Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Prime-pull immunisation with yeast-based vaccine modifies cell trafficking and induces T-cell immunity in the bovine mammary gland

Célya Danzelle
ISP, INRAE, Université de Tours, UMR1282
Pablo Gomes Noleto
ISP, INRAE, Université de Tours, UMR1282
Patricia Cunha
ISP, INRAE, Université de Tours, UMR1282
Florence B. Gilbert
ISP, INRAE, Université de Tours, UMR1282
Yves Le Vern
ISP, INRAE, Université de Tours, UMR1282
Olivier Boulesteix
PFIE, INRAE, UE1277 Nouzilly, France
Pierre Germon
ISP, INRAE, Université de Tours, UMR1282
Pascal Rainard
ISP, INRAE, Université de Tours, UMR1282
Marc Blondel
Université de Brest; Inserm UMR1078; Etablissement Français Du Sang (EFS) Bretagne; CHRU Brest, Hôpital Morvan, Laboratoire de Génétique Moléculaire
Ignacio Caballero-Posadas
ISP, INRAE, Université de Tours, UMR1282
Rodrigo Prado Martins
rodrigo.prado-martins@inrae.fr

PFIE, INRAE, UE1277 Nouzilly, France

Article

Keywords: yeast-based vaccine, cell-mediated immunity, mammary gland, mastitis, dairy cattle

Posted Date: August 7th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9360454/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at npj Vaccines on September 26th, 2026.
See the published version at https://doi.org/10.1038/s41541-026-01584-9.

Abstract

Mastitis remains a major challenge for the dairy industry and the limited efficacy of commercially available vaccines is attributed to their inability to induce strong local cell-mediated immunity. As alternatives, yeast shows great promise, acting as antigen vectors, production platforms and natural adjuvants inducing robust cellular responses. We have previously shown that strategies to counterbalance the inherent immunogenicity of yeast-based vaccines are necessary to enhance host response to vaccinal antigens. In this study, we vaccinated cows with an improved bivalent *Saccharomyces cerevisiae*-based formulation using a prime-pull regimen and employed multiple approaches to provide deep insight into the impact of immunisation on the establishment of immune mechanisms in the mammary gland. We provide evidence that our vaccination strategy modulates cell trafficking and promotes memory T-cell responses without compromising mammary gland integrity. Our results also demonstrate that immunising all quarters may not be necessary to achieve protection at the whole mammary gland.

Introduction

Mastitis, an inflammation of the mammary gland, is primarily caused by bacterial infections and represents the most widespread disease in dairy cattle worldwide. Mastitis not only compromises animal health and welfare but also leads to substantial economic losses due to decreased milk yield, altered milk quality and increased veterinary costs. It remains the primary indication for antibiotic administration in dairy breeding, raising major concerns about antimicrobial resistance[1]. Commercial vaccines against mastitis have demonstrated limited protective efficacy[2] which is attributed to their predominant effect on eliciting antibody-mediated protection at the expense of inducing robust cellular immune response. However, latest studies show that the induction of robust cellular immune responses, particularly through Th17 and possibly Th1 lymphocytes, is crucial for effective protection of the mammary gland [3–6].

Yeast-based vaccines are promising alternatives to conventional vaccines for several reasons. First, yeast may serve both as a production and delivery system, offering a simple and cost-effective platform. Yeast is also convenient for the production of vaccine formulations for food-producing animals, as species like *Saccharomyces cerevisiae* are Generally Recognized As Safe (GRAS) organisms. Most importantly, yeast-based vaccines do not require an external adjuvant, as the yeast cell wall itself acts as an adjuvant due to its highly immunogenic nature [7,8]. In fact, yeast cell wall carbohydrates are capable of inducing Th1, Th17, and cytotoxic T-cell responses *in vivo*, surpassing the Th2-type response elicited by conventional adjuvanted vaccines[9,10] .

Besides vaccine formulation, the adoption of appropriate immunisation strategies is essential for successful development of vaccination approaches. Vaccination schemes for livestock are mostly based on systemic prime-booster administrations, which are more suitable for mass immunisation, but may be less effective in stimulating local immunity[11]. However, immunisations delivered in proximity to

target organs can promote the establishment of tissue-resident immune populations and enhance local protection[12,13]. Previous evidence suggests that systemic immunisation alone fails to generate robust memory responses in the mammary gland, highlighting the need for alternative strategies[5]. In addition, the combination of different routes of administration has been reported to improve protective responses at mucosal sites[14]. In this context, prime-pull immunisation regimens represent a promising approach to overcome this limitation by combining systemic priming with localized immune activation, in which pathogen-specific T cells are primed systemically and subsequently recruited to the target tissue through local administration of chemoattractants or booster doses[15,16].

We have previously shown that *S. cerevisiae*-based vaccines are safe for both intramuscular and intramammary administration in dairy cows, but strategies to counterbalance their inherent immunogenicity in order to enhance host response to vaccinal antigens are still necessary[17]. In the present study, we developed an improved *S. cerevisiae*-based bivalent model formulation exploring the expression of two antigens, previously validated in cattle, both at the surface and intracellularly. We implemented a prime-pull immunisation strategy to investigate how this yeast-based vaccine formulation shapes immune cell populations within the mammary gland, with the aim of improving our understanding of tissue-specific immune responses in this still poorly characterised compartment. We provide the first detailed single-cell characterization of the local recall immune responses of the mammary gland following prior immunisation. We further demonstrate that intramammary homologous boosting influences local immune cell trafficking, and that booster administration in only half of mammary quarters seems sufficient to elicit immune modulation across the whole mammary gland.

Material and methods

Ethics statement and animal experimentation

Animal handling and experimentation were conducted with the approval of the Ethics Committee of Val de Loire (France, DGRIs agreements APAFIS #35905-2022060113398545 v1) in strict accordance with all applicable provisions established by the European directive 2010/63/UE. All experimental procedures were approved by the Loire Valley ethical review board (CEEA VdL, committee number 19).

Yeast-based platform engineering and VAXIO2 production

DNA sequences encoding a truncated version of *Gallus gallus* ovalbumin[18] and the outer membrane protein A (OmpA) of *Escherichia coli*, were individually inserted into the pYD1 (Invitrogen), in-frame with the *S. cerevisiae* Aga2p coding sequence, or p425GPD vector backbones[19]. Recombinant DNA constructs were cloned and amplified in *E. coli* DH5a (New England Biolabs). *S. cerevisiae* strain EBY100 was cultivated in YPD broth to OD_{600} of 0.6–0.8 and transformed with 1 µg of either pYD1-OVA and p425GPD-OVA or pYD1-OmpA and p425GPD-OmpA using the LiAc/SS carrier DNA/PEG method to generate the VAXIO-OVA and VAXIO-OmpA strains (Fig. 1A). For vaccine production, VAXIO-OVA and

VAXIO-OmpA strains were inoculated in minimal broth (0.67% Yeast Nitrogen Base with ammonium sulfate (w/v), 2% raffinose (w/v) and dropout amino acids mixture (76 mg/l each) without tryptophan and leucin) at an OD_{600} of 0.2 and protein expression was induced by adding 2% galactose when the cultures reached an OD_{600} of 0.5. After galactose addition, the cultures were allowed to grow for an additional 6 h, heat-inactivated at 56°C for 30 min and centrifuged at 3500 *g* for 10 minutes at room temperature. Yeast pellets were washed twice with wash buffer (DPBS without Ca^{+2} and Mg^{+2}, 2% FBS (v/v), 2 mM EDTA) and kept dry at − 20°C until vaccination. Equal amounts of VAXIO-OVA and VAXIO-OmpA cells were mixed for the production of VAXIO2. Prime doses contained 5.10^9 CFU of VAXIO-OVA and 5.10^9 CFU of VAXIO-OmpA resuspended in 3 ml of DPBS. Booster doses contained 2.10^9 CFU of VAXIO-OVA and 2.10^9 CFU of VAXIO-OmpA resuspended in 4 ml of DPBS.

Western blot

VAXIO-OVA, VAXIO-OmpA, EBY100 and EBY100 transformed with empty vectors (EBY100-pYD1, EBY100-p425GPD, EBY100-pYD1-p425GPD) were cultivated in raffinose and galactose-containing media as previously described. Yeast strains were cultivated at an OD_{600} of 1.0, centrifuged at 3500 rpm for 10 minutes and lysed with the CelLytic Y reagent (C4482, Sigma-Aldrich) following the manufacturer's condition. Ten micrograms of total protein were separated by electrophoresis and blotted under standard conditions using internally produced rabbit polyclonal antibodies anti-OVA or anti-OmpA at 0.5 µg/ml in PBS 5% skimmed milk. A goat anti-rabbit IgG (H + L) antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch, 1:5000) was used to generate immunocomplexes revealed with enhanced chemiluminescence (Super Signal West reagent, Thermo Scientific). Membranes were scanned using a Fusion FX imager (Vilber).

Vaccination with VAXIO2 and clinical monitoring

Six dried-off Holstein cows with quarter milk somatic cell counts below 200,000 cells/mL, and free of intramammary infection by major mastitis pathogens (*Staphylococcus aureus*, *Escherichia coli*, or *Streptococcus* spp.) prior to dry-off, were enrolled in the immunisation protocol conducted in the facilities of the Plateforme d'Infectiologie Expérimentale (PFIE, UE-1277, INRAE, France, doi: 10.15454/1.5572352821559333E12). In compliance with the 3R principles, the same animals used in a preliminary study described elsewhere[17] were used in this trial. For prime immunisation, VAXIO2 was administrated intramuscularly at the prescapular region and, 15 days later, two booster doses were intramammarily administered in the right mammary quarters. For each immunisation, the presence of systemic and local reactogenicity signs was evaluated and recorded by a single operator using a scoring grid published elsewhere[17].

Blood immune response to vaccination

Peripheral blood mononuclear cells (PBMC) and serum were routinely collected at the indicated time points. All PBMC samples were kept in liquid nitrogen and then defrosted and stimulated at the same time as follows: 3.10^5 cells were mixed resuspended in 200 µl of WBA medium (RPMI 10% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 100 nM non-essential amino acids, 50 µM β-mercaptoethanol, 10 mM HEPES) containing 10^6 CFU of heat-inactivated EBY100, 1 µg of pyrogen-free ovalbumin (Calbiochem) or 1 µg of recombinant OmpA (internally produced) in a 96 well round bottom plate (Nunc). Crude WBA medium was used as negative control. Cultures were kept for 48 h at 38.5°C with 5% CO_2 and supernatants were collected for IFNγ (Mabtech, 3119-1H-20) and IL-17 measurement by ELISA[20]. For the estimation of total antibody titers to OVA, OmpA and EBY100 in blood serum, microtiter plates (Nunc Immunoplate Maxisorp) were coated by overnight incubation with 2 µg/ml of either ovalbumin, OmpA or EBY100 protein lysate (50 µl/well) in phosphate-buffered saline (PBS). Then, ELISA assays were carried out as described in Rainard *et al.*, 2015.

Intramammary stimulations

Three intramammary stimulations were conducted at days 30, 73 and 106 after prime vaccination. For this, 10 µg of pyrogen-free ovalbumin (Calbiochem) and 10 µg of pyrogen-free recombinant OmpA (internally produced) were mixed in 3 ml of DPBS without Ca^{2+} and Mg^{2+} (Gibco) containing 1 mg of pyrogen-free bovine serum albumin (Sigma). At days 30 and 73, antigen solution was administrated to all mammary quarters of the six experimental animals. At day 106, only the front quarters of one cow were stimulated. At each stimulation, 10 ml of mammary secretion were collected immediately before and 1 day after intramammary injections for flow cytometry (days 30, 31, 73 and 74) and single-cell analysis (day 106).

Flow cytometry

Mammary secretions were diluted in DPBS without Ca^{2+} and Mg^{2+} in a 1:1 ratio and centrifuged for 20 minutes at 1400 *g* at 20°C. The fat layer and supernatant were removed and cell pellets were washed with 10 ml of FACS buffer (DPBS without Ca^{2+} and Mg^{2+} supplemented with 2% (v/v) normal goat serum and 2 mM EDTA) and resuspended in the same buffer for further analysis. Surface and intracellular markers were labelled using the antibodies described in **Supplementary Table 1** and according to a previous study[21]. Dead cells were stained using the Fixable Viability Dye eFluor 450 (eBioscience). Cells were examined by flow cytometry using a BD LSR Fortessa™ X-20 flow cytometer (Becton Dickinson) and data were analysed using the Kaluza software (Beckman Coulter). Dead cells were excluded from the analysis and the gating strategy employed is shown in **Supplementary Figs. 1 and 2**.

Cell sorting and scRNA-seq analysis

One cow was intramammarily stimulated as previously described at day 106 and euthanised at day 107 for mammary secretion and tissue sampling from front quarters. For the production of cell suspensions, mammary secretions were processed as previously described and tissue samples from different mammary gland compartments (glandular tissue, gland cistern and teat sinus, 300 mg/compartment) were mixed and digested according to a previous study [22]. Subsequently, secretions and tissue cell suspensions were processed using a MoFlo Astrios EQ flow cytometry sorter (Beckman Coulter, Brea, CA, USA) to exclude neutrophils ($CD45^+G1^+$) and collect the remaining live $CD45^+$ cells. Reagents and gating strategy used for cell sorting are described in the **Supplementary Table 1** and **Supplementary Fig. 3**, respectively. To obtain single cell 3' libraries for Illumina sequencing, 10^4 cells were recovered in a 50 µL PCR tube and processed according to the manufacturer's protocol (Chromium Single Cell 3' Reagent Kit, v3.1 Chemistry).

Raw data were analysed using Cell Ranger Single-cell software, pipeline version 7.0.1, and the raw reads were aligned to the *Bos taurus* genome (ARS-UCD1.2, GCA_002263795.2) to generate gene-barcode matrices. Cell Ranger output was analysed with the Seurat package v.5.2.1 [23] using R v.4.3.2 and ambient RNA present in the cell suspension was removed using DecontX [24]. Each dataset was filtered to retain cells with at least 1,000 detected genes and no more than three times the median absolute deviation (MAD) above the median number of detected genes, a minimum of 2000 UMI counts and less than 10% of mitochondrial sequences. Doublet cells were identified using a consensus approach combining scDbIFinder[25] and the hybrid method (cxds_bcds_hybrid) implemented in scds [26], whereby cells flagged as doublets by either method were removed. After normalization and variance stabilization with SCTransform (v2), and dimensionality reduction by Principal Component Analysis, data were visualized using Uniform Manifold Approximation and Projection (UMAP) embedding based on the first 30 principal components. Global integration was performed after normalization and identification of the 3000 most variable features of each dataset. Integration was performed using the Reciprocal PCA (RPCA) integration method, as implemented in the Seurat function IntegrateLayers. Cell clusters were obtained using the FindClusters function. Manual annotation was performed on each cell cluster based on the expression of canonical marker genes reported in the relevant scientific literature (**Supplementary Tables 2 and 3**).

Preparation and ex vivo stimulation of mammary tissue explants

At day 108, teat explants from three VAXIO2-immunised cows were prepared as previously described[22]. Briefly, teats were collected from an unboosted and a boosted mammary quarter (front left and right) immediately after euthanasia, transported to the laboratory in a Hank's balanced salt solution (HBSS, H6648, SigmaAldrich, Irvine, United Kingdom) with antibiotics (100 IU/mL penicillin combined with 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (A5955, SigmaAldrich, Saint Louis, USA)) and dissected under sterile conditions for the collection of 6-mm-diameter explants from the teat sinus using biopsy punches. Explants were washed twice in DPBS supplemented with penicillin, streptomycin and amphotericin B and then individually transferred to 24-well plates containing 1 mL of complete medium

(Advanced DMEM/F-12 supplemented with 10% heat-inactivated endotoxin-free FBS, 2 mmol/L L-glutamine (Gibco, USA), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B). Explants were maintained at 37°C in a humidified atmosphere of 5% CO_2 until stimulation (< 1h). Explants were stimulated with 1 µg of pyrogen-free ovalbumin (Calbiochem), 1 µg of pyrogen-free recombinant OmpA (internally produced) or 10^6 CFU of EBY100. Crude complete medium was used as control. After 24 h at 37°C in a humidified atmosphere of 5% CO_2, culture supernatants were collected and stored at − 20°C for cytokine quantification (< 1 month). CXCL8 (Mabtech 3114-1H-20) and CXCL9 (Kingfisher DIY0661B) release was measured by ELISA using commercially available kits according to the manufacturer's instructions. To compensate differences in explant weight, results were normalized to tissue mass and expressed as picogram per milligram of tissue.

Ex vivo immune cells migration assay

Teat sinus explants from unboosted and boosted mammary quarters were prepared as previously described and their capacity to attract immune cells was tested *ex vivo*. For this, PBMC from the same three VAXIO2-immunised cows were obtained and stained with CellTrace™ Violet (Thermo Fisher Scientific) according to the manufacturer's conditions. Migration assays were performed using transwell inserts (3 µm pore size) in a 24-well plate. Mammary explants were placed in the lower chamber with 1 mL of complete medium containing OVA (2 µg/ml) + OmpA (1µg/ml) or 10^7 CFU of EBY100 or crude medium. Then, 10^6 stained PBMC resuspended in 500 µL of complete medium were added to the upper chamber. After 24 h at 37°C in a humidified atmosphere of 5% CO_2, explants were carefully removed, washed in DPBS to eliminate non-adherent cells, and digested for flow cytometry analysis as previously described[22]. The presence of migrated PBMC within the explant tissue was analysed by flow cytometry based on CellTrace™ Violet fluorescence and data were expressed as the number of migrated PBMC per explant.

Statistical analyses

Data were analysed and plotted using GraphPad Prism, version 10.5 (GraphPad Software Incorporation). Wilcoxon signed-rank and Mann-Whitney U tests were used to compare paired and unpaired samples, respectively. Data shown represent the median and interquartile range. Asterisks denote statistically significant difference as follows: *p < 0.05, **p < 0.01, ****p < 0.0001.

Results

Construction and validation of a yeast-based vaccine platform enabling simultaneous intracellular and surface expression of heterologous antigens

We have previously developed and evaluated *in vivo* a vaccine formulation based on the *S. cerevisiae* strain EBY100 engineered to express OVA as a model antigen by surface display, using the vector PYD1. Our preliminary results showed that immunised cows did not respond to OVA, while the immune

response was predominantly directed toward endogenous yeast antigens[17]. These observations suggested that strategies enabling to counterbalance the immunodominance of yeast are necessary to exploit this microorganism as a vaccine platform. Initially, we hypothesized that antigen levels produced by our previous platform might have been insufficient. To address this limitation, we introduced to our platform an additional construct enabling intracellular OVA expression under a constitutive promoter, in complement to the galactose-inducible surface expression. We designated this dual-expression vaccine strain as VAXIO-OVA (Fig. 1, left) Previous studies have also shown inter-individual variability in the immune response of cows to OVA immunisation, with the occurrence of both low and high responders[20]. To improve the immunogenicity of our prototype vaccine, we set out to develop a bivalent formulation by incorporating OmpA, a membrane protein from *E. coli* previously studied by our group[27]. For this, we engineered EBY100 to express OmpA both on the surface and intracellularly, giving rise to the VAXIO-OmpA strain (Fig. 1, right). Expression of OVA and OmpA on the surface and intracellularly was confirmed by Western blotting (Fig. 1B) and a 1:1 mixture (VAXIO2) of both *S. cerevisiae* vaccine strains (VAXIO-OVA and VAXIO-OmpA) was used for the *in vivo* evaluation.

Clinical evaluation and systemic immune response of dairy cows to immunisation with VAXIO2

Six dairy cows were immunised with VAXIO2 according to the prime and pull approach[16,28]. VAXIO2 was first administered intramuscularly to induce a systemic response and 15 days later, a booster dose was delivered into the right mammary quarters to elicit local protective immunity (Fig. 2A). As shown in Fig. 2B, none of the animals developed fever or any other reactogenicity signs following the prime administration. After booster administration, fever was not detected, but transitory signs of inflammation were observed in the mammary gland from 6 to 48 h after the intramammary administrations (Fig. 2C).

Next, we evaluated anti-OVA, anti-OmpA and anti-EBY100 antibody titres in blood serum from 0 to 84 days post-prime (dpp). Significant increase of both anti-OVA and anti-OmpA titres was detected at 43 and 56 dpp, as well as at 84 dpp for anti-OmpA. Higher anti-EBY100 titres were observed from the beginning of the protocol and peaked earlier, at 28 dpp, consistent with prior exposure of the experimental animals to yeast antigens (Fig. 2D-E)[17]. To evaluate systemic T-cell responses, whole blood from the immunised animals was stimulated at the indicated time points with either EBY100 or recombinant OVA and OmpA for the quantification of IL-17 and IFNγ release by ELISA. Production of IL-17 in response to EBY100 was detected at day 0 and remained relatively stable throughout the analysed time points. In contrast, OVA and OmpA stimulation elicited little to no IL-17 production, except for OVA, which showed an increase at day 28, followed by a drop at 43 dpp (Fig. 2F-G). Blood IFNγ response to EBY100 decreased to background levels at 84 dpp, after an increase at 56 dpp, and low levels of response to OVA or OmpA were observed (Fig. 2H-I).

Immunisation with VAXIO2 elicits antigen-specific inflammatory and T-cell responses in the mammary gland

Previous studies have highlighted the role of neutrophils in the protection of the mammary gland against bacterial infections[3,29] and the modulation of neutrophilic inflammation via mechanisms of type 3 cell-mediated immunity, as a target for vaccine strategies to prevent mastitis[5]. In order to assess the capacity of VAXIO2 to induce neutrophilic inflammation upon antigen recall, immunised cows were intramammarily stimulated with recombinant OVA and OmpA at 30 dpp and mammary secretions were collected before and after infusions for an analysis of cell populations by flow cytometry (Fig. 3A-B). As shown in Fig. 3C, neutrophils levels were low in both boosted and unboosted quarters prior to stimulation, but increased significantly in both conditions following stimulation, indicating the occurrence of OVA-OmpA-specific inflammation. In addition, $CD14^+$ (Fig. 3D) and MHC-II^+ (Fig. 3E) cell populations increased in both boosted and unboosted quarters after stimulation. Intramammary stimulation with vaccinal antigens also induced a trafficking of distinct $CD3^+$ cell populations to the mammary secretion. As depicted in Fig. 3F-G, local stimulation led to an increase of $CD3^+CD14^+$ and $CD3^+$MHC-II^+ cells in all mammary quarters, with a higher increase in boosted quarters. $CD3^+CD14^+MHC^+$ cells were more abundant in the mammary secretion after stimulation but no differences between boosted and unboosted quarters were observed (Fig. 3H). In parallel, $CD3^+$ cells were consistently more abundant in boosted quarters after stimulation, but not in the unboosted ones (Fig. 3I).

At 73 dpp, we repeated intramammary stimulations and mammary secretion samplings, as described in Fig. 3A and **Supplementary Fig. 4A**, in order to examine T-cell populations in greater depth by surface and intracellular staining coupled to flow cytometry (Fig. 4A). Local stimulation at 73 dpp generated a neutrophilic recruitment to the mammary secretion at 74 dpp, similar to the one observed at 31 dpp (**Supplementary Fig. 4B).** The number of T-cells (Fig. 4B), as well as IL-17 and IFNγ-producing T-cells (Fig. 4C-D) was also increased in mammary secretions after stimulation. Further analysis of T-cell populations revealed that $CD4^+$ T cells were more abundant after stimulation regardless of the quarter status (Fig. 4E). However, IL-17^+ and IFNγ^+ $CD4^+$ T cells were significantly enriched only in boosted quarters (Fig. 4F-G). $CD8^+$ T cells (Fig. 4H) were more abundant after stimulation in all quarters and no changes were observed for IL-17 producing $CD8^+$ cells (Fig. 4I), although IFNγ^+ $CD8^+$ T cells were significantly increased in boosted quarters (Fig. 4J). Notably, γδ T cells showed a marked increase after stimulation in all quarters (Fig. 4K), with higher abundance of IL-17^+ γδ T cells (Fig. 4L) and IFNγ^+ γδ T cells (Fig. 4M) in both conditions after stimulation. A greater rise of IL-17^+ γδ T cells in boosted quarters than in the unboosted ones was also observed.

Single-cell RNA sequencing reveals that local stimulation with vaccinal antigens triggered antigen presentation and effector/memory T-cell responses in the mammary gland of cows immunised with VAXIO2

Next, we carried out single-cell RNA sequencing (scRNA-seq) to refine our previous observations on the mammary secretion and provide new data on the cell populations found in the mammary tissue. For this, one cow was intramammarily stimulated with the vaccinal antigens (OVA mixed with OmpA) on the front

right (boosted) and left (unboosted) mammary quarters at 106 dpp (91 days after booster). After 24h, the cow was euthanised and mammary secretion and tissue were collected from the stimulated quarters for the production of cell suspensions. Subsequently, cell suspensions were depleted of neutrophils and epithelial cells and remaining live $CD45^+$ cells were collected by cell sorting (**Supplementary Fig. 3**) for scRNA seq analysis (Fig. 5A).

After quality filtering, cell clusters were identified using a shared nearest-neighbour approach and visualized in two-dimensions via Uniform Manifold Approximation and Projection (UMAP) and 18 major cell clusters were annotated based on the average expression of canonical marker genes (Fig. 5B and 5C, **Supplementary Table 2, Supplementary Fig. 5**). As observed in previous experiments using flow cytometry (Fig. 3), single cell analysis confirmed the presence of a $CD3^+CD14^+MHCII^+$ population in the bovine mammary gland transcriptionally closer to macrophages than to T-cells (Fig. 5C, **Supplementary Figs. 5 and 6**). Moreover, an activation of antigen presentation mechanisms was deduced by the strong upregulation of BOLA-DRA and other MHCII encoding genes by these cells and other antigen-presenting cells (APC) populations (Fig. 5C, **Supplementary Fig. 7**).

Lymphoid clusters displayed a strong expression of CD7, CD69, CD28 and ICOS indicative of recent activation and tissue-resident memory (TRM)-like state. Of note, upregulation of the regulatory receptor CTLA-4 was only observed in $FOXP3^+CD4^+$ T-cells. In agreement with our analysis by flow cytometry, γδ T-cells were confirmed as the main source of IL17 and the overexpression of IL23R by these cells, $CD8^+$ MAIT cells and cDC confirms previous evidence denoting local induction of type-3 cell-mediated immunity upon stimulation with vaccinal antigens (Fig. 5C).

To gain deeper insight into these observations, we repeated two-dimensional cell clustering to analyse tissue and secretion data sets from unboosted and boosted mammary quarters separately. As shown in Fig. 5D, mammary secretion is predominantly composed of macrophages, B-cells and type 2 conventional dendritic cells (cDC2), whereas mammary tissue is enriched in distinct T-cell populations including cytotoxic innate like T-cells, tissue-resident memory $CD4^+$ T-cells ($CD4^+$ TRM) and γδ T-cells. Few differences were observed between cell populations found in boosted or unboosted quarters, in both tissue and secretion compartments. However, $CD3^+CD14^+MHCII^+$ cells, as well as cytotoxic innate like T-cells, γδ T-cells and $CD4^+$ TRM were observed to be more abundant in the secretion of boosted quarters. Interestingly, the T-cell populations more abundant in the secretion of boosted quarters seem to be more present in the tissue of unboosted quarters suggesting that, in absence of local booster, these cells might be retained in tissue compartment. Consequently, it can be inferred that local booster might modify chemiotaxis and cell trafficking in the mammary gland.

Local booster with VAXIO2 impacts cell trafficking in the bovine mammary gland upon antigen-specific stimulation

We took advantage of scRNA-seq analysis to investigate the expression of chemokines and chemokine receptors across the different cell populations identified in the secretion and tissue of unboosted and

boosted mammary quarters (Fig. 6A). Higher expression CCL5 could be observed by $CD3^+CD14^+MHCII^+$ cells and B-cells in boosted tissue, as well as by quiescent innate-like T-cells and NK cells in boosted secretion. Similarly, CCL3 was upregulated by $CD3^+CD14^+MHC^+$ cells, effector memory CTL, $FOXP3^+CD4^+$ cells and plasma cells in boosted secretion. These chemokines interact with CCR5, which was upregulated by highly proliferative, γδ and $FOXP3^+CD4^+$ T-cells in boosted secretion. CXCL9 was upregulated only by cDC found in boosted tissue, whereas CXCL16 was more expressed by $CD3^+CD14^+MHC^+$ cells, $FOXP3^+CD4^+$ cells, NK cells and macrophages $CD276^+$ in boosted secretion. CXCR6, which interacts with CXCL16, is more expressed by γδ and $FOXP3^+CD4^+$ T-cells in boosted secretion. These results suggest that local boosting elicits robust pro-inflammatory and chemotactic responses favouring the local recruitment and retention of effector T cells and myeloid cells.

To address this hypothesis, we adapted an *ex vivo* model previously described by our team[22] to compare the chemiotactic capacity of tissue from unboosted and boosted mammary quarters. Three VAXIO2 immunised cows were euthanised at day 108 and teat explants from the front right (boosted) and left (unboosted) mammary quarters were obtained for *ex vivo* stimulation and cell mobility tests (Fig. 6B). As depicted in Fig. 6C, local booster improved the capacity of mammary tissue to attract blood immune cells in steady state (mock control) and upon stimulation of the recombinant vaccinal antigens. Additionally, tissue explants from boosted mammary quarters released higher CXCL9 levels after stimulation with vaccine antigens and vector (Fig. 6D), whereas no differences were observed for CXCL8 (Fig. 6E), which validates evidence provided by sc RNA seq analysis.

Discussion

In a previous study, we have shown that a vaccine formulation based on *S. cerevisiae* expressing heterologous proteins only on its surface does not induce strong immune responses to target antigens, probably due to the immunodominance of endogenous yeast components[17]. To compensate for this constraint, we have developed an improved formulation (VAXIO2) by establishing a dual expression system allowing both intracellular expression and surface display of the reporter antigens OVA and OmpA.

Clinical monitoring of cows vaccinated with VAXIO2 enabled us to confirm that *S. cerevisiae*-based vaccine formulations are safe for intramuscular and intramammary administration in dairy cattle, as previously shown[17]. After the booster administration, we observed a mild local inflammation of the udder that was limited and resolved rapidly, supporting the overall safety of the tested formulation. Moreover, our results indicated that engineering of *S. cerevisiae* for concurrent intracellular and surface heterologous protein expression enhances recognition of vaccinal antigens, as evidenced by measurable systemic anti-OVA and anti-OmpA antibody responses. Although low OVA or OmpA-specific IFNγ and IL17 responses were observed in blood, intramammary administration of vaccinal antigens induced a local neutrophilic inflammation. Previous observations associate the prime and pull approach with improved tissue migration of antigen-specific T-cells and establishment of local memory

mechanisms[12,28], which supports the notion that VAXIO2, coupled with this immunisation strategy, favoured the trafficking and the establishment of activated and memory T-cells in the mammary gland, rather than their persistence in the systemic circulation.

Accumulating evidence indicates that innate-like immune defences mediated by IL-17, resulting in neutrophils migration to the mammary gland, represent a major target for vaccines against mastitis[4,29,30]. In this sense, an efficient vaccination strategy should promote the reactivation of resident memory T cells upon antigen exposure, leading to IL-17-mediated epithelial antimicrobial response, neutrophil recruitment to the mammary gland lumen via CXCL8 release and enhanced bacterial clearance[31]. In this study, local antigen-specific recall responses after immunisation led to an increase of neutrophils, antigen-presenting cells (APC) and distinct $CD3^+$ populations in the mammary secretion. Interestingly, we uncovered that γδ T-cells ($WC1^+$) are major sources of IL-17 and IFNγ after stimulation of the mammary gland. Mammary γδ T-cells have been highlighted as the main mediators of IL-17-based innate protection against *S. aureus*-induced mastitis in mice[29] and an increase in activated γδ T-cells expressing memory surface markers has been reported in the milk of cows with subclinical mastitis caused by *S. aureus*[32]. Cattle present large proportions of γδ T-cells when compared to human and mice. Although these cells have been shown to contribute to different immune mechanisms such as phagocytosis, antigen presentation and regulation of T-cell activity in cattle[33], their role in the response to vaccination is not completely understood and has been underestimated in vaccine development. Until recently, γδ T-cells were considered as innate lymphoid cells. Nevertheless, their functional activation has been associated to vaccination-induced protection against bacterial infections in different hosts and evidence demonstrates the adaptive character of their immune memory responses[34,35]. In this context, the results of this study show that our immunisation strategy can induce γδ T-cells recall responses in the bovine mammary gland.

$WC1^+$ γδ T-cells, rather than $FoxP3^+$ T cells, have been previously described as regulatory populations in bovine blood[36]. Interestingly, we demonstrate that $WC1^+$ γδ T cells within the bovine mammary gland display a markedly proinflammatory phenotype, characterized by IL-23R, IL-17 and IL-26 expression, whereas a $CD4^+$ $IL2R(CD25)^+$ $FoxP3^+$ T-cell subset exhibits a regulatory profile, including high expression of CTLA-4, CD28, ICOS, and IL6ST. Previous evidence also indicates that, beyond immune suppression, $CD4^+$ $CD25^+$ $FoxP3^+$ T-cells can modulate Th17 responses, thereby contributing to host protective immunity against fungal infections while restraining sustained IL-17-mediated inflammation at later stages[37]. In this context, the coexistence of inflammatory and regulatory T-cell subsets upon local antigenic challenge suggests that vaccination with a yeast-based formulation may favour the establishment of antigen-experienced $FoxP3^+$ T-cells that fine-tune IL-17-based immune mechanisms in the mammary gland leading to a neutrophilic inflammation, while limiting collateral tissue damage. Consistent with this interpretation, the mild and transient clinical signs of inflammation after local stimulation (clinical monitoring not shown) further supports the notion that the evaluated yeast-based

formulation promotes a balanced immune environment in which protective inflammation is efficiently controlled, thereby ensuring tissue protection without compromising the integrity of the mammary gland.

Interestingly, our observations indicate that, although partial booster (2 quarters out of 4) is sufficient to induce a neutrophilic migration in all mammary quarters upon antigen-specific stimulation, local booster seems to modify the mediators and or the kinetics of cell trafficking and improve protective responses, at the mammary quarter level. Our *ex vivo* migration test using mammary tissue and blood cells from VAXIO2-immunized cows showed that migration upon stimulation with vaccinal antigens is improved in boosted quarters. Accordingly, a previous study exploring an immunisation scheme based on intraperitoneal (IP) and IM systemic immunisations followed of a partial IMM booster (2 out of 4 quarters) demonstrated that higher numbers of CD3-positive cells and antibody-producing cells are observed in boosted quarters[38]. Our results also provide novel insights into the cellular immune landscape of the bovine mammary gland by combining the characterisation of both immune cells recruited into the gland lumen and residing within the mammary tissue. scRNA-seq analysis revealed that molecules involved in tissue retention of effector and memory T-cells such as CXCL16-CXCR6[39] and CCL5-CCR5[40] tended to be more expressed by different cell populations in boosted secretion or tissue. Moreover, levels of IL-17 and IFNγ-producing γδ T-cells were increased in both boosted and unboosted quarters, but local booster was shown to be necessary to increase the presence of Th17 ($CD4^+$ $IL17^+$), Th1 ($CD4^+$ $IFN\gamma^+$) and Tc1 ($CD8^+$ $IFN\gamma^+$) cells in the mammary secretion upon intramammary stimulation, probably due to the local expansion and activation of Trm cells. In agreement with this hypothesis, previous observations by Cebron et al.[5] suggested that a booster immunisation via the intramammary route after a systemic IM prime is necessary to induce Th17-polarization of $CD4^+$ T-cells in the bovine mammary gland.

We also uncovered that $CD3^+$ cell populations expressing myeloid/APC-associated markers (CD14 and/or MHCII) were increased upon intramammary stimulation with vaccinal antigens, particularly in boosted quarters. scRNA seq analysis indicated that these cells are transcriptionally related to macrophages, express a broad repertoire of chemokine and chemokine receptor encoding genes, and are more abundant in mammary secretion compared to mammary tissue. In contrast to other macrophage populations, these cells expressed CCL5 at levels comparable to those observed in different lymphoid cell populations present in mammary tissue and secretion. This particularity suggests a potential role in modulating the recruitment of CCR5-expressing effector T-cell subsets, notably γδ and $FoxP3^+$ T-cells, which were enriched in secretions from boosted quarters. Consistent with our findings, CD3-expressing macrophages have been implicated in host response to *Mycobacterium tuberculosis* infection through modulation of chemotaxis [41]. We also observed that cDC2 expressing higher levels of CXCL9 were enriched in the tissue compartment of boosted mammary quarters, in line with the results of our *ex vivo* model of mammary tissue restimulation. As a mediator of type 1 cellular immune responses[42], higher expression of CXCL9 in boosted tissue can be also associated with the significant

increase of IFNγ-producing $CD4^+$ and $CD8^+$ T-cells after *in vivo* intramammary stimulation with the vaccinal antigens observed only in boosted quarters.

While local booster administration appears to reinforce memory immune mechanisms, compensatory responses are also evident in unboosted quarters. This is supported by comparable levels of *in vivo* neutrophil recruitment and *ex vivo* CXCL8 release between boosted and unboosted quarters. In cattle, the right and left mammary quarters are connected to an ipsilateral supra mammary lymph node through lymphatic ducts. Upon activation, bovine $CD4^+$ and $CD8^+$ T-cells can migrate between mammary quarters as well as to ipsilateral and contralateral supra mammary lymph nodes[43]. Based on these observations, it can be inferred that following intramammary antigen stimulation, activated T-cells from boosted quarters and their draining lymph-node may have migrated to unboosted quarters and contributed to the induction of the observed immune responses.

In a pioneering study, Harp et al[44] demonstrated that mammary lymphocytes originate from the periphery in cows, suggesting that the mammary gland is not part of the common mucosal system in this species. Further evidence, such as the absence of mucosal addressin molecule-1 (MAdCAM-1), a molecule involved in lymphocyte homing to mucosae, also suggested that the bovine mammary gland does not act as a mucosal tissue[45]. However, our results indicate that, despite these particularities, the bovine mammary gland behaves as a mucosal immune site with active local memory mechanisms that can be modulated by vaccination. As pathogen-specific memory T cell responses are preferentially maintained at the sites of initial effector T cell recruitment after natural infections[14], efficient mastitis vaccines should promote the migration of activated T cells and the formation of memory subsets in the mammary gland. Corroborating previous observations showing that combining different routes of administration induces durable *in situ* T cell immunity at mucosal sites[12,14], we provide evidence that systemic immunisation with a yeast-based vaccine followed by a homologous IMM booster modulates cell trafficking and leads to the establishment memory T-cell responses in the mammary gland. In this context, yeast represents promising platforms for efficient mastitis vaccines as they can prime T cell responses[46] and be intramammarily administered without compromising mammary gland integrity or its function in milk production.

These findings have important implications for vaccination strategies against mastitis, indicating that although local booster delivery enhances site-specific immune responses, immunising all quarters may not be necessary to achieve protection at the mammary gland as whole. From a veterinary and industrial perspective, this suggests that limiting the number of booster doses could reduce immunisation costs while maintaining effectiveness, thereby improving the feasibility of vaccination strategies exploiting the intramammary route in cattle.

Declarations

Contributions

Conceptualization: RPM. Animal handling and clinical evaluation: OB and RPM. Experimental work and data analysis: CD, RPM, PC, ICP, PGN, FBG, YLV and PG. Resources: RPM, PR, PG, FBG and MB. Writing-Original Draft Preparation: CD, RPM. Writing-Review & Editing: CD, RPM, ICP, PC, FBG, PR, PG and MB. Supervision: RPM and PC. Funding Acquisition: RPM.

Competing interests

The authors declare no competing interests.

Funding

This work was supported by the Agence Nationale de la Recherche (ANR-20-CE20-0023) and INRAE Département Santé Animale.

Author Contribution

Conceptualization: RPM. Animal handling and clinical evaluation: OB and RPM. Experimental work and data analysis: CD, RPM, PC, ICP, PGN, FBG, YLV and PG. Resources: RPM, PR, PG, FBG and MB. Writing-Original Draft Preparation: CD, RPM. Writing-Review & Editing: CD, RPM, ICP, PC, FBG, PR, PG and MB. Supervision: RPM and PC. Funding Acquisition: RPM.

Acknowledgement

The authors are grateful to the staff of the PFIE, especially to the study managers and animal handlers in charge of this study: Mickael Riou, Anne Pinard, Alain Delis and Elodie Moyer. We also thank Alix Sausset and Isabelle Fleurot for their assistance in the preparation of samples for single cell RNA analysis. We thank Dane Wittrup and Robin Fahraeus for the pYD1 and pcDNA3-OVA cloning vectors, respectively.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

References

1. Morales-Ubaldo, A. L. *et al.* Bovine mastitis, a worldwide impact disease: Prevalence, antimicrobial resistance, and viable alternative approaches. *Veterinary and Animal Science* 21, 100306 (2023).

2. Mata, F., Jesus, M. S., Pinto, R. P. & Mata, A. A meta-analysis of the mastitis vaccination efficacy in dairy cattle. *Open Vet J* 13, 179–187 (2023).
3. Zhao, Y. *et al.* Shifted T Helper Cell Polarization in a Murine Staphylococcus aureus Mastitis Model. *PLOS ONE* 10, e0134797 (2015).
4. Porcherie, A. *et al.* IL-17A Is an Important Effector of the Immune Response of the Mammary Gland to Escherichia coli Infection. *J Immunol* 196, 803–812 (2016).
5. Cebron, N. *et al.* Th17-related mammary immunity, but not a high systemic Th1 immune response is associated with protection against E. coli mastitis. *npj Vaccines* 5, 108 (2020).
6. Rainard, P. *et al.* Type 3 immunity: a perspective for the defense of the mammary gland against infections. *Veterinary Research* 51, 129 (2020).
7. Angrand, G. *et al.* Sneaking Out for Happy Hour: Yeast-Based Approaches to Explore and Modulate Immune Response and Immune Evasion. *Genes (Basel)* 10, 667 (2019).
8. Tan, Y. *et al.* Yeast as carrier for drug delivery and vaccine construction. *Journal of Controlled Release* 346, 358–379 (2022).
9. Berner, V. K., duPre, S. A., Redelman, D. & Hunter, K. W. Microparticulate β-glucan vaccine conjugates phagocytized by dendritic cells activate both naïve CD4 and CD8 T cells in vitro. *Cellular Immunology* 298, 104–114 (2015).
10. Huang, H., Ostroff, G. R., Lee, C. K., Specht, C. A. & Levitz, S. M. Robust Stimulation of Humoral and Cellular Immune Responses following Vaccination with Antigen-Loaded β-Glucan Particles. *mBio* 1, e00164-10 (2010).
11. Hodgins, D. C. *et al.* Mucosal Veterinary Vaccines. in *Mucosal Immunology* 1337–1361 (Elsevier, 2015). doi:10.1016/B978-0-12-415847-4.00068-9.
12. Sinha, D., Gopalakrishna, P. K., Paul, S. & Longet, S. A user's guide to designing efficient and safe mucosal vaccines: Challenges & potentials. *Oxford Open Immunology* 6, iqaf007 (2025).
13. Zhang, Z. *et al.* Mucosal immunity and vaccination strategies: current insights and future perspectives. *Mol Biomed* 6, 57 (2025).
14. Farber, D. L., Yudanin, N. A. & Restifo, N. P. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* 14, 24–35 (2014).
15. Knight, F. C. & Wilson, J. T. Engineering Vaccines for Tissue-Resident Memory T Cells. *Advanced Therapeutics* 4, 2000230 (2021).
16. Herry, V. *et al.* Local immunization impacts the response of dairy cows to Escherichia coli mastitis. *Sci Rep* 7, 3441 (2017).
17. Danzelle, C. *et al.* Saccharomyces cerevisiae as a platform for vaccination against bovine mastitis. *Vaccine* 42, 126385 (2024).
18. Apcher, S., Daskalogianni, C., Manoury, B. & Fåhraeus, R. Epstein Barr Virus-Encoded EBNA1 Interference with MHC Class I Antigen Presentation Reveals a Close Correlation between mRNA Translation Initiation and Antigen Presentation. *PLoS Pathog* 6, e1001151 (2010).

19. Mumberg, D., Müller, R. & Funk, M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 156, 119–122 (1995).
20. Rainard, P. *et al.* Antigen-Specific Mammary Inflammation Depends on the Production of IL-17A and IFN-γ by Bovine CD4 + T Lymphocytes. *PLOS ONE* 10, e0137755 (2015).
21. Cunha, P. *et al.* Expansion, isolation and first characterization of bovine Th17 lymphocytes. *Sci Rep* 9, 16115 (2019).
22. Noleto, P. G. *et al.* Punch-excised explants of bovine mammary gland to model early immune response to infection. *J Anim Sci Biotechnol* 14, 100 (2023).
23. Hao, Y. *et al.* Integrated analysis of multimodal single-cell data. *Cell* 184, 3573–3587.e29 (2021).
24. Yang, S. *et al.* Decontamination of ambient RNA in single-cell RNA-seq with DecontX. *Genome Biology* 21, 57 (2020).
25. Germain, P.-L., Lun, A., Garcia Meixide, C., Macnair, W. & Robinson, M. D. Doublet identification in single-cell sequencing data using scDblFinder. *F1000Res* 10, 979 (2021).
26. Bais, A. S. & Kostka, D. scds: computational annotation of doublets in single-cell RNA sequencing data. *Bioinformatics* 36, 1150–1158 (2020).
27. Rainard, P., Répérant-Ferter, M., Gitton, C., Gilbert, F. B. & Germon, P. Cellular and humoral immune response to recombinant Escherichia coli OmpA in cows. *PLoS ONE* 12, e0187369 (2017).
28. Shin, H. & Iwasaki, A. A vaccine strategy that protects against genital herpes by establishing local memory T cells. *Nature* 491, 463–467 (2012).
29. Pan, N. *et al.* Mammary γδ T cells promote IL-17A-mediated immunity against Staphylococcus aureus-induced mastitis in a microbiota-dependent manner. *iScience* 26, 108453 (2023).
30. Rainard, P., Gilbert, F. B., Martins, R. P., Germon, P. & Foucras, G. Progress towards the Elusive Mastitis Vaccines. *Vaccines* 10, 296 (2022).
31. Rainard, P., Foucras, G. & Martins, R. P. Adaptive Cell-Mediated Immunity in the Mammary Gland of Dairy Ruminants. *Front. Vet. Sci.* 9, (2022).
32. Tucker, N. *et al.* Bovine blood and milk T-cell subsets in distinct states of activation and differentiation during subclinical *Staphylococcus aureus* mastitis. *Journal of Reproductive Immunology* 156, 103826 (2023).
33. Guzman, E. & Montoya, M. Contributions of Farm Animals to Immunology. *Front. Vet. Sci.* 5, (2018).
34. Dantzler, K. W., de la Parte, L. & Jagannathan, P. Emerging role of γδ T cells in vaccine-mediated protection from infectious diseases. *Clin Transl Immunology* 8, e1072 (2019).
35. Suen, T. K. *et al.* A dual nature of γδ T cell immune memory responses. *eLife* 14, e104887 (2025).
36. Guzman, E. *et al.* Bovine γδ T Cells Are a Major Regulatory T Cell Subset. *J Immunol* 193, 208–222 (2014).
37. Pandiyan, P. *et al.* CD4 + CD25+Foxp3 + Regulatory T Cells Promote Th17 Cells In Vitro and Enhance Host Resistance in Mouse *Candida albicans* Th17 Cell Infection Model. *Immunity* 34, 422–434 (2011).

38. Hodgkinson, A. J., Carpenter, E. A., Smith, C. S., Molan, P. C. & Prosser, C. G. Effects on adhesion molecule expression and lymphocytes in the bovine mammary gland following intra-mammary immunisation. *Vet Immunol Immunopathol* 131, 110–116 (2009).
39. Mabrouk, N. *et al.* CXCR6 expressing T cells: Functions and role in the control of tumors. *Front. Immunol.* 13, (2022).
40. Kohlmeier, J. E. *et al.* The chemokine receptor CCR5 plays a key role in the early memory CD8 + T cell response to respiratory virus infections. *Immunity* 29, 101–113 (2008).
41. Beham, A. W. *et al.* A TNF-Regulated Recombinatorial Macrophage Immune Receptor Implicated in Granuloma Formation in Tuberculosis. *PLoS Pathog* 7, e1002375 (2011).
42. Strazza, M. & Mor, A. Consider the Chemokines: a Review of the Interplay Between Chemokines and T Cell Subset Function. *Discovery Medicine* 24, 31–39 (2017).
43. Kimura, K., Harp, J. A., Goff, J. P. & Olsen, S. C. Lymphocytes from one side of the bovine mammary gland migrate to the contra lateral gland and lymph node tissue. *Veterinary Immunology and Immunopathology* 108, 409–415 (2005).
44. Harp, J. A., Runnels, P. L. & Pesch, B. A. Lymphocyte recirculation in cattle: Patterns of localization by mammary and mesenteric lymph node lymphocytes. *Veterinary Immunology and Immunopathology* 20, 31–39 (1988).
45. Hodgkinson, A. J., Carpenter, E. A., Smith, C. S., Molan, P. C. & Prosser, C. G. Adhesion molecule expression in the bovine mammary gland. *Veterinary Immunology and Immunopathology* 115, 205–215 (2007).
46. Levitz, S. M., Huang, H., Ostroff, G. R. & Specht, C. A. Exploiting fungal cell wall components in vaccines. *Semin Immunopathol* 37, 199–207 (2015).

Figures

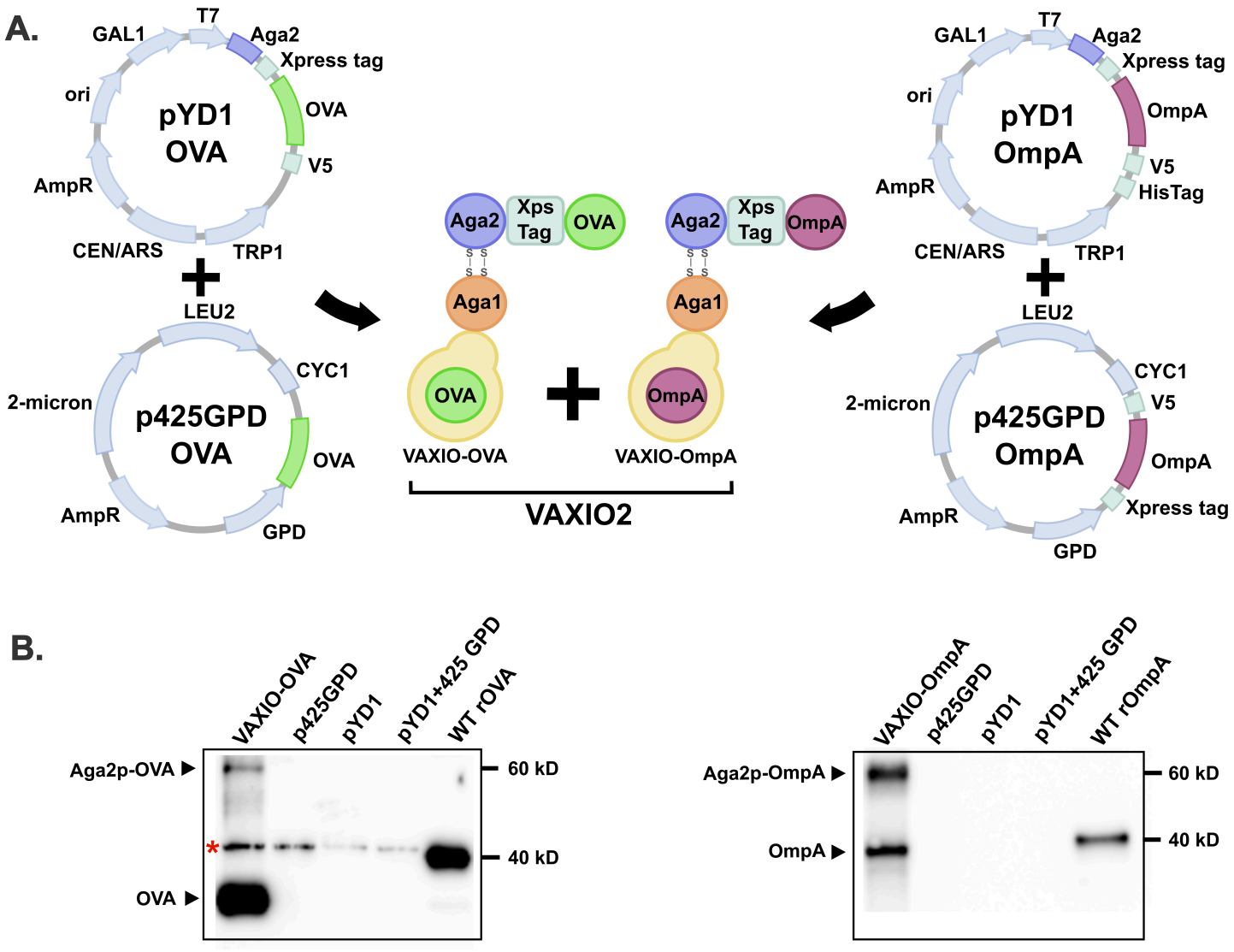


Figure 1

Design and validation of a bivalent yeast-based vaccine (VAXIO2). A. Schematic representation of dual heterologous antigen expression by *Saccharomyces cerevisiae*. Surface display of OVA or OmpA was promoted by the Aga1p–Aga2p system, after transformation of the EBY100 strain with the plasmids pYD1-OVA or pYD1-OmpA. For concurrent intracellular expression, EBY100 carrying pYD1-OVA or pYD1-OmpA were retransformed with p425GPD-OVA or p425GPD-OmpA. EBY100 strain transformed with OVA (VAXIO-OVA) or OmpA (VAXIO-OmpA) constructs were mixed at equivalent amounts to generate the prototype vaccine VAXIO2. **B.** Western blot analysis of OVA and OmpA expression. EBY100 strains carrying empty plasmids (p425GPD, pYD1 and pYD1+p425GPD) were included as negative controls. Recombinant WT OVA (rOVA) and OmpA (rOmpA) were used as positive controls. Red asterisk indicates a non-specific band.

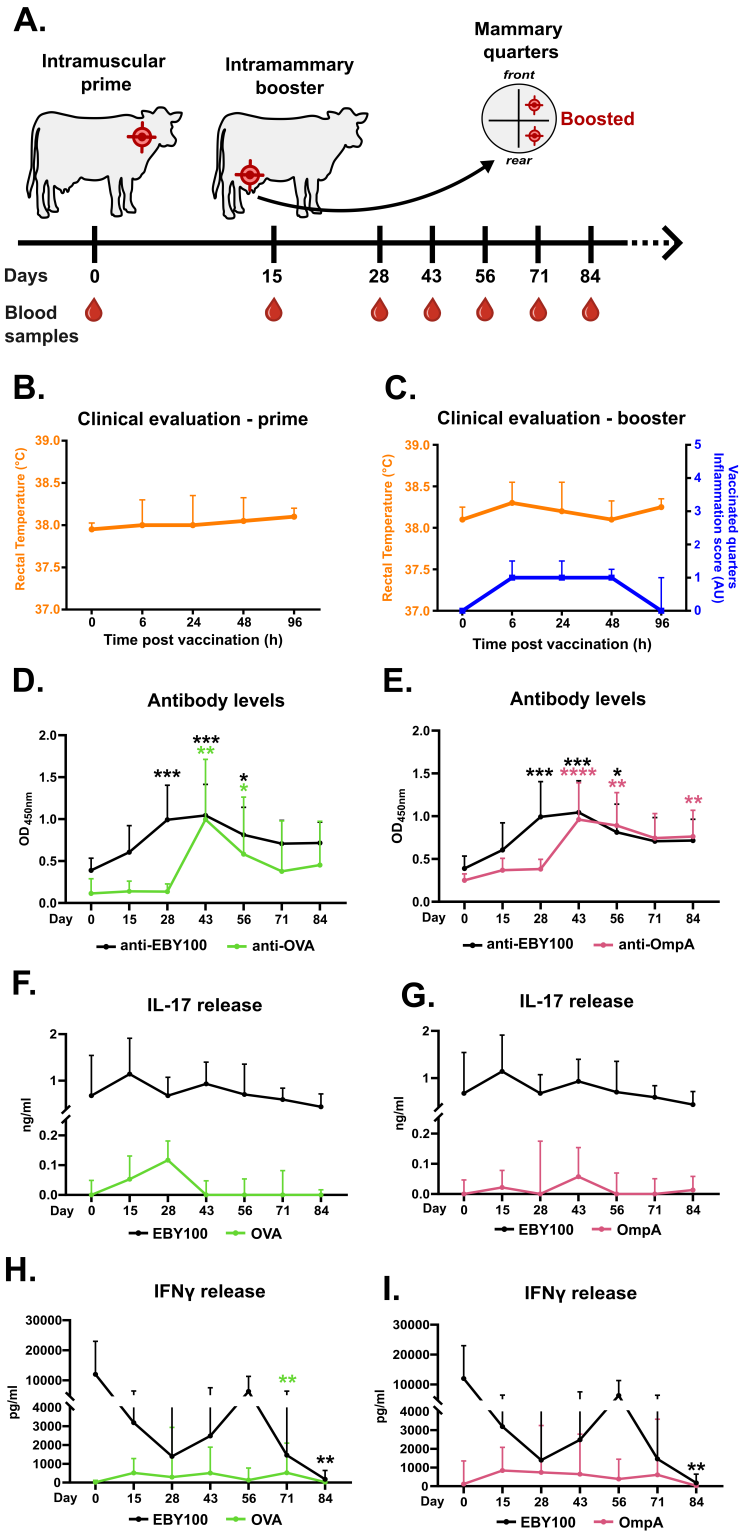


Figure 2

***In vivo* evaluation of VAXIO2. A.** Cartoon depicting the immunisation protocol and blood collection time points. **B.** Monitoring of rectal temperature after prime. **C.** Rectal temperature and mammary gland evaluation after booster. **D-E.** Analysis of serum total antibodies anti-EBY100, anti-OVA and anti-OmpA by ELISA. Blood from vaccinated animals was collected at indicated time points and stimulated in vitro with OVA, OmpA, or empty EBY100. IL-17 (**F–G**) and IFN-γ (**H–I**) release in the supernatant was

measured by ELISA. Asterisks denote statistically significant difference between indicated time points versus day 0.

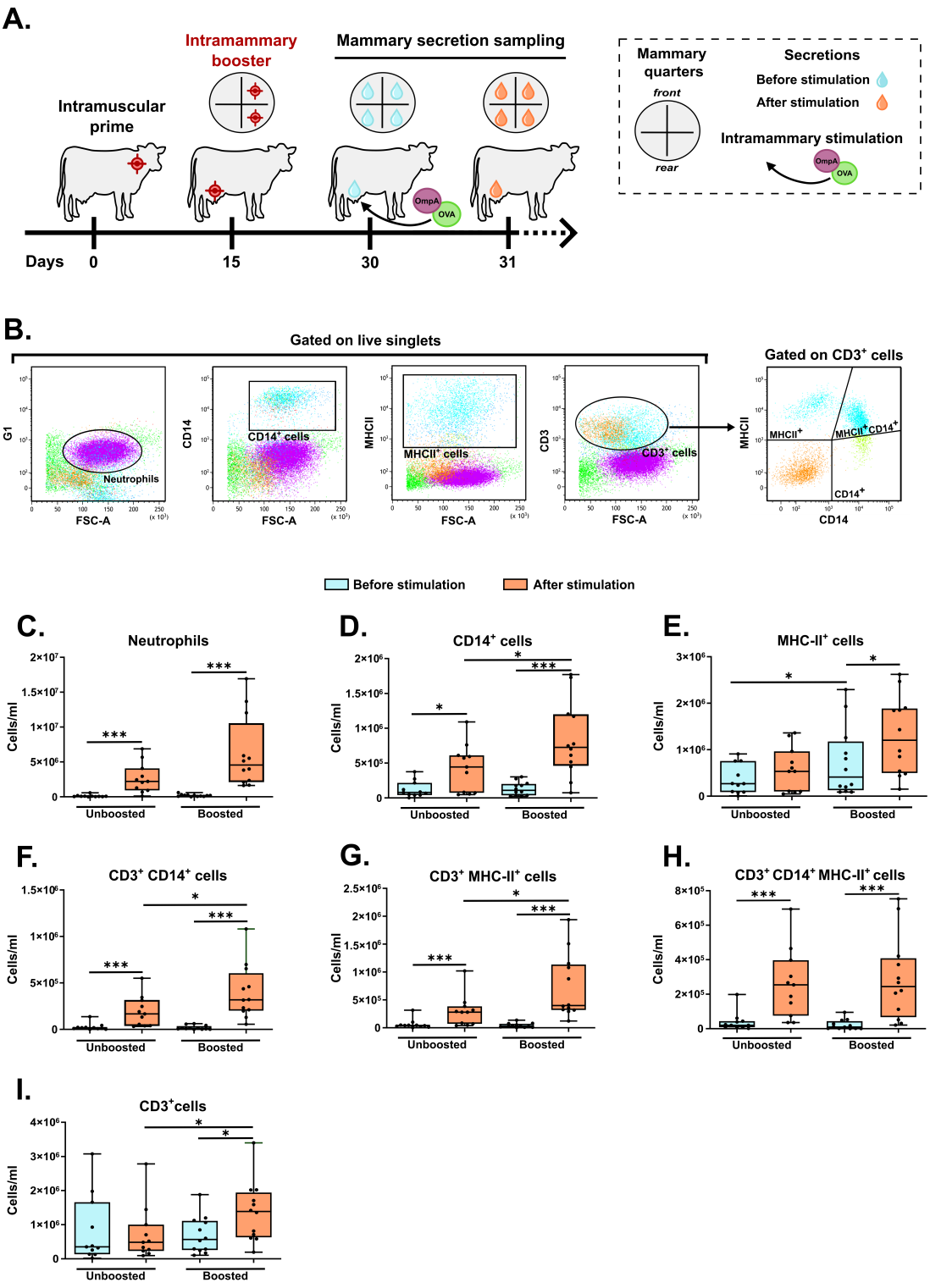


Figure 3

Mammary response to local stimulation with vaccinal antigens: overview of major cell populations. A. Scheme of intramammary stimulation with recombinant OVA and OmpA after immunisations (15 days

after the booster). Mammary secretions were collected before (day 30) and after (day 31) stimulation. Cells from unboosted and boosted secretions were stained for flow cytometry analysis as indicated in **B**. The number of the following cell types per ml of secretion was estimated: $G1^+$ neutrophils (**C**), $CD14^+$ (**D**), MHC-II^+ (**E**), $CD3^+CD14^+$(**F**), $CD3^+MHCII^+$ (**G**), $CD3^+CD14^+MHCII^+$ (**H**) and CD3+ (**I**). Data were analysed using Mann–Whitney for comparisons between boosted and control quarters, and Wilcoxon for paired comparisons within quarters. Data are shown as medians with interquartile ranges (n = 12). Asterisks indicate statistically significant differences between groups (*p < 0.05, **p < 0.01, *p < 0.001).

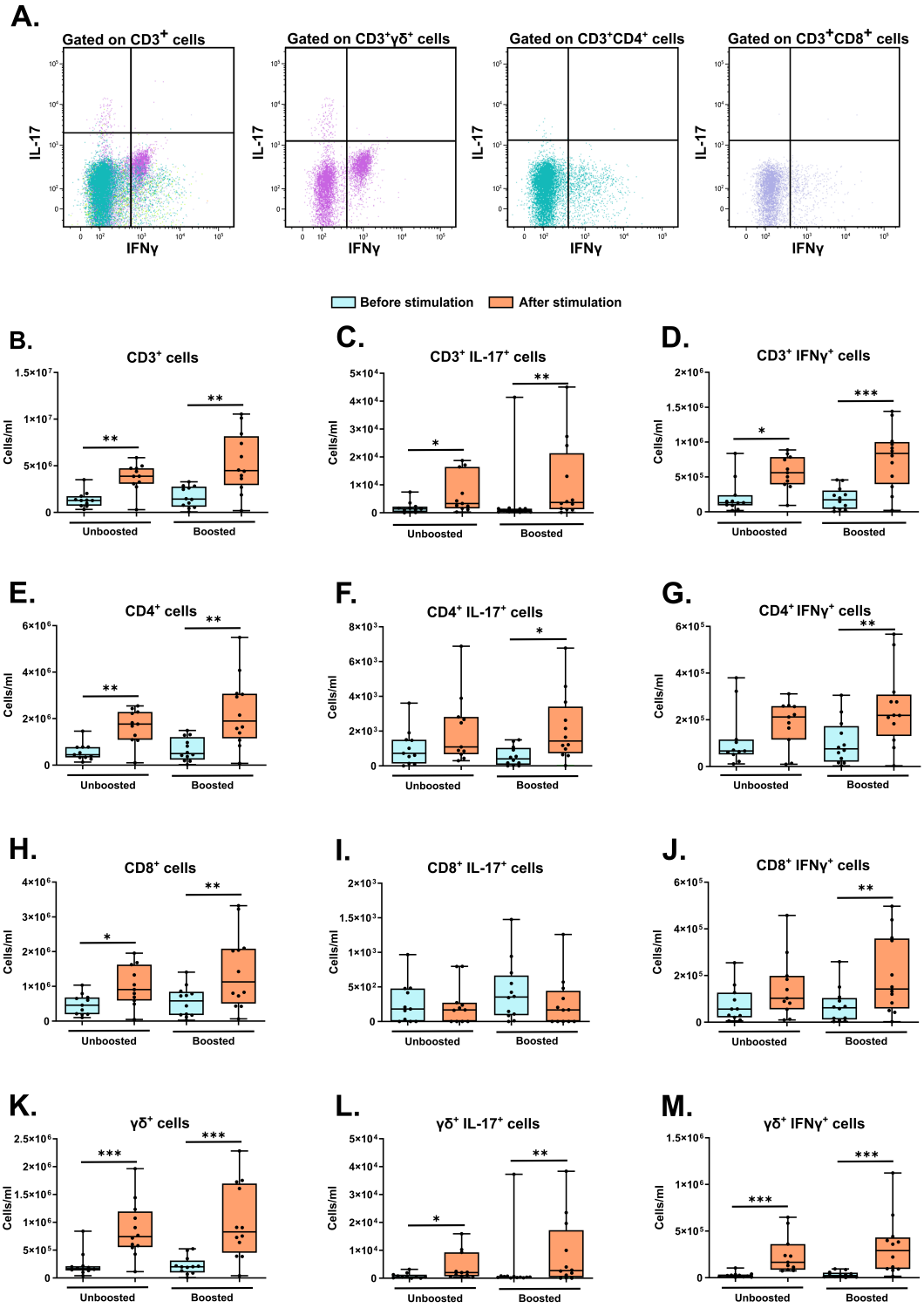


Figure 4

Mammary response to local stimulation with vaccinal antigens: overview of T-cell populations. Intramammary stimulation with recombinant OVA and OmpA were repeated 45 days after the booster. Mammary secretions were collected before (day 73) and after (day 74) stimulation. Cells from unboosted and boosted secretions were stained for flow cytometry analysis as indicated in **A**. The number of the following cell types per ml of secretion was estimated: $CD3^+$ (**B**), $CD3^+IL\text{-}17^+$ (**C**),

$CD3^+IFN\gamma^+$ (**D**), $CD4^+$ (**E**), $CD4^+IL\text{-}17^+$ (**F**), $CD4^+IFN\gamma^+$ (**G**), $CD8^+$ (**H**), $CD8^+IL\text{-}17^+$ (**I**), $CD3^+IFN\gamma^+$ (**J**), $\gamma\delta^+$ (**K**), $\gamma\delta^+IL\text{-}17^+$ (**L**), $\gamma\delta^+IFN\gamma^+$ (**M**). Data were analysed using Mann–Whitney for comparisons between boosted and control quarters, and Wilcoxon for paired comparisons within quarters. Data are shown as medians with interquartile ranges (n = 12). Asterisks indicate statistically significant differences between groups (*p < 0.05, **p < 0.01, *p < 0.001).

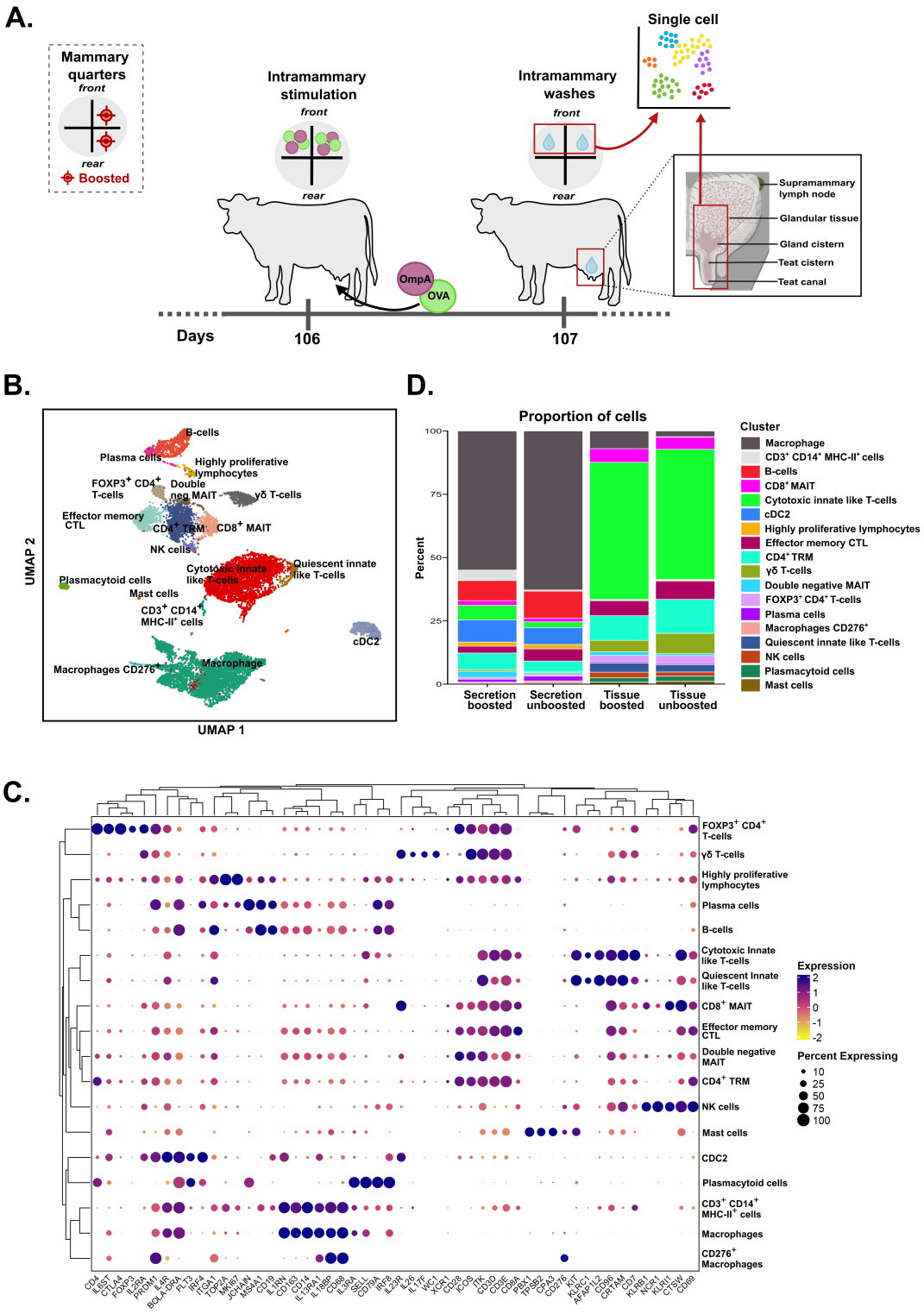


Figure 5

Local booster modifies the composition and trafficking of cell populations in the mammary gland. A. Scheme depicting the experimental approaches used to study changes in mammary gland upon local stimulation with vaccinal antigens. Three animals were intramammarily stimulated with recombinant OVA and OmpA at day 106 (91 days after booster) and euthanised at day 107 for mammary secretion and tissue collection. Leukocytes other than neutrophils (CD45+ G1-) were collected by flow-cytometry cell sorting and analysed by single cell RNA sequencing (scRNA-seq). **B.** Cell clusters visualization in two-dimensions via Uniform Manifold Approximation and Projection (UMAP). Eighteen major cell clusters were annotated based on the average expression of canonical marker genes. **C.** Dot plots showing the expression of surface and activation markers by each cell cluster. Dot size and color denote the percentage of cells within a cell type and the expression level, respectively. **D.** Distribution of cell clusters in boosted and unboosted mammary secretion and tissue.

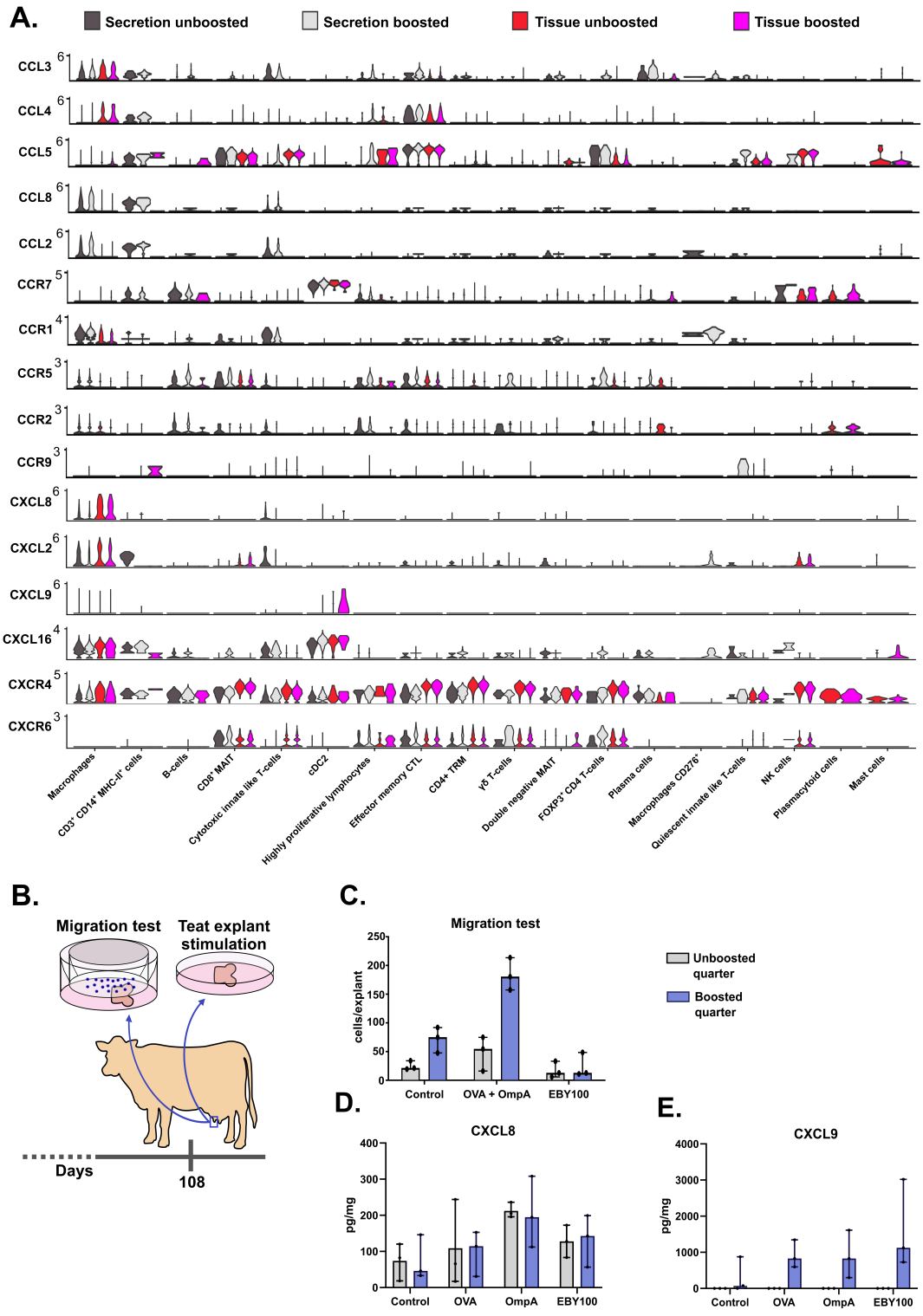


Figure 6

Expression of cell trafficking mediators and activation markers by cell populations in secretion and tissue from boosted and unboosted mammary quarters. A. Violin plots depicting the expression of chemokine and chemokine receptors coding-genes. **B.** Scheme depicting the experimental approaches used to study changes in cell trafficking after immunisation. Three animals were euthanised at day 108 (93 days after booster) and teat explants from unboosted and boosted mammary quarters were

collected for *ex vivo* stimulation and migration tests. **C.** Teat explants were stimulated with empty medium, vaccine antigens (recombinant OVA and OmpA) or empty vector (EBY100) and their capacity to induce the migration of Cell-Trace® stained autologous PBMC was evaluated by flow cytometry. **D-E.** Teat explants were stimulated with empty medium, recombinant OVA, recombinant OmpA or EBY100 and cytokine release of CXCL8 (**D**) and CXCL9 release (**E**) was analysed by ELISA.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplFig42026.04.08.png
- SupplFig12026.04.08.png
- SupplFig72026.04.08.png
- SupplFig62026.04.08.png
- SupplFig52026.04.08.png
- SupplFig22026.04.08.png
- SupplFig32026.04.08.png
- SupplementaryTable12025.10.31.pdf
- SupplementaryTable22026.02.28.pdf
- SupplementaryTable3geneexpressioncellclusters.xlsx