98	0.874	NS
IL-17	0.0017	0.0003	**0.0185**	99	0.757	NS
log_{10} CFU	0.1573	<0.0001	0.7776	99	0.886	NS

NS = Variance between cows was not significant ($P > 0.05$).

Supplementary Table S2: ANOVA results of parameters measured in the first 24 h post challenge, including significance (*P* values) for the strain and time effects, and the strain × time interaction (S × T), number of measurements (n), R^2 and the percentage of variance in the trial attributed to variance between cows. In bold, statistically significant results ($P < 0.05$) between strains throughout time (S × T). Results refer only to glands challenged with one of the three MPEC strains studied, without control glands and glands challenged with the environmental strain K71.

	strain	time	S × T	n	r^2	Variance between cows (%)
log_{10} SCC	0.1072	<0.0001	**0.0240**	40	0.944	NS
$TLR4^+CD18^+$	0.3625	<0.0001	**0.0040**	40	0.891	NS
$CD18^+G1^+$PMN	0.5375	<0.0001	0.7981	40	0.799	NS
TNF blood	0.0149	0.3422	0.5422	40	0.872	NS
TNF milk	0.8268	<0.0001	0.4072	39	0.796	NS
IL-1β	0.5545	0.0024	0.6607	39	0.689	NS
IL-4	0.6312	0.4038	0.8733	39	0.439	NS
IL-6	0.0378	<0.0001	**0.0030**	38	0.959	NS
IL-17	0.3682	<.0001	**0.0405**	39	0.760	NS
log_{10} CFU	0.3682	<0.0001	0.2473	40	0.928	38.8

NS = Variance between cows was not significant ($P > 0.05$).

Dynamics of leukocytes and cytokines secretion in milk following intra-mammary challenge with three distinct MPEC strains

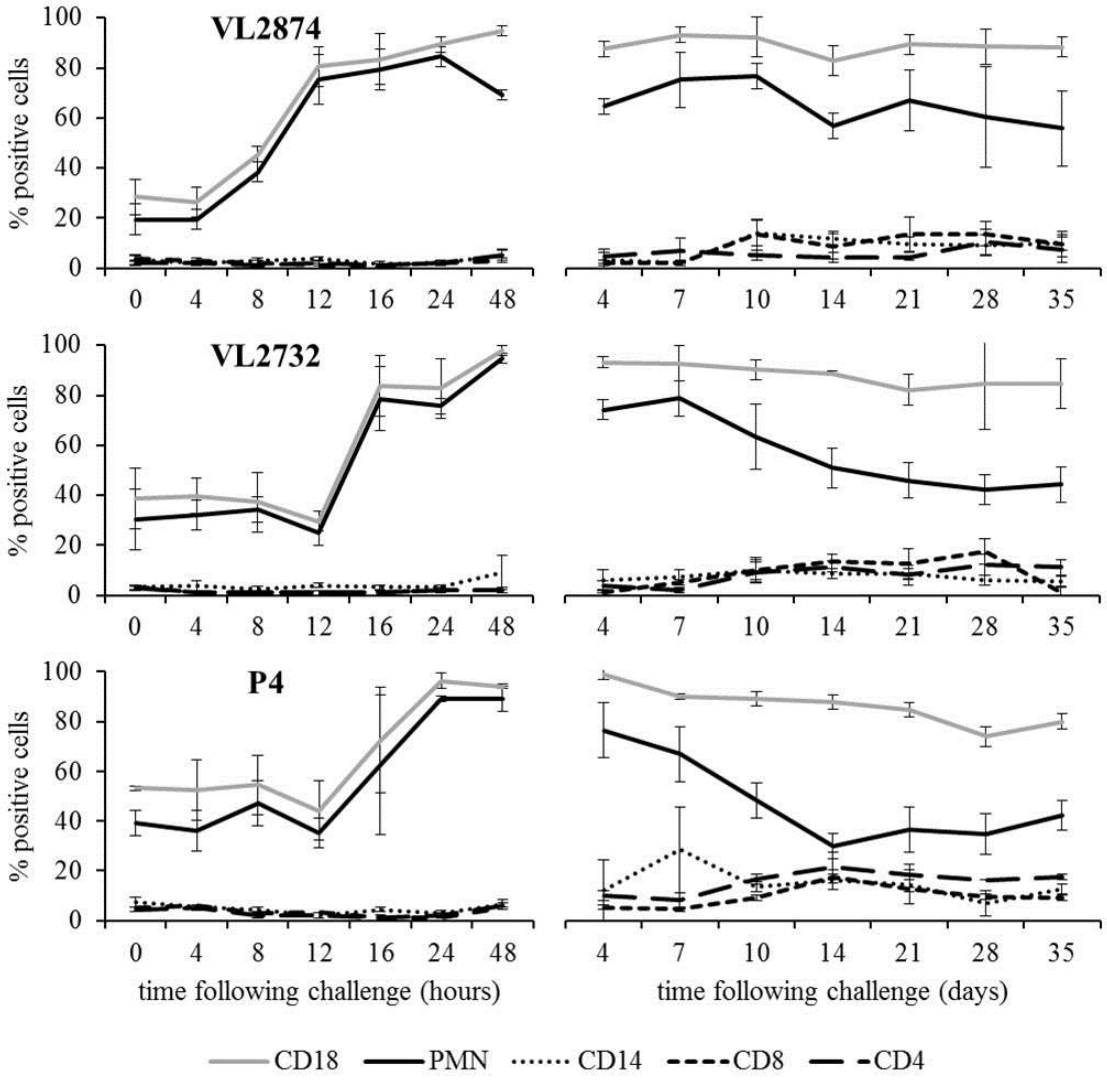
With all three MPEC strains leukocytes became 90% of the cells in milk by 24 h and remained above pre-infusion levels throughout the study. However, different leukocytes dynamics were observed with each MPEC strain. In milk from glands challenged with VL2874, PMN remained the dominating leukocytes until the end of the study, despite a low increase of mononuclear cells from about 10 days post challenge (DPC). In contrast, glands challenged with VL2732 showed significant decrease of PMN from 14 DPC, concomitantly to a slow increase in mononuclear cells. In glands challenged with P4, the shift towards mononuclear cells was more accentuated and was observed at 7 DPC, concomitantly to bacterial clearance from milk (Fig. 1). In glands challenged with strain K71, leukocytes levels did not increase following bacterial infusion and somatic cell types remained unchanged, comprising mostly epithelial cells.

The LPS co-receptor CD14 was found mainly on macrophages; with only about 10% $CD14^+$ PMN ($G1^+CD14^+$ double-positive PMN). A sharp increase in $CD14^+$ PMN was recorded in milk from glands challenged with VL2874 on 2 DPC, and later on 4 DPC in milk from glands challenged with VL2732 and P4 (Fig. 2), and could be attributed to secondary binding of soluble CD14 to PMN. Levels of double-positive PMN declined on 10 DPC until the end of the study in glands challenged with VL2732 and P4, but remained above 20% of PMN in glands challenged with VL2874.

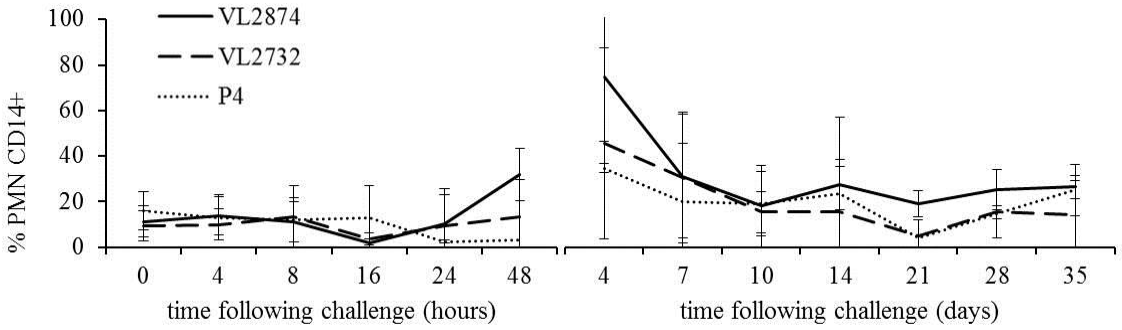
The T lymphocytes CD4 and CD8 distribution was fairly equivalent during the first 48 post-challenge (Fig. 3). The CD4:CD8 ratio significantly increased at 4 DPC, and fluctuated around

0.5 thereafter, except in glands challenged with P4, in which the CD4:CD8 ratio fluctuated around 1.5 and CD4 remained the dominant lymphocytes detected.

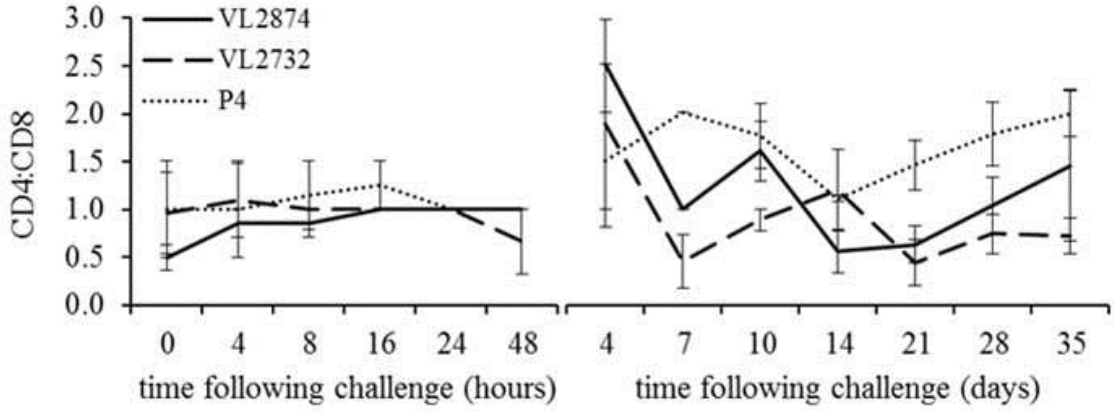
The levels of secreted cytokines in milk followed an overall expected pattern. Peak TNFα was observed at 16 h, followed by IL-1β at 24 h and IL-6 at 48 h (Fig. 4). A peak of IL-4 was recorded 8 h after bacterial infusion in glands challenged with VL2874 and VL2732, but not with P4. In glands challenged with P4, the cytokine response was either lower (IL-4, IL1β, IL6) or delayed (TNFα). Significant higher levels in milk yield were recorded in glands challenged with P4. Peak IL-17 was recorded at 7 DPC, coinciding with peak CD4:CD8 ratio, and followed by a decrease in PMN percentage and SCC.



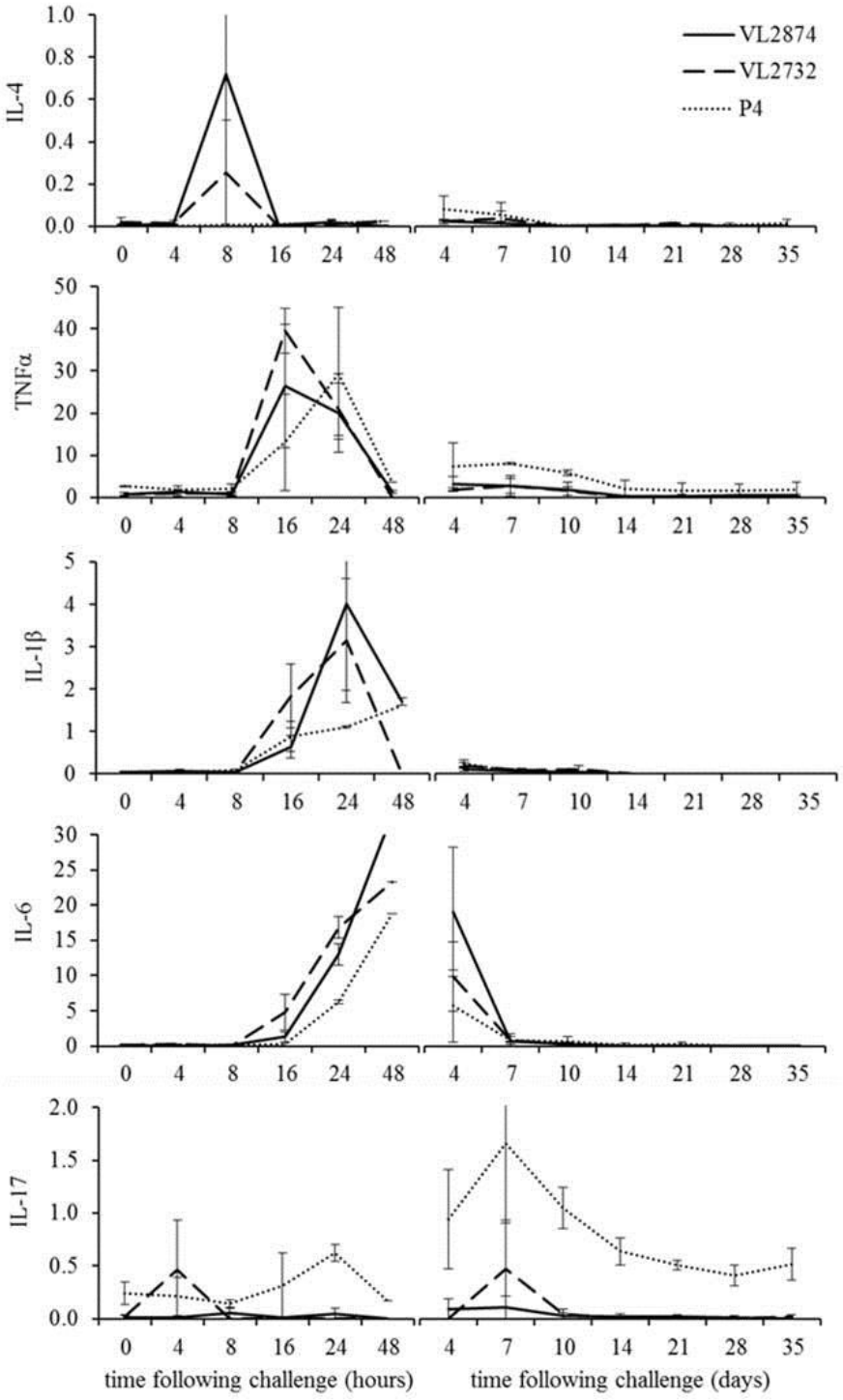
Supplementary Figure S1. Percentage of leukocytes ($CD18^+$), neutrophils (PMN), macrophages ($CD14^+$) and lymphocytes ($CD4^+$ and $CD8^+$) of glands challenged with three distinct MPEC strains (VL2874, VL2732 and P4), showing mean and SE within 48 hours (left panel) and 35 days (right panel) following challenge.



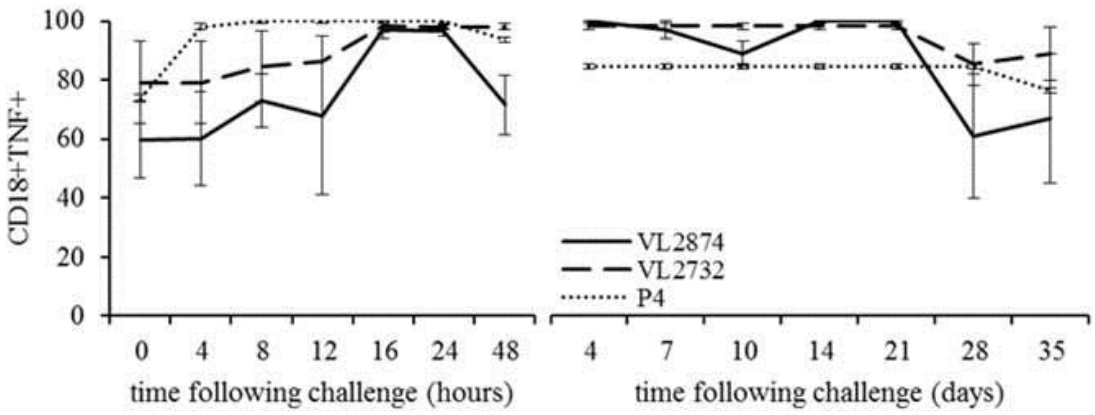
Supplementary Figure S2. $CD14^{+}G1^{+}$ PMN in milk of glands challenged with three distinct *E. coli* strains (VL2874, VL2732 and P4), showing percentage mean and SE within 48 hours (left panel) and 35 days (right panel) following challenge.



Supplementary Figure S3. T lymphocytes CD4:CD8 ratio in milk of glands challenged with three distinct *E. coli* strains (VL2874, VL2732 and P4), showing mean and SE within 48 hours (left panel) and 35 days (right panel) following challenge.



Supplementary Figure S4. Secreted cytokines levels (IL-4, TNFα, IL-1, IL-6, IL-17) in milk of glands challenged with three distinct *E. coli* strains (VL2874, VL2732 and P4), showing mean and SE within 48 hours (left panel) and 35 days (right panel) following challenge.



Supplementary Figure S5. Intra-cellular TNF in leukocytes in milk of glands challenged with three distinct MPEC strains (VL2874, VL2732 and P4), showing mean and SE within 48 hours (left panel) and 35 days (right panel) following challenge.