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Research paper

Maximizing the immunological output of the cervicovaginal explant model

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ABSTRACT

In the field of sexually transmitted infections (STI), the cervicovaginal explant (CVEx) model, not only provides the opportunity to study the different immunological arms present in these tissues under steady state conditions, but also their response against ex vivo infection with relevant pathogens. The methodology associated to the establishment of the HIV infection model in the cervicovaginal tissue was described in detail by Grivel et al. earlier (Grivel and Margolis, 2009). With this model as a foundation, we illustrate different approaches to obtain a large number of immunological readouts from a single piece of tissue, thus maximizing the immunological output obtained. Additionally, we discuss several ideas to study some of the immunological subsets present in this mucosal tissue by enriching them with the addition of distinct chemokines or specifically inducing their activation. Importantly, most of the methodology and concepts proposed here can be applied to study the immune subsets resident in other tissues. In the field of mucosal immunology, the possibility of studying resident immune subsets from tissue explants offers a great opportunity to understand the real players against invading pathogens and localized pathologies. Furthermore, this model allows for addressing the therapeutic benefit of modulating the activity of certain molecules and immune subsets against invading pathogens, which may infer their contribution to pathogen control and direct novel therapeutic interventions.

1. Introduction

The ability to assess ongoing mucosal immune responses is critical for understanding host-pathogen responses and would assist mucosal vaccine development. In the case of the female genital tract immune responses, assays to determine the magnitude and quality of the immune response in mucosal tissues largely rely on sampling of peripheral blood and thus provide an incomplete picture of localized immune control. This localized immune response consists of resident immune subsets of innate and adaptive nature, such as mucosal-associated invariant T cells (MAIT) or resident memory T cells (T_{RM}), which could potentially protect against the nascent infection. Then, as the first wave of the immune response has been initiated and pro-inflammatory and chemoattractant molecules are released, recruited immune subsets from circulation will further contribute to fight against infection while it is still contained.

Ex vivo tissue culture has revolutionized cell biology (Al-Lamki et al., 2017). The first in vitro model of human cervical explant (CVEx) was established by Palacio et al. to study HIV infection (Palacio et al., 1994). Since then, particularly in the last 15 years, the human CVEx has developed as a new tool to study HIV transmission and to pre-clinically test microbicides (Anderson et al., 2010). Different models have been developed, including a polarized CVEx model described in 2000 by Collins et al. (2000), where a piece of ectocervix was placed on the top of a transwell chamber, with the mucosa facing up, surrounded by a sealing of agarose. All these models can provide early indicators of drug efficacy, allowing acceleration of promising microbicide candidates into the next pre-clinical phase (Richardson-Harman et al., 2009). In fact, the CVEx model has already demonstrated predictability of clinical outcome. The increased risk of HIV transmission caused by cellulose sulfate and nonoxynol-9 demonstrated in phase 3 efficacy trials would have been revealed and, thus, expensive clinical trials avoided, if the promising compounds would have been previously tested in the CVEx model, which predicted no efficacy or even increased susceptibility due to toxicity (Dezzutti and Hladik, 2013).

However, reproducibility is an issue, since many variables affect viral growth and pharmacological effects of a given preventative candidate (efficacy) (Richardson-Harman et al., 2009). Anderson et al. (2010) pointed out some of the variables confounding results obtained from clinical specimens collected from anonymous hysterectomies:

Abbreviations: CVEx, Cervicovaginal explant; STI, Sexually transmitted infections; MAIT, Mucosal-associated invariant T cells; TRM, Resident memory T cells; HSV-2, Herpesvirus-2; IFN- γ, Interferon-γ; LPS, lipopolysaccharide; TLR, Toll-like receptor; aGC, α-galactosylceramide; iNKT, Invariant Natural Killer cells

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unknown clinical history, pre-surgical hormone therapy, pre-surgical topical microbicide application, variability of the hormonal state, ectocervix vs endocervix origin, variation in number, types and location of immune cells, tissue deterioration in culture, use of amphotericin B, missing physiological variables (circulation, microflora, mucus, etc.). All these factors ultimately lead to a high degree of variability due to the intrinsic complexity of the model, which may require large sets of experiments to obtain reproducible results.

To limit that, efforts to standardize culture conditions, challenge virus and endpoint measurements have been performed in the field of microbicides against HIV (Richardson-Harman et al., 2009). On the other hand, there are ways to minimize such intrinsic variability, for other purposes beyond microbicide testing, mainly on the bases of including extensive controls for quality assurance (i.e. viability) and data normalization to the individual controls (Grivel and Margolis, 2009). Further, if menstrual cycle phase at the time of the hysterectomy can be inferred before setting-up the CVEx culture, different experiments can be grouped based on proliferative or secretory phase, or on non-cycling patients (amenorrhea/menopause), so the hormonal influence on the immune subsets is minimized. However, tissue culture is still limited by a number of other fundamental problems that include the lack of a circulatory system, the yield of certain immune cell subsets and the effect of tissue digestion in some analyses. Further, the logistics of coordinating collection of this type of samples are complex, as Gynecologists, Pathologists and possibly other services (tumor bank) may be involved in the circuit.

Although HIV infection and microbicide efficacy has been the main focus of this model, CVEx culture can be particularly useful to study innate responses and resident immune subsets dynamics in response to specific stimulus and other mucosal pathogens beyond HIV. Considering that immune response occurs in multiple distinct anatomical compartments over protracted periods of time, we can certainly not address the effectiveness of recruited immune subsets versus resident ones, yet we can evaluate initial critical immune cells and factors for pathogen containment as well as address the benefit of activating or inactivating certain subsets towards immunotherapy development. Overall, the main advantages of using these models against microbial infections are: the effects on barrier function can be addressed, the physiologically relevant target cells are present and, mainly, the effects of resident and innate immunity as well as factors characteristic of the genital tract environment can be addressed (Anderson et al., 2010). The female genital tract shares many features with other mucosal sites, but it also contains some unique features due to being a reproductive organ. This is another argument to perform certain experiments in the CVEx model itself, where for example the overall effect of hormones on the different resident cells can be determined.

In peripheral sites, tissue-resident memory T cells provide superior protection compared to circulating memory T cells (Cauley and Lefrancois, 2013; MacKay and Gebhardt, 2013; Shin and Iwasaki, 2013). These cells have been shown to stably reside in peripheral tissues and do not enter the circulation for prolonged periods of time. In the mouse model of intravaginal herpesvirus (HSV)-2 infection, the group led by Iwasaki has demonstrated that circulating memory $CD8^+$ T cells are mostly dispensable for protection against genital HSV-2 infection, and that interferon-γ (IFN-γ) production is a crucial effector mechanism by which the CD8 T_{RM} cells control HSV-2 (Shin et al., 2016). In our own series of studies in the live-attenuated vaccine model in non-human primates, the presence of antiviral effector $CD8^+$ T cells in the vagina of immunized monkeys correlates with protection from uncontrolled viremia after pathogenic challenge with simian immunodeficiency virus (Genesca et al., 2008). Similar observations are found for Chlamydia (Johnson and Brunham, 2016) and even cancer, where infiltration of tumors by $CD103^+CD8^+$ T cells correlated with better clinical outcome in patients with lung, ovarian and bladder cancer (Sun et al., 2016). Thus, vaccine development should focus on generating and stimulating these type of cells, while correlates of protection should be determined in tissues instead of blood.

One of the first mentions to resident memory cells appears in 1981, when researchers investigating the dynamics of the T cell responses against *Listeria monocytogenes* in a rat model suggested that antigen-specific lymphocytes, during the early phase of the infection, spontaneously extravasate and settle in tissues as long-lived memory cells (Jungi and Jungi, 1981). Fourteen years after, Ibraghimov et al. report phenotypically distinct resident $CD4^+$ alpha beta T cells in the female genital tract of mice that rapidly proliferate in response to stimuli (Ibraghimov et al., 1995). Few years later, while reviewing the role of intraepithelial lymphocytes (IEL), the concept of a "Third Way" in immunology is proposed (Hayday et al., 2001). This idea emphasizes the power of local mechanisms to deal with local issues (Hayday et al., 2001). Yet, is after 2012 when T_{RM} research expands and better defines the phenotype and functionality of these populations (Ariotti et al., 2012; MacKay et al., 2012; Wakim et al., 2012). Since then, similar emphasis on the diversity and particularities of tissue resident subsets due to their environment are being discussed in other cell types such as macrophages (Mowat et al., 2017) or natural killer cells (Peng and Tian, 2017). Considering that in the genital tract, lymphoid aggregates containing $CD19^+$ and $CD20^+$ B cells as well as $CD3^+$, $CD4^+$ and $CD8^+$ T cells are present (Johansson et al., 1999), non-conventional T cell subsets are also abundant (in some cases more frequent than in blood) (Qualai et al., 2016) and different antigen presenting cells (Duluc et al., 2014) are also abundant, it seems that the use of the CVEx model to study the role of the different immune players, their activation and function in situ is rather unlimited.

An improved characterization of resident immune subsets would seem essential if we are to understand how immune responses perform at mucosal surfaces and how to boost them during immune interventions. Herein, we discuss some of the possible readouts and analyses that can be obtained from culturing a mucosal tissue like the cervix. Interestingly, we consider that the methodology described in this protocol can be applied to other biopsies and explants obtained from other peripheral tissues. This may help guide how to maximize immunological readouts from any tissue culture system.

2. Immunological interventions and outputs

From the basic cervicovaginal explant model (Grivel and Margolis, 2009), several analyses can simultaneously be performed to obtain different readouts from the same experiment. This may be applied to determine a given parameter by different methodologies, thus confirming the obtained results and giving strength to the overall experiment. Alternatively, different immune parameters may be determined in the conditioned medium (supernatant), in the cells that emigrate out of the tissue, or in the remnant tissue, providing a broader picture of, for example, the effect of a given stimulus.

2.1. Immune stimulation

Hormones, cytokines, chemokines, bacterial compounds like lipopolysaccharide (LPS) and other Toll-like receptor (TLR) ligands are examples of molecules that will impact the immune populations present in the cervicovaginal mucosa. They can be added to the tissue culture medium to study, for example, their effect as potential adjuvants or when simulating initial danger signals initiated by a pathogen (pathogen-associated molecular patterns). After several hours or days of stimulation we can determine the activation profile of these stimulus via different techniques like flow cytometry phenotyping of the immune subsets (after tissue digestion), immunohistochemistry (IHC) or immunofluorescence (IF) phenotyping, gene expression profiling (RNA extraction and PCR) or determine the cytokines and molecules secreted by the tissue in response to the stimulus (secretome) by ELISA or similar techniques.