Development of *in Vitro* Recurrent Aphtous Stomatitis (RAS) Immuno-competent model

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Introduction

Recurrent aphthous stomatitis (**RAS**) is a common ulcerative disease of the oral mucosa characterized by an increase of the mucosal permeability and disorders in the immune response modulation, in particular with a Th1 polarization, pro-inflammatory cytokines induction and apoptosis (1-2).
The inflammatory process involves the activation of IL-8, IL-6 and IL-1β, with an imbalanced ratio Th1/Th2 that causes a delayed re-epithelization of the ulcer. Treatments of Recurrent Aphthous Ulcers (RAU) aim to rebalance the Th1/Th2 response towards the establishment of a tolerant immune response.

Objective

To recapitulate the features of RAS *in vitro* by using 3D human reconstituted oral mucosa (HOE) model produced on special polycarbonate CMM inserts and co-cultured with monocytes (THP-1 cells).

The induction was based on a superficial localized ulcer associated to a pro-inflammatory stimulus that resulted in THP-1 cell migration through the tissue.

Materials & Methods:

As schematized in **Figure 1**, HOE-CMM have been abraded with algerbrush and challenged with the systemic incubation in 1,5 ng/mL TNF-α. Then tissues have been co-incubated with THP-1 cultured on the CMM polycarbonate membrane insert for 4h. After that, the treatment with reference products for aphtous lesions (e.g. anti-inflammatory steroids) were applied topically for 24h.
The tissue response was evaluated by:

- qRT-PCR to quantify inflammatory genes (IL-1 β, TLR-2) expression
- TUNEL assay to identify apoptotic cells in the ulcer
- Hematoxylin Eosin (H&E) staining to assess tissue morphological changes, monitor ulcers reduction during the recovery period, cells migration and re-epithelization

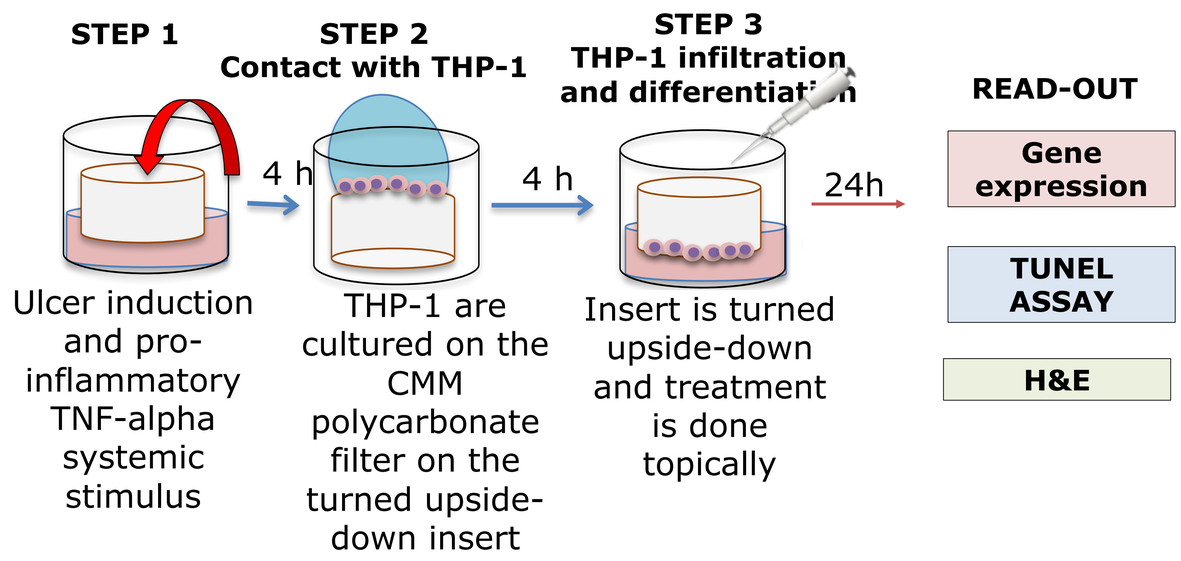


Figure 1. Experimental design and parameters to analyze.

GENE EXPRESSION OF RAS MODEL

The expression of inflammatory genes in the RAS immuno-competent model after 24h is reported in **Figure 2**.

- IL-1β has been evaluated because it is secreted by lymphocytes T-helper 1 and is involved in the immunomodulation of RAS, causing inflammation and tissue destruction.
- TLR2 has been considered because it stimulates the production of pro-inflammatory cytokines (through NF-kB activation) in monocytes and epithelial cells. Deficiency of TLR-2 activity is considered one of the main causes of the establishment of the pathology, leading to dysregulation of the inflammatory response and of the control of barrier integrity.

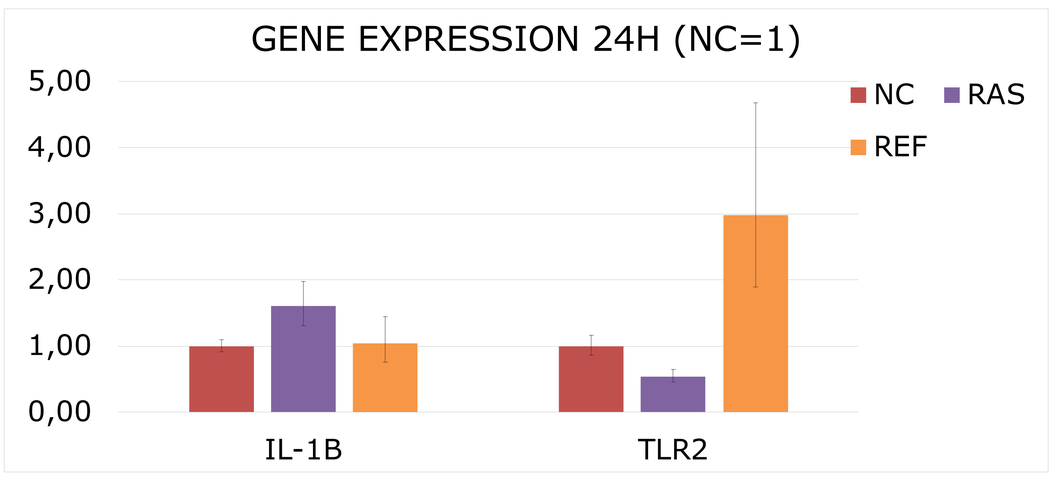
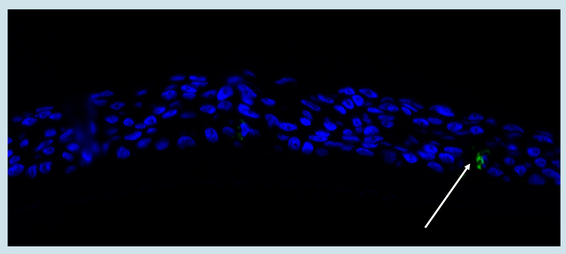
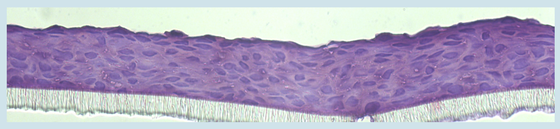


Figure 2. Gene expression in the RAS immunocompetent model after 24h recovery.

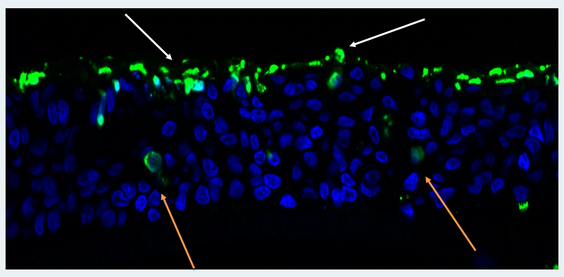
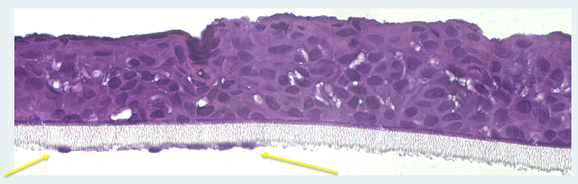
After 24h recovery in the RAS model (injured and inflammed HOE incubated with THP-1 and treated in STEP 3 with saline solution)
- IL-1β is slightly up-regulated (RQ: 1.61)
- TLR-2 is slightly down-regulated (RQ: 0.54)

suggesting that tissue is inflammed and the pathology is being established.
The reference product (REF: **Triamcinolone acetonide** caused an increase in TLR-2 (RQ: 2.97) counteracting inflammatory status.

HOE MORPHOLOGY AND APOPTOTIC CELLS AFTER 24H

TUNEL ASSAY: apoptotic cells	H&E: tissue morphology
HOE (Negative control)	
	

The HOE tissue shows a not significant number of apoptotic cells (white arrow) consistent with a HOE tissue cultivated in standard conditions.

RAS (Positive control)	
	

The RAS model has induced many apoptotic cells in the squamous layer (white arrows) while the infiltration of THP-1 cells in the tissue has not determined a significant damage as shown by the few apoptotic cells detected in the middle layer (orange arrows). As confirmed by H&E, at 24h HOE presents signs of the abrasion as reduced cell to cell connection: however, the injury at squamous layer level has been mostly recovered at this time. The yellow arrows indicate THP-1 cells in direct contact with polycarbonate filter and not yet infiltrated.

References:

1. Barros FM et al. (2010) Possible Association between Th1 Immune Polarization and Epithelial Permeability with Toll-Like Receptors 2 Dysfunction in the Pathogenesis of the Recurrent Aphthous Ulceration. Ulcers, Article ID 163804
2. Chen L et al. (2010) Positional differences in the wound transcriptome of skin and oral mucosa. BMC Genomics. 12;11:471.

Conclusions:

The RAS features have been recapitulated *in vitro* as evidenced by the apoptotic cells detection on the HOE superficial layer, down-regulation of TLR-2 and concomitant IL-1β release upon THP-1 stimulation in induced inflammatory conditions, indicating the establishment of a Th-1 polarization.
The lesional inflamed HOE-CMM cell migration model appears a biologically relevant model to assess the efficacy of new potential anti-inflammatory products designed to accelerate the healing process of the aphtous lesion.