Lactic acid dampens inflammatory responses elicited by microbial TLR agonists from vaginal and cervical epithelial cells

Gilda Tachedjian[1,2,3,4], Anna C. Hearps[1,2], Daniela Srbinovski[1,3], David Tyssen[1], Muriel Aldunate[1,3], Raffi Gugasyan[1], Deborah Anderson[5], Richard A. Cone[6].

[1]Centre for Biomedical Research, Burnet Institute, Melbourne, Australia; [2]Department of Infectious Diseases and [3]Department of Microbiology, Monash University, Melbourne, Australia; [4]Department of Microbiology and Immunology at the Peter Doherty Institute for Infection and Immunity, University of Melbourne, Melbourne, Australia; [5]Department of Obstetrics and Gynecology, Boston University School of Medicine, Boston, MA, USA; [6]Department of Biophysics, Johns Hopkins University, Baltimore, MD, USA.

Background

- The female reproductive tract (FRT) is a primary route of transmission of sexually transmitted infections (STI) including HIV.
- *Lactobacillus spp.* dominate the microbiota of the healthy FRT.
  - Produce lactic acid (LA, both L and D isoforms) to ≈1%.
  - Associated with positive reproductive and sexual health outcomes.
- The FRT is lined with epithelial cells which are a physical and immunological barrier to infection (Fig.1).

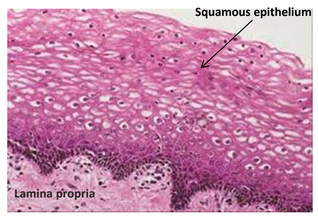

Figure 1. Epithelial structure of the FRT

- Inflammation in the FRT increases the risk of STI and HIV acquisition[1].
  - Inflammatory FRT imbalances such as bacterial vaginosis increases susceptibility to STI/HIV by 2-3 fold[2].
- Lactobacilli impair pathogen mediated inflammation from FRT cells[3].
- We have shown LA is virucidal against HIV[4], but the impact of LA on pathogen-induced inflammation from FRT epithelial cells is unknown.

Methods

The effect of LA (pH 3.9) on the viability and inflammatory response of epithelial FRT cells was assessed.

- Vaginal (VK2), endocervical (End) and ectocervical (Ect) epithelial cell lines and cervicovaginal primary cells were used.
- Cells were treated in transwells (physiologically relevant format).
- Cell viability (MTS assay) and monolayer integrity (diffusion of fluorescent dextrans, Fig. 2) were determined following treatment.
- Cytokine release from FRT epithelial cells stimulated with toll-like receptor (TLR) agonists ± L-LA was determined using a Luminex-based multiplex assay (ProCartaplex, eBiosciences).
- The effect of D-LA and L-LA at neutral pH was also determined and compared to media pH adjusted to low pH with HCl.

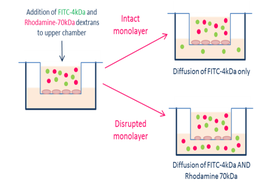

Figure 2. Assessment of monolayer integrity by dextran diffusion.

Results

Virucidal concentrations of LA are relatively non-toxic and do not disrupt FRT epithelial cell monolayers.

- L-LA and D-LA up to 0.3% (pH3.9) have minimal effect on FRT epithelial cell line viability (Fig. 3A).
  - Low pH alone (pH 3.9, adjusted with HCl) was non-toxic.
- Treatment of epithelial cell lines with 0.3% L- or D-LA, or low pH alone does not significantly alter dextran diffusion (B&C).

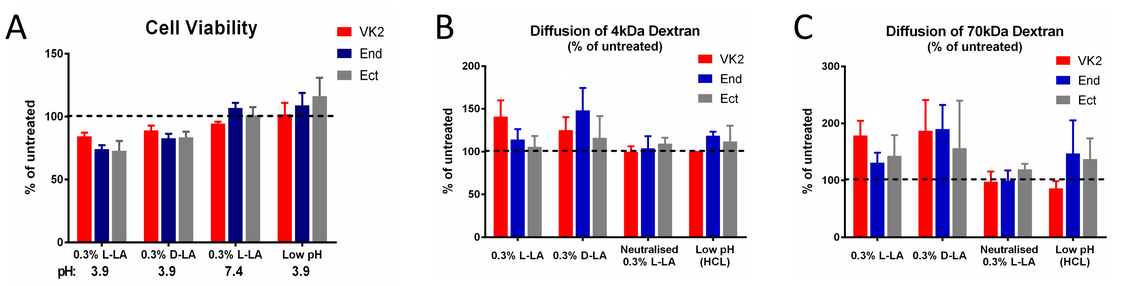

Fig 3 Epithelial cell lines were cultured in transwells for 18h in the presence of 0.3% L- or D-LA (pH 3.9), or low pH (HCl, pH 3.9) added to the upper chamber. Cell viability was assessed by MTS **(A)** and monolayer integrity by diffusion of 4kDa-FITC **(B)** and 70kDa-Rhodamine **(C)** dextrans. Data expressed as a % of untreated cells. Mean ± SEM shown from n≥3 independent assays.

LA induces an anti-inflammatory response from primary FRT epithelial cells and cell lines

- 0.3% L-LA (pH 3.9) induces production of the anti-inflammatory cytokine IL-1RA from primary and FRT epithelial cell lines (Fig 4A).
- Similar effect seen with D-LA, but not L-LA at neutral pH (pH 7.4) or low pH alone (HCl, Fig. 4B).

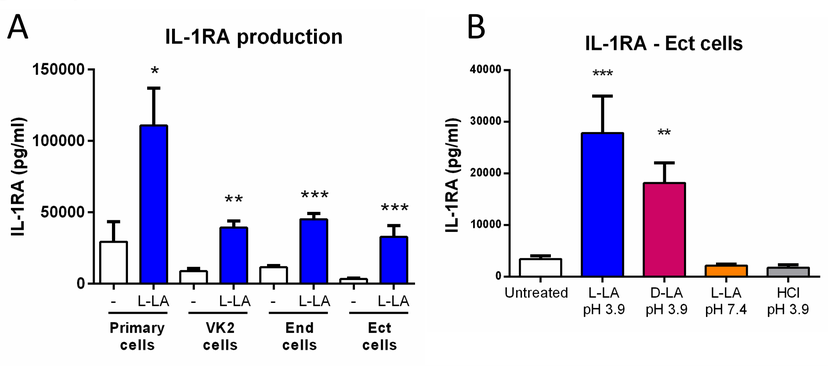

Fig 4 (A) IL-1RA production from the indicated cells was measured 18 h post-stimulation with 0.3% L-LA (pH 3.9). **(B)** IL-1RA produced from Ect cells stimulated with either 0.3% L- or D-LA (both pH 3.9), neutralised 0.3% L-LA (pH 7.4) or low pH (HCl, pH 3.9 maintained during stimulation). Mean ± SEM shown from ≥3 independent assays. *p<0.05, **p<0.01, ***p<0.001 as compared to untreated by Mann-Whitney test.

LA induces an anti-inflammatory state in FRT epithelial cells which inhibits TLR-induced inflammation.

- 0.3% L-LA inhibits the pro-inflammatory response to TLR agonists.
  - Observed in FRT epithelial cell lines (Fig. 5A-F) and primary cells (G&H).
  - Similar effect seen with D-LA but not neutralised L-LA or low pH (not shown).
- Pre-treatment of cells with L-LA protects cells from subsequent TLR-induced inflammation (Fig. 5I&J).

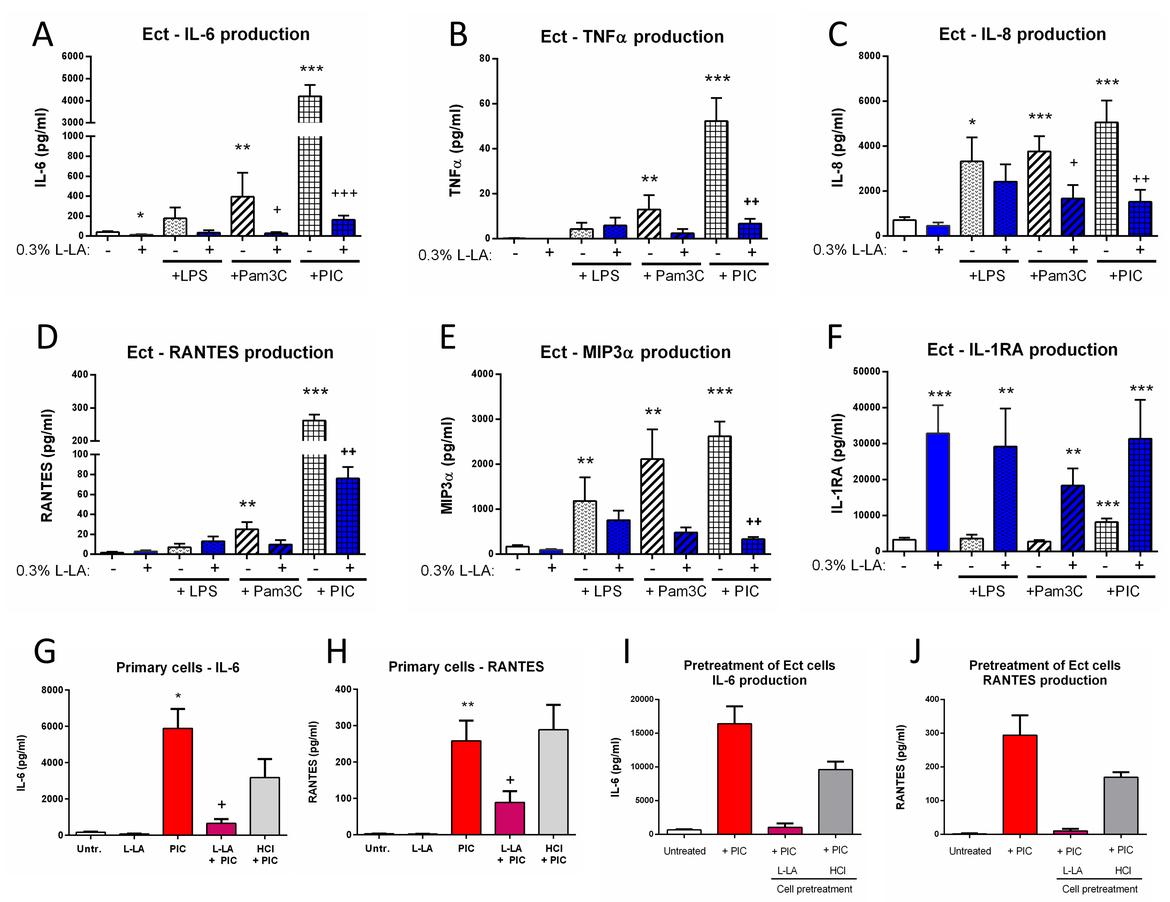

Figure 5: (A-F) Production of immune mediators from Ect cells after stimulation with the TLR agonists lipopolysaccharide (LPS; TLR4), Pam3CSK4 (Pam3C; TLR2) or Poly(I:C) (PIC, TLR3) for 18 h ± 0.3% L-LA. Similar results were seen in VK2 and End epithelial cells. Production of IL-6 **(G)** and RANTES **(H)** from primary cervicovaginal epithelial cells stimulated with PIC ± 0.3% L-LA or low pH (HCl) as above. Similar results were seen with IL-8, TNFα and MIP3α. Production of IL-6 **(I)** and RANTES **(J)** from Ect cells pre-stimulated for 1 h with 0.3% L-LA (pH 3.9), HCl (pH 3.9) or left unstimulated prior to PIC stimulation. Similar results were seen with IL-8, TNFα and MIP3α. Mean ± SEM shown from ≥3 independent assays (except I & J; from 2 experiments). *p<0.05, **p<0.01, *p<0.001 as compared to untreated; +p<0.05, ++p<0.01, +++p<0.001 as compared to TLR stimulated by Mann-Whitney test.

Conclusions and Significance

- Virucidal, relatively non-toxic concentrations of LA (0.3%) elicit an anti-inflammatory response from cervicovaginal epithelial cells of the FRT and reduce the TLR-induced production of pro-inflammatory cytokines and chemokines known to activate/recruit HIV target cells.
- D-LA had a similar anti-inflammatory effect, but L-LA at neutral pH nor low pH (HCl adjusted) media alone did not.
- Pre-treatment of cells with LA was able to induce an anti-inflammatory state that protected from later TLR challenge.

These results suggest the potential for LA to be used in topical microbicides to maintain an anti-inflammatory state in the FRT, and help reduce inflammation, cell activation and subsequent HIV and STI susceptibility

References

1. Masson L. *et al*, Clin Infect Dis, 2015:61(2):260-9.

2. Aldunate M. *et al*, Front Physiol, 2015;6:164.

3. Rose WA. *et al*, PLoS One, 2012;7(3):e32728.

4. Aldunate M. *et al*, J Antimicrob Chemother, 2013;68(9):2015-25.