In the murine and bovine maternal mammary gland signal transducer and activator of transcription 3 is activated in clusters of epithelial cells around the day of birth

Laura J. A. Hardwick[1,2,§], Benjamin P. Davies[1,§], Sara Pensa[3,4], Maedee Burge-Rogers[1], Claire Davies[5], André Figueiredo Baptista[1], Robert Knott[1,6], Ian McCrone[1], Eleonora Po[1], Benjamin W. Strugnell[7], Katie Waine[7,8], Paul Wood[1,9], Walid T. Khaled[3,4], Huw D. Summers[10], Paul Rees[10,11], John W. Wills[1*], Katherine Hughes[1*]

1. Department of Veterinary Medicine, University of Cambridge, Cambridge, UK.
2. Current address: Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, UK.
3. Department of Pharmacology, University of Cambridge, Cambridge, UK.
4. Wellcome-MRC Cambridge Stem Cell Institute, University of Cambridge, Cambridge, UK.
5. The Fold Farm Vets Ltd, Tyne Green, Hexham, Northumberland, UK.
6. Current address: Bristol Veterinary School, University of Bristol, Langford, UK.
7. Farm Post Mortems Ltd, Durham, UK.
8. Current address: Faculty of Veterinary Medicine, University of Calgary, Calgary, AB T3R 1J3, Canada.
9. Current address: SRUC Aberdeen, Craibstone Estate, Bucksburn, Aberdeen, UK.
10. Department of Biomedical Engineering, Swansea University, Swansea, UK.
11. Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

§Equal contribution.

*Correspondence: Katherine Hughes, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK. Email kh387@cam.ac.uk

John W. Wills, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge, CB3 0ES, UK. Email jw2020@cam.ac.uk

ORCID IDs

Benjamin Davies: https://orcid.org/0000-0001-9808-8626

Eleonora Po: https://orcid.org/0000-0002-9777-6707

John Wills: https://orcid.org/0000-0002-4347-5394

Katherine Hughes: https://orcid.org/0000-0002-3331-1249

Statements and declarations

Funding: LJAH was funded by a Peterhouse Research Fellowship. BPD is funded by an Anatomical Society PhD Studentship awarded to KH. This research was funded by a grant awarded to KH by the British Veterinary Association Animal Welfare Foundation Norman Hayward Fund (NHF_2016_03_KH).

Competing interests: Katherine Hughes is an Editorial Board Member for the Journal of Mammary Gland Biology and Neoplasia.

Ethics approval: The Ethics and Welfare Committee of the Department of Veterinary Medicine, University of Cambridge, reviewed the study plan relating to the use of tissue from bovine post mortem examinations for the study of mammary gland biology (references: CR223 and CR625). For mouse work, all animals were treated according to local ethical committee and UK Home Office guidelines.

Consent: Owners of animals provided informed consent for tissue to be collected for research purposes.

Authors' contribution statements: LJAH and KH designed the study. KH supervised the study. LJAH, BPD and KH conducted the experiments. Data was collected and/or analysed

by LJAH, BPD, MB-R, CD, AFB, RK, IMC, EP, BWS, KW, PW and KH. The laboratory of WTK provided the murine tissue. Mouse experiments were conducted by SP. HDS, PR, and JWW performed the computational image analysis and spatial statistics. The first draft of the manuscript was written by KH. All authors critically revised the manuscript and read and approved the final manuscript and author contribution statements.

Abstract

Signal transducers and activators of transcription (STAT) proteins regulate mammary development. Here we investigate the expression of phosphorylated STAT3 (pSTAT3) in the mouse and cow around the day of birth. We present localised colocation analysis, applicable to other mammary studies requiring identification of spatially congregated events. We demonstrate that pSTAT3-positive events are multifocally clustered in a non-random and statistically significant fashion. Arginase-1 expressing cells, consistent with macrophages, exhibit distinct clustering within the periparturient mammary gland. These findings represent a new facet of mammary STAT3 biology, and point to the presence of mammary sub-microenvironments.

Keywords

Macrophage, mammary, parturition, STAT3, udder, spatial statistics

71 The mammary gland exhibits extensive postnatal development [1-3]. Signal transducers and
72 activators of transcription (STAT) proteins are classically activated by phosphorylation, and
73 play key roles in regulating this development [4]. STAT3 is particularly associated with post
74 lactational regression (involution) and there is striking up-regulation of phosphorylated
75 STAT3 (pSTAT3) following the onset of involution [5-8]. During involution STAT3
76 constitutes a key regulator of cell death [5, 9, 10] and modulates the mammary
77 microenvironment [11]. A pulse of expression of pSTAT3 protein is also observed on the day
78 of birth in mice although this has received less focus than the prolonged activation of STAT3
79 accompanying involution [6].
80
81 It is well-established that there is a periparturient period of immunosuppression, and cows are
82 very susceptible to mastitis at this time [12]. Given the susceptibility of the mammary gland
83 to mastitis around the day of birth, and that we have previously demonstrated that during
84 involution mammary epithelial STAT3 regulates genes associated with the acute phase
85 response and has immunomodulatory effects [11], we considered that better understanding of
86 the distribution of pSTAT3-expressing cells will impact understanding of the periparturient
87 mammary immune microenvironment. We therefore sought to investigate the expression of
88 pSTAT3 in the murine and bovine mammary gland around the day of birth.
89
90 We first examined immunohistochemical expression of pSTAT3 in murine mammary tissue
91 from 17.5 d gestation and 2 d lactation. These time points flank the previously observed pulse
92 of mammary pSTAT3 expression that was recorded at 0 d lactation, but not at 15 d gestation
93 or 5 d lactation [6]. Murine mammary levels of pSTAT3 expression are extremely variable at
94 17.5 d gestation and 2 d lactation, with large parts of the gland exhibiting minimal pSTAT3
95 expression. However, where nuclear pSTAT3 expression is present in luminal epithelial cells,

96 indicating activated STAT3, expression is frequently restricted to individual mammary
97 alveoli or clusters of alveoli (Fig. 1 and Online Resource 1). This distribution is similar to
98 previously reported patterns of transferrin gene expression in rats [13]. In view of this
99 observation, we wished to determine whether a similar pattern of pSTAT3 expression was
100 observed in bovine mammary tissue. The gestation length of cows is affected by breed but is
101 approximately 279-290 d, so we examined tissue from cows between 248 d gestation and 46
102 d lactation (Online resource 2).
103
104 The udder of cows in the last third of gestation, and in early lactation, exhibits variable levels
105 of mammary alveolar development and expansion. pSTAT3 expression levels also vary
106 dramatically within a single mamma, between different mammae of the same animal, and
107 between animals of similar reproductive stage. However, we noted foci with pSTAT3
108 expression patterns similar to those of the mouse, with pSTAT3 expression localised to
109 individual ducts or groups of alveoli (Figure 1, Online resource 3).
110
111 Given the variable levels of alveolar expansion that we observed in the udder, and the
112 demonstration by other investigators that a transient increase in STAT3 phosphorylation can
113 be observed in mammary epithelial cells subjected to mechanical stress mimicking
114 involution-associated distension [14], we considered it possible that the level of mammary
115 alveolar dilation was affecting pSTAT3 expression. However, expression of pSTAT3 is
116 unaffected by alveolar dimensions (Online resource 4).
117
118 Examination of tissue sections suggested that mammary alveoli are frequently composed of a
119 majority of either pSTAT3-positive or pSTAT3-negative luminal epithelial cells. We
120 interrogated this observation by devising a grading scheme for mammary alveolar pSTAT3

121 expression (Methods and Fig. 1c). We applied the grading scheme to 25 randomly selected
122 mammary alveoli from 12 mammae from 7 individual cows, comprising a total analysis of
123 300 randomly selected mammary alveoli. This revealed that the frequency distribution of
124 mammary alveolar grades is skewed towards either low (grade 1) or high (grade 4) grade and
125 that there is a relative paucity of alveoli with a relatively even balance of cells expressing
126 pSTAT3 and not exhibiting pSTAT3 expression (grades 2 and 3) (Fig. 1d). This indicates that
127 in most mammary alveoli, there is a predominance of either pSTAT3-negative or pSTAT3-
128 positive cells, and therefore suggests that there may be an alveolar-level commitment to a
129 pSTAT3 transcriptional profile.
130
131 We wished to further investigate the qualitative observation that pSTAT3-positive mammary
132 alveoli were clustered. To analyse any potential alveolar associations, we randomly selected
133 alveoli exhibiting any degree of pSTAT3 positivity and analysed the positivity of all adjacent
134 alveoli in the same lobule. Mammary alveoli exhibiting any degree of luminal epithelial
135 pSTAT3 positivity have a significantly higher proportion of pSTAT3-positive neighbouring
136 alveoli than those mammary alveoli that are composed entirely of luminal epithelial cells in
137 which pSTAT3 expression is not detected (Fig. 1e). This may reflect the arrangement of
138 neighbouring alveoli in terminal duct lobular units that are drained by the same branch of the
139 mammary ductal tree.
140
141 We have previously utilised spatial statistical analyses (Getis-Ord GI*) to demonstrate
142 mammary congregation of positive immunohistochemical events [2]. In this study we
143 performed a similar spatial analysis, adopting a local colocation quotient metric (LCQ) and
144 further refining the image analysis by removal of the spaces created by the mammary
145 ductular and alveolar lumina, where positive events cannot occur, whilst retaining the ability

to detect positive events occurring in shed cells within the lumina. This confirmed that pSTAT3-positive immunohistochemical events are multifocally clustered in a non-random and statistically significant fashion within the mammary parenchyma (Fig. 2).

We wished to determine whether there was a relationship between mammary expression of pSTAT3 and immune cell distribution within the gland, specifically focusing on macrophages as a pilot investigation. Macrophages have distinct spatial and temporal dynamics in the mammary gland of pre-pregnant sheep [2] and we have previously demonstrated that, during involution of the murine mammary gland, macrophage phenotype is modulated by epithelial pSTAT3 signalling [11].

Ionised calcium binding adaptor molecule 1 (IBA1) is involved in macrophage membrane ruffling and expression is common to macrophages and microglia [15]. Using dual staining immunohistochemistry (IHC) we demonstrated that cells expressing IBA1 are distributed in a relatively uniform pattern irrespective of the pSTAT3-status of adjacent mammary alveoli (Fig. 3a and b). Arginase-1 expression is associated with an immunomodulatory phenotype in macrophages [16]. In our pilot investigation, we noted that cells expressing arginase-1 are less evenly distributed within the periparturient mammary gland, exhibiting distinct clustering. When present, arginase-1 expression colocalises with IBA1 expression, allowing inference that these cells are likely immunomodulatory macrophages (Fig. 3c and d). Overall, these data point to the periparturient mammary gland having different sub-microenvironments where the transcriptional profile of the mammary alveoli, and the composition of the immune cell compartment, may vary.

Taken together, these observations shed light on an aspect of mammary STAT biology that has previously received less attention than other facets of STAT activity related to the mammary postnatal developmental cycle [4]. The finding that pSTAT3 is expressed predominantly in the mammary luminal epithelium during the periparturient period raises important questions regarding the function of this transcription factor at this developmental stage, particularly given its well-known role during post lactational regression [5, 9-11]. Although structural and functional differences between the periparturient and involution stages of mammary development may suggest a lack of commonality between these two postnatal developmental time points, a subset of STAT3 target genes that are upregulated during involution also exhibit upregulation on the day of birth. These include leucine rich alpha-2 glycoprotein 1 and CD14, although the latter finding is inconsistent between studies [11, 17, 18]. It is therefore possible that STAT3 upregulation around the day of birth may modulate the immune milieu of the gland.

The concept of heterogeneous mammary expression of proteins during pregnancy and early lactation in the ruminant has been previously established. Hotspots, and less frequently, gradients, of alpha lactalbumin, alpha-S1-casein and lactoferrin expression have been documented in the mammary gland of sheep and cattle [19]. Given that we demonstrate multifocal hotspots of pSTAT3 expression, this may suggest that local autocrine and paracrine influences are of significance in sculpting the periparturient glandular microenvironment. This, and the uneven distribution of immunomodulatory arginase-1 expressing macrophages, are likely important in the light of the overall susceptibility of the ruminant gland to periparturient mastitis [20].

Although the alveolar dimensions are similar between STAT3-expressing and non-expressing mammary alveoli, this does not rule out the possibility that during the periparturient period, sub-compartments of the mammary parenchyma may be at differing phases of alveolar development, lactational stasis, or even overt involution. It is possible/likely that STAT3 may have differing functions in different contexts, similar to other STATS [21]. For example, it is possible that pSTAT3 expression may be associated with transient upregulation of immune factors at the commencement of lactation, but in other mammary sub-compartments there may, in parallel, be localised initiation of involution associated with sustained non-drainage of secretion from specific terminal duct lobular units. Importantly, this study suggests that the mammary microenvironment likely has local sub-microenvironments.

In this analysis we capitalise on the use of spatial statistics to demonstrate significant clustering of mammary pSTAT3 expression. Assessment of the spatial distribution of cells is a powerful tool in cell biology and histopathology [22], particularly so when studying heterogeneous tissue in which marked spatial variation, and the presence of sub-microenvironments, limits the value of whole-population statistics. Importantly, in this study the spatial analysis takes account of the structure of the mammary gland where the presence of lumina of ducts and alveoli poses a particular challenge in assessment of clustering of positive immunohistochemical events as the regions of the image not occupied by cells need to be accounted for. The localised colocation analysis presented here will be applicable to other mammary studies where identification, quantification and interrogation of significant, spatially congregated events is required.

This study has several limitations. The use of mammary tissue from non-experimental cows means that the animals sampled were of several different breeds or crosses, although the

majority were Holstein Friesian cows. Lactating cows would have had variable intervals since last milking. All the cows had concurrent morbidities, including in two cases foci of mastitis. Concurrent inflammation may influence the immune phenotype of the gland. However, in one of the two mastitis cases (case 5) both the right and left fore quarters were included in the analyses and only the left fore quarter had mastitis. In this case, both quarters had minimal pSTAT3 epithelial staining and thus the presence of an inflammatory focus appeared to have no impact. In the other case of mastitis (case 7) there was no microscopic correlation of pSTAT3 positivity with foci of inflammation. Despite the limitations of this study, the presence of clusters of pSTAT3-positive epithelial cells in the cows is strikingly similar to the tissues derived from healthy experimental mice at the defined time points of 17.5 d gestation and 2 d lactation.

It is also noteworthy that in timepoints around the day of birth pSTAT3 expression is not restricted to luminal epithelial cells and is noted in other cell populations including presumed myoepithelial cells and infiltrating immune cells (Fig. 1c). It may be informative to interrogate pSTAT3 expression in different mammary cellular compartments in future investigations.

This analysis raises interesting questions for future investigations, most specifically examining correlation of pSTAT3 expression with expression of STAT3 target genes. Single cell transcriptomic technologies have already been widely adopted in the mammary field [23] and spatial transcriptomics would be well suited to investigation of the role of clustered pSTAT3 expression in the periparturient gland and the definition of mammary sub-microenvironments.

Our study reveals similarities between the mouse and the cow, lending weight to the assertion that ruminants are valuable non-traditional models of mammary developmental processes [24]. Our work demonstrates that around the day of birth, in the murine and bovine mammary gland there is mammary alveolar-level commitment to a pSTAT3 transcriptional profile and pSTAT3-positive mammary alveoli are frequently grouped, as are arginase-1 expressing macrophages. pSTAT3 is an important regulator of the mammary microenvironment in other contexts. This finding therefore represents a new facet of mammary STAT3 biology meriting further functional investigation.

Materials and methods

Animals

Mammary tissue was collected from C57BL/6 mice at 17.5 days gestation and 2 days lactation following standard husbandry procedures. Udder tissue was collected from cows that were submitted to the diagnostic veterinary anatomic pathology post mortem service of the University of Cambridge or from cows that were euthanised by veterinarians in practice (Online resource 2). The cause of death of the animal was recorded as part of the post mortem examination procedure and/or preceding clinical investigations. No information was available regarding time since last milking or suckling.

Histology, immunohistochemistry and analyses

Mammary tissue was fixed in 10% neutral-buffered formalin. Tissues were processed using a standard methodology and 5 μm tissue sections were cut and stained with haematoxylin and eosin.

IHC followed a standard protocol using a PT link antigen retrieval system with high pH antigen retrieval solution (both Dako Pathology/Agilent Technologies, Stockport, UK). For dual IHC an ImmPRESS® Duet Double Staining Polymer Kit (Vector Laboratories) was used. Antibodies for pSTAT3 (1:100, rabbit monoclonal antibody #9145, Cell Signaling Technology; or 1:100, mouse monoclonal antibody #4113, Cell Signaling Technology), IBA1 (1:1200, rabbit monoclonal antibody, ab178846, Abcam or 1:800 mouse monoclonal antibody, MABN92, Merck) and Arginase-1 (1:250 mouse monoclonal antibody, ab215894, Abcam) were incubated overnight at 4ºC and secondary antibodies were incubated for thirty minutes at room temperature. Negative control slides were treated with isotype- and species-matched immunoglobulins. Slides were counterstained using Mayer's Haematoxylin for 3 minutes.

Slides were scanned at 40× using a NanoZoomer 2.0RS, C10730, (Hamamatsu Photonics, Hamamatsu City, Japan) and were analysed with the associated viewing software (NDP.view2, Hamamatsu Photonics).

A random selection of either positive or negative alveoli were selected on a scanned slide at low magnification (25 of each per slide). Alveolar dimensions were measured using NDP.view2 and positive alveoli were assigned a grade 1-4. Grade 1 alveoli were those exhibiting 25% or less pSTAT3-positive luminal epithelial cells, grade 2 alveoli exhibited 26-50% pSTAT3-positive luminal epithelial cells, grade 3 alveoli exhibited 51-75% pSTAT3-positive luminal epithelial cells, and grade 4 alveoli were those with 76% or more pSTAT3-positive luminal epithelial cells. The number of immediately adjacent alveoli were counted and those with positive epithelial cells noted and converted to a percentage of positive neighbours for the initially selected alveolus.

pSTAT3: Local correlation quotient spatial statistics

The centroid locations of pSTAT3+ and pSTAT3- nuclei alongside masks for tissue 'void areas' unpopulated by cells were extracted using pixel-classification machine learning using the freely available Ilastik and CellProfiler softwares using methods described in previous works [25] Statistically significant clustering of pSTAT3+ events relative to what would be expected by random chance were identified using the local correlation quotient (LCQ) statistic [26, 27] defined as:

$$\mathrm{LCQ} = \frac{n_B/n_A}{(N_B-1)/(N_A-1)} \qquad \text{(equation 1)}$$

Where N_A is the global number of all nuclei and N_B is the global number of pSTAT3+ nuclei. n_A is the local number of all nuclei and n_B the local number of pSTAT3+ nuclei. The size of the local area was dynamically set for each cell according to the local cell density. A Gaussian spatial filter was used with a bandwidth equal to the distance to the 10^{th} nearest neighbour. To enable reproducibility, all image-data, image analysis steps in Ilastik and CellProfiler as well as the MATLAB code used to calculate the LCQ measure are available for download from the BioStudies database under accession number S-BSST1025 (https://www.ebi.ac.uk/biostudies/studies/S-BSST1025).

Acknowledgements

The authors would like to thank Debbie Sabin, Ross Barker and Emma Ward of the Department of Veterinary Medicine, University of Cambridge, for their excellent technical expertise in the preparation of tissue sections, and Mathew Rhodes, of the same department,

for technical assistance in the post mortem room. The authors gratefully acknowledge the Ethics and Welfare Committee of the Department of Veterinary Medicine, University of Cambridge, for their review of the study plan relating to the use of tissue from bovine post mortem examinations for the study of mammary gland biology (references: CR223 and CR625). Parts of this data were presented in oral abstract form at the Anatomical Society Summer Meeting 2023 in Bangor, Wales (25-27 July 2023; presentation date 26 July 2023).

References

1. Hughes, K., *Comparative mammary gland postnatal development and tumourigenesis in the sheep, cow, cat and rabbit: Exploring the menagerie.* Semin Cell Dev Biol, 2021. **114**: 186-195.
2. Nagy, D., C.M.C. Gillis, K. Davies, A.L. Fowden, P. Rees, J.W. Wills, and K. Hughes, *Developing ovine mammary terminal duct lobular units have a dynamic mucosal and stromal immune microenvironment.* Commun Biol, 2021. **4**(1): 993.
3. Vang, A.L., T. Bresolin, W.S. Frizzarini, G.L. Menezes, T. Cunha, G.J.M. Rosa, L.L. Hernandez, and J.R.R. Dorea, *Longitudinal analysis of bovine mammary gland development.* J Mammary Gland Biol Neoplasia, 2023. **28**(1): 11.
4. Hughes, K. and C.J. Watson, *The spectrum of STAT functions in mammary gland development.* JAKSTAT, 2012. **1**(3): 151-8.
5. Chapman, R.S., P.C. Lourenco, E. Tonner, D.J. Flint, S. Selbert, K. Takeda, S. Akira, A.R. Clarke, and C.J. Watson, *Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3.* Genes Dev, 1999. **13**(19): 2604-16.
6. Kritikou, E.A., A. Sharkey, K. Abell, P.J. Came, E. Anderson, R.W. Clarkson, and C.J. Watson, *A dual, non-redundant, role for LIF as a regulator of development and*

STAT3-mediated cell death in mammary gland. Development, 2003. **130**(15): 3459-68.

7. Singh, K., I. Vetharaniam, J.M. Dobson, M. Prewitz, K. Oden, R. Murney, K.M. Swanson, R. McDonald, H.V. Henderson, and K. Stelwagen, *Cell survival signaling in the bovine mammary gland during the transition from lactation to involution.* J Dairy Sci, 2016. **99**(9): 7523-7543.

8. Singh, K., C.V.C. Phyn, M. Reinsch, J.M. Dobson, K. Oden, S.R. Davis, K. Stelwagen, H.V. Henderson, and A.J. Molenaar, *Temporal and spatial heterogeneity in milk and immune-related gene expression during mammary gland involution in dairy cows.* J Dairy Sci, 2017. **100**(9): 7669-7685.

9. Kreuzaler, P.A., A.D. Staniszewska, W. Li, N. Omidvar, B. Kedjouar, J. Turkson, V. Poli, R.A. Flavell, R.W. Clarkson, and C.J. Watson, *Stat3 controls lysosomal-mediated cell death in vivo.* Nat Cell Biol, 2011. **13**(3): 303-9.

10. Sargeant, T.J., B. Lloyd-Lewis, H.K. Resemann, A. Ramos-Montoya, J. Skepper, and C.J. Watson, *Stat3 controls cell death during mammary gland involution by regulating uptake of milk fat globules and lysosomal membrane permeabilization.* Nat Cell Biol, 2014. **16**(11): 1057-1068.

11. Hughes, K., J.A. Wickenden, J.E. Allen, and C.J. Watson, *Conditional deletion of Stat3 in mammary epithelium impairs the acute phase response and modulates immune cell numbers during post-lactational regression.* J Pathol, 2012. **227**(1): 106-17.

12. Sordillo, L.M. and K.L. Streicher, *Mammary gland immunity and mastitis susceptibility.* J Mammary Gland Biol Neoplasia, 2002. **7**(2): 135-46.

13. McCracken, J.Y., A.J. Molenaar, R.J. Wilkins, and M.R. Grigor, *Spatial and temporal expression of transferrin gene in the rat mammary gland.* J Dairy Sci, 1994. **77**(7): 1828-34.

14. Quaglino, A., M. Salierno, J. Pellegrotti, N. Rubinstein, and E.C. Kordon, *Mechanical strain induces involution-associated events in mammary epithelial cells.* BMC Cell Biol, 2009. **10**: 55.

15. Ohsawa, K., Y. Imai, H. Kanazawa, Y. Sasaki, and S. Kohsaka, *Involvement of Iba1 in membrane ruffling and phagocytosis of macrophages/microglia.* J Cell Sci, 2000. **113 (Pt 17)**: 3073-84.

16. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions.* Immunity, 2010. **32**(5): 593-604.

17. Clarkson, R.W., M.T. Wayland, J. Lee, T. Freeman, and C.J. Watson, *Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression.* Breast Cancer Res, 2004. **6**(2): R92-109.

18. Stein, T., J.S. Morris, C.R. Davies, S.J. Weber-Hall, M.A. Duffy, V.J. Heath, A.K. Bell, R.K. Ferrier, G.P. Sandilands, and B.A. Gusterson, *Involution of the mouse mammary gland is associated with an immune cascade and an acute-phase response, involving LBP, CD14 and STAT3.* Breast Cancer Res, 2004. **6**(2): R75-91.

19. Molenaar, A.J., S.R. Davis, and R.J. Wilkins, *Expression of alpha-lactalbumin, alpha-S1-casein, and lactoferrin genes is heterogeneous in sheep and cattle mammary tissue.* J Histochem Cytochem, 1992. **40**(5): 611-8.

20. Oliver, S.P. and L.M. Sordillo, *Udder health in the periparturient period.* J Dairy Sci, 1988. **71**(9): p. 2584-606.

21. Molenaar, A.J., T.T. Wheeler, and M.R. Grigor, *Nuclear localisation of the transcription factor Stat5b is associated with ovine milk protein gene expression during lactation but not during late pregnancy or forced weaning.* Histochem J, 2000. **32**(5): 265-74.
22. Summers, H.D., J.W. Wills, and P. Rees, *Spatial statistics is a comprehensive tool for quantifying cell neighbor relationships and biological processes via tissue image analysis.* Cell Rep Methods, 2022. **2**(11): 100348.
23. Twigger, A.J. and W.T. Khaled, *Mammary gland development from a single cell 'omics view.* Semin Cell Dev Biol, 2021. **114**: 171-185.
24. Hughes, K., *Studying Mammary Physiology and Pathology in Domestic Species Benefits Both Humans and Animals.* J Mammary Gland Biol Neoplasia, 2023. **28**(1): 18.
25. Wills, J.W., J. Robertson, H.D. Summers, M. Miniter, C. Barnes, R.E. Hewitt, A.V. Keita, J.D. Soderholm, P. Rees, and J.J. Powell, *Image-Based Cell Profiling Enables Quantitative Tissue Microscopy in Gastroenterology.* Cytometry A, 2020. **97**(12): 1222-1237.
26. Cromley, R.G., D.M. Hanink, and G.C. Bentley, *Geographically weighted colocation quotients: Specification and application.* The Professional Geographer, 2014. **66**(1): 138-148.
27. Wills, J.W., J. Robertson, P. Tourlomousis, C.M.C. Gillis, C.M. Barnes, M. Miniter, R.E. Hewitt, C.E. Bryant, H.D. Summers, J.J. Powell, and P. Rees, *Label-free cell segmentation of diverse lymphoid tissues in 2D and 3D.* Cell Rep Methods, 2023. **3**(2): 100398.

415 **Figure legends**

416 **Fig. 1 Around the day of birth there is polarisation of alveoli towards either a low- or**
417 **high- proportion of pSTAT3 positive alveolar epithelial cells.** (a, b) Murine tissue from
418 17.5 dG (a) and 2 dL (b). IHC for pSTAT3 (brown) with haematoxylin counterstain. Arrow
419 indicates rare pSTAT3 positive alveolus. Scale bar = 40 μm (a) and 80 μm (b). Images are
420 representative of 5 mice (2 mice 17.5 dG; 3 mice 2 dL). (c) Example images (case 1)
421 illustrating bovine grading scheme used to denote proportion of pSTAT3 positive alveolar
422 luminal epithelial cells within an alveolus (*). Grade 1: 25% or less positive luminal
423 epithelial cells; grade 2: 26 - 50% positive; grade 3: 51-75% positive; grade 4: 76 - 100%
424 positive. IHC for pSTAT3 (brown) with haematoxylin counterstain. Scale bar = 30 μm. (d)
425 Frequency histogram showing distribution of grades of 300 bovine alveoli selected at random
426 from immunohistochemically stained slides from 12 mammary quarters (25 alveoli per
427 quarter) from 7 cows. (e) pSTAT3 positive mammary alveoli have a higher proportion of
428 pSTAT3-positive neighbouring alveoli. Results represent mean % of neighbouring alveoli
429 that are positive for 25 alveoli analysed from each of 12 quarters from 7 cows (negative
430 alveoli) and 7 quarters from 4 cows (positive alveoli). *** $p<0.001$. dG, days gestation; dL,
431 days lactation.
432
433 **Fig. 2 During late gestation and early lactation the bovine mammary gland exhibits**
434 **hotspots of pSTAT3 expression.** IHC for pSTAT3 (a, c, e, g) and accompanying spatial
435 statistical analyses (local colocation quotient) (b, d, f, h) demonstrating regions with
436 significant spatial congregation of pSTAT3+ cells. Mammary gland from cows 248 dG (a, b),
437 1 dL (c, d) 8 dL (e, f) and 46 dL (g, h); dG, days gestation; dL, days lactation. a, c, e, g
438 Haematoxylin counterstain. Scale bar = 200 μm.
439

Fig. 3 During late gestation and early lactation, IBA1-positive and ARG1-positive cells, consistent with macrophages, are present in the bovine mammary gland. IHC for pSTAT3 and IBA1 (a, b), pSTAT3 and ARG1 (c) and IBA1 and ARG1 (d). (a) Arrow indicates alveolus with pSTAT3 positive cells. (c) Arrow indicates cluster of ARG1 positive cells. (d) Blue arrows indicate IBA1-positive macrophages. White arrow indicates dual IBA1- and ARG1-positive cell. * indicates non-specific staining of mammary secretory product. Mammary gland from cows 248 dG (a, b), 8 dL (c) and 1 dL (d); dG, days gestation; dL, days lactation. Haematoxylin counterstain. Scale bar = 80 μm. Images are representative of 4 cows.