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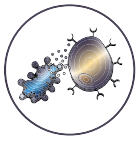

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Review Article

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Modulation of Mucosal Antiviral Immune Response at the Female Genital Tract by Immunobiotic Lactic Acid Bacteria

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Abstract

The female genital tract (FGT) has unique characteristics, which have evolved to adequately carry on its vital function of reproduction. Thus, on the one hand the FGT has to be tolerogenic enough not to reject the allogeneic sperm and fetus in order to ensure procreation and; on the other hand it should be reactive enough for clearing viral, bacterial, fungal and parasitic pathogens. To increase complexity, there is a constant exposure of mucosal cells to an endogenous microbiota [1-3], and to the direct and indirect action of sexual hormones (estradiol and progesterone) [4]. The immunity of the FGT has not been studied as extensively as the immunity of the gut, and the antiviral response at this important mucosal site is even less understood. In line with this, immune modulation by lactic acid bacteria (LAB) offers a brand new field of research. In this review, we discuss some recent advances in the understanding of viral infections at the FGT, the use of TLR-ligands as possible therapeutic tools, and the hallmarks during the infection process, which may be used for modulation of the antiviral responses using immunobiotic and/or recombinant LAB expressing viral antigens.

Keywords

Female genital mucosa; TLR-ligand; Mucosal immunity; Microbiota; Immunobiotics

The Female Genital Tract Mucosa

The female genital tract consists of two different types of mucosal surfaces. The lower genital tract (ectocervix and vagina) is a type II mucosal surface, and it differs from the respiratory and intestinal mucosa mainly because it consists on a stratified epithelium, it produces IgG rather than IgA and it lacks inductor sites such as the Peyer's patches (PPs). In contrast, the upper female genital tract (oviducts, ovaries, uterus, and endocervix) consists on a type I mucosal surface, being monolayered as most of the epithelia constituting the Common Mucosal Immune System. The cervical transition zone bridges both regions and it is especially susceptible to infections and cancer. It was long believed that the upper FGT was sterile, but recent studies have shown that there is a constant exposure to antigenic material (especially commensal bacteria), which is transported to the uterine lumen by peristaltic waves [5,6]. The relatively low incidence of upper FGT infections suggests that this mucosa is more reactive than the vaginal mucosa [7,8]. Furthermore, quantitative and qualitative differences in the expression of pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) in the FGT coincides with its tolerogenic tendency in the lower tract and with higher reactive mucosal immune responses in the upper part [9].

Many pathogens have adapted their transmission mechanisms to take advantage of behaviours that are essential for the survival of the host species, such as eating, breathing and sexual reproduction.

The main viral sexual transmitted diseases are human papilloma virus (HPV), human immunodeficiency virus (HIV), and Herpes simplex virus type 2 (HSV-2). Each virus has its unique kinetics of infection, pathology and host evasion mechanisms. In a similar way, the genital mucosal immunity reacts differently towards each of them. The study of viral infectious cycles and the concomitant host response as well as the development of microbicides and vaccines are limited to the use of animal models because of obvious ethical and practical issues. While HIV research uses SIV and non-human primates as main models of infections, HSV-2 research is mainly based on mouse models, whereas HPV studies rely on the cottontail rabbit papillomavirus model. The advantages and disadvantages of the specific animal models as well as their differences to human FGT have been reviewed elsewhere [10-13]. Furthermore, the mechanisms of antiviral immunity in the genital mucosa have attracted relatively little attention compared to those in other mucosal surfaces such as gastrointestinal and respiratory tracts. Thus, viral infection processes in the FGT are complex and will only be introduced in this review in general terms highlighting some aspects of HSV-2 infection and those aspects involved in antiviral immunity, which may be targeted by immunobiotic bacteria as discussed more extensively in this text. The response to viral infection could be summarized in four main stages: a) viral sensing and the activation of innate defenses, b) linking innate and adaptive responses, c) elimination of virus by effector mechanisms and, d) the establishment of long-term memory [14].

Innate Immunity in the Context of FGT Viral Infections

PRRs related to viral recognition are expressed at endosomes (TLR3, 7, 8 and 9) or in the cytosol such as the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and the RIG-I-like receptor family (RLRs) (RIG-I (retinoic acid inducible gene 1) and MDA5 (melanoma differentiation associated gene 5) of epithelial cells and dendritic cells (DCs) of the upper and lower female FGT. These PRRs play a key role in the first recognition of the viral pathogen [9]. Other TLRs expressed in the cell surface, including TLR-2 and TLR-6 have been implicated in viral sensing of specific viruses. Viral recognition may be cell-intrinsic if detected by the infected cell itself, or cell-extrinsic when the recognizing cell (mainly macrophages and DCs) is not infected itself but endocytoses viral components such as nucleic acids, which may be recognized by endosome-associated PRR [14,15]. Upon activation of TLRs by

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viral antigens in the epithelial cells, the signal can be transduced via two pathways, the MyD88-dependent and the MyD88-independent pathways. The MyD88-dependent pathway is triggered by all TLRs except for TLR-3. This pathway ends up in the translocation of NF-κB into the nucleus and activates the transcription of inflammatory cytokines and natural antimicrobial peptides [16]. Inflammatory cytokines and chemokines are crucial for the recruitment and activation of innate immune cells. Defensins are microbicidal *per se* but they also exert other important effects: it has been shown that they are endogenous activators of macrophages via TLR-1 and 2 [17], and that they are able to directly inhibit HIV entry to cells *in vitro* [18]. The MyD88-independent pathway, converges in the activation of IRF3 and results in the transcription of type-1 interferons (IFN-α and IFN-β) as well as the activation of NF-κB [16]. The most important innate antiviral defense initialized by this signaling pathway is the synthesis of type I IFNs. Firstly, type I IFNs initiate an antiviral state in non-infected cells by the activation of genes which directly inhibit viral replication; and secondly, they activate natural killer (NK) cells and plasmacytoid DCs (pDCs). NK cells produce IFN-γ and induce apoptosis of virus-infected cells [19]. Mature pDCs are the principal type I IFN producers exerting a positive feed-back in the antiviral state (Figure 1).

Innate immunity is critical for controlling the first stages of viral infection, but the activity of pDCs and NK cells may not be sufficient for complete viral clearance and therefore, the activation of adaptive immunity is fundamental for full protection [19]. Both intrinsic and extrinsic types of recognition by PRRs are linked and are necessary for proper Th1 activation. For instance, in a HSV-2 infection, the activation of DCs needs a certain mucosal microenvironment given by epithelial cells in order to activate Th1 responses in an effective way [20].

Linking Innate and Adaptive Immune Responses to Viral Infections

As mentioned before, DCs are key players in connecting the innate and adaptive immunity. In the FGT, as described for other mucosal sites, macrophages and DCs are the principal antigen presenting cells (APCs). Under normal conditions, the main population found in the FGT mucosa is Langerhans cells located within the epithelium and submucosal DCs located beneath the epithelium (Figure 1) [21]. In the steady state, Langerhans cells and submucosal DCs are highly phagocytic and express several pattern recognition receptors (PRRs) that can recognize a wide array of microorganisms. After pathogen recognition through PRRs, DCs and Langerhans cells undergo a maturation programme and migrate to the draining lymph nodes to prime naive T and B cells. Both cells are tolerogenic in the absence of pathogens (Figure 2A). In case of infection, or in an inflammatory state, other blood-derived populations of APCs such as pDCs and monocyte-derived DCs can also be found in the FGT mucosa after pathogen challenge [21,22]. The current paradigm of immune induction to infectious agents at body surfaces covered by squamous epithelium such as the vagina is that Langerhans cells encounter pathogens within the epithelium, take up antigens from pathogens, and migrate to the draining lymph nodes to prime naive T cells. Therefore, some observations suggest that Langerhans cells provide critical antiviral defense functions and suggest that treatments to augment their activity may be useful therapeutic tools. There are at least four populations of Langerhans cells in the FGT epithelium that

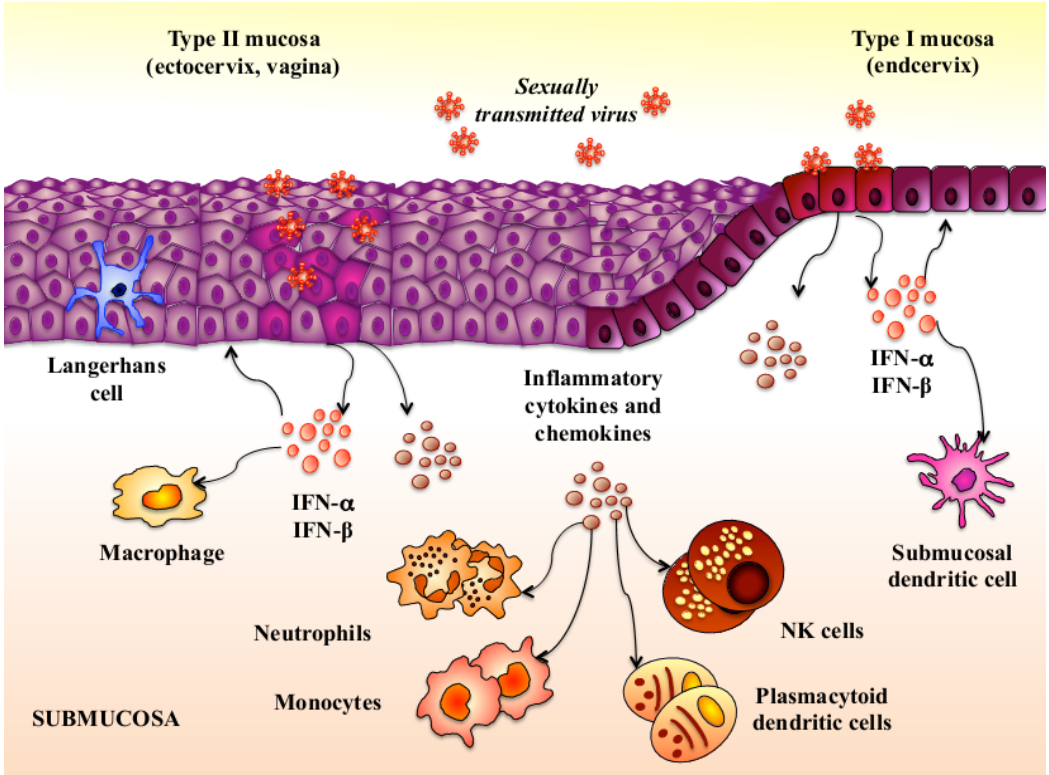

Figure 1: Scheme of the structure of mucosa and submucosa of the female genital tract showing the main components involved in the antiviral innate response.

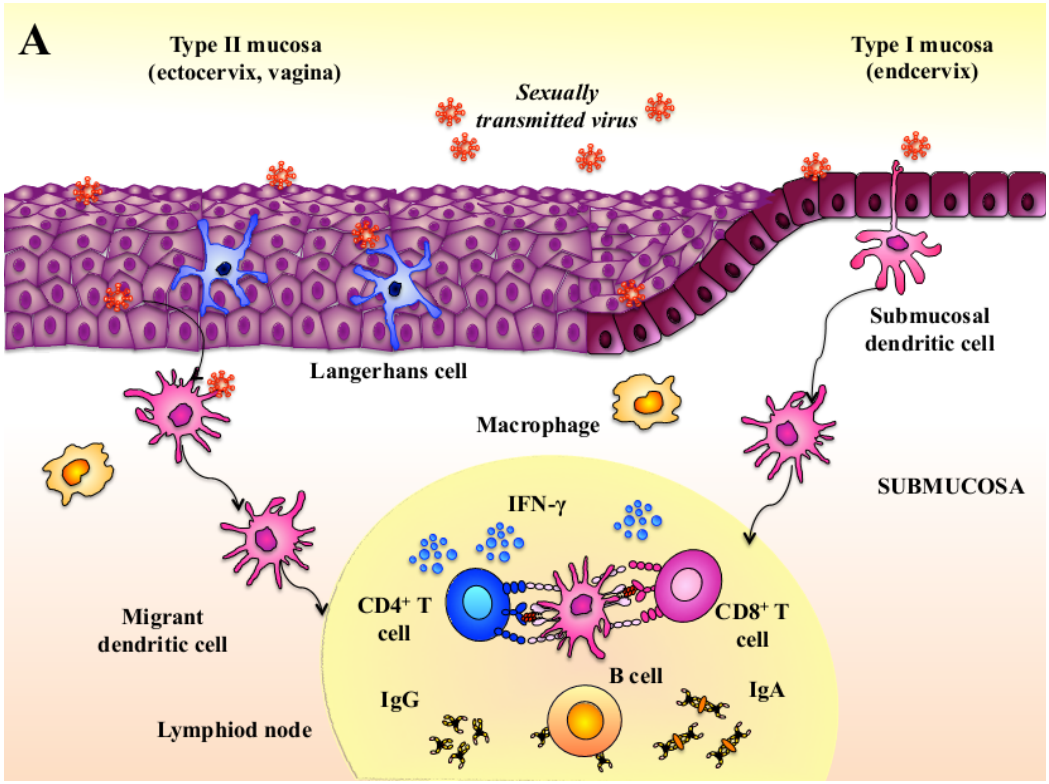

Figure 2A: Antiviral adaptive immune response at the mucosal surface of the female genital tract A) Adaptive Humoral Response.

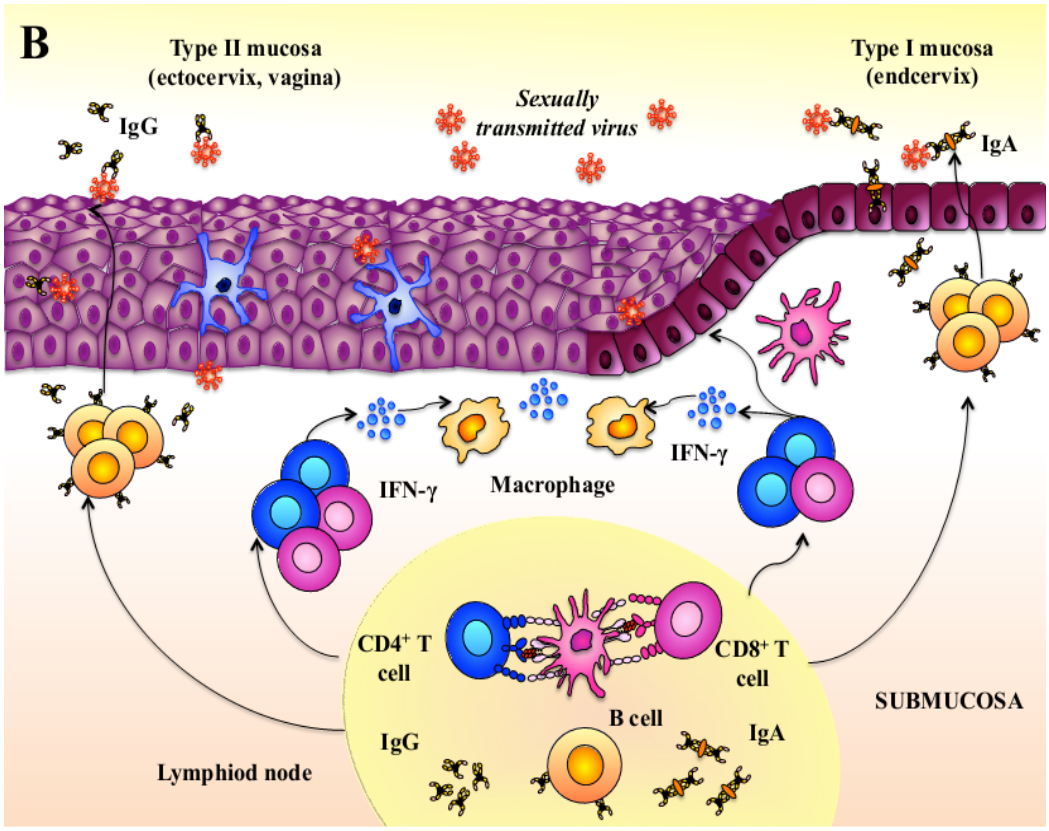

Figure 2B: Antiviral cellular adaptive immune response.

have been identified: by immunohistochemistry (I-A$^+$/F4/80$^+$, I-A$^+$/F4/80$^-$, I-A$^-$ /CD205$^+$, and I-A+/CD205$^-$) [23] or by flow cytometry (CD11b$^+$F4/80^{high}, CD11b$^+$F4/80^{int}, and CD11b$^-$ F4/80$^-$) [24]. However, it is not known whether these populations have specific functions in the immune responses of the vaginal mucosa. It has been shown that *in vitro* generated Langerhans cells have cytotoxic activity against cervical epithelial cells expressing HPV16 E6 and E7 [25]. On the contrary, it was reported that HSV-2 infection of the vaginal epithelium results in the complete lysis of the cells in this layer, destroying Langerhans cells within the first 48 hours after challenge [26]. Therefore, Langerhans cells seem to have little or no protective effects in HSV-2 infection.

In addition to Langerhans cells, the FGT sub-mucosal DCs express PRRs with capability of binding to a variety of pathogens including viruses (Figure 2A) [27]. Usually, DCs in the FGT are tolerogenic but in the presence of viral antigens, they are able to mount a strong and immediate effector immune response. In fact, it is speculated that non-infected DCs are more important antigen presenters than infected DCs and epithelial cells (Figure 2A) [14]. Depending on the virus, specific DC populations will be responsible of priming CD4$^+$ T cells. For instance, in HSV-2 infection, migratory CD11b$^+$ DCs and not lymph node resident CD8a$^+$ are responsible for priming CD4$^+$ T cells (Figure 2A) [26]. It was observed a rapid recruitment of submucosal CD11c+ DCs within 24 hours after the challenge with HSV-2, followed by a subsequent appearance of CD11c$^+$/CD11b$^+$ DCs presenting the viral peptides in the draining lymph nodes by hour 48 post-infection [26]. In contrast, it has been reported that DCs have a limited role in the host response to HPV infection, as they are not activated by uptake of HPV capsids [28]. T-cell priming occurs exclusively in the vaginal draining lymph nodes (inguinal and iliac LNs) and is followed by a naïve lymphocyte influx in the vaginal mucosa (Figure 2A). One of the major characteristics of plasmacytoid dendritic cells (pDCs) is the secretion of high levels of type 1 IFN in response to viral infection, therefore pDCs play a key role in antiviral immunity. Following intravaginal HSV-2 infection, pDCs are recruited to the vaginal tissue and produce large amounts of IFN-α [29]. pDCs recognize HSV-2 through the TLR9 to provide the first line of immune defense. Furthermore, this subset is required for CpG oligodeoxynucleotide-mediated protection against lethal intra-vaginal HSV-2 challenge [30]. In support of a role for pDCs in the defense against HSV, severe inflammation and tissue destruction was seen in pDC deficient mice after genital infection with HSV-2, although mice deficient in TLR9 showed more profound tissue damage [29].

Humoral Immunity in the Context of FGT Viral Infections

The humoral immune compartment of the human genital tract exhibits features which are unique and functionally different from other compartments of the mucosal immune system. The main immunoglobulin (Ig) isotype found in the lumen of the upper FGT is IgA as in other mucosal tissues. This is not the case for vagina and ectocervix, where the main Ig isotype present in cervicovaginal secretions is IgG [31] (Figure 2B). These differences are related to the transcytosis and the presence or absence of the corresponding Fc receptors, which allow the transport of the respective Ig across epithelial cells. To add complexity to the humoral immune system in the FGT, it was reported that hormonally mediated variations modulate the expression of receptors on epithelial cells involved in Ig transport and profoundly influence Ig levels in the vaginal fluid. Even though the antibodies found in cervicovaginal secretions may directly bind free virus particles as well as cell-associated viruses (Figure 2B), the contribution of these antibodies to virus clearance is still discussed [32]. While IgG produced in response to HPV systemic vaccines confers protection [28], the effectiveness of IgA and IgG neutralizing antibodies in the context of HSV-2 and HIV infections remains controversial [19,33]. In this regard, it seems that specific HSV-2 antibodies play a role in defense by neutralizing viruses, but on the other hand are not sufficient for conferring full protection [34,35].

Cellular Immunity in the Context of FGT Viral Infections

Although neutralizing antibodies are protective against infections with many viruses such as HPV, induction of T cell-mediated immunity, particularly antigen-specific CD4$^+$ Th1 cells, is critical for full protection in infections such as HIV and HSV-2. Th1 cell-mediated immunity is indispensable for the destruction of intracellular pathogens and is driven primarily by T lymphocytes. T cells are located in the stroma of the vagina, cervix and uterus both below the epithelium and also dispersed within epithelial cells where they are known as intraepithelial lymphocytes [32]. The cellular adaptive response is crucial for effectively resolving HSV-2 infection. This has been shown in mouse models as well as in human studies where deficiencies in T cell immunity resulted in very severe outcomes [36]. CD4$^+$ T cells play an important role in the clearance of HSV-2. These cells assist CD8$^+$ T cells in their migration to vaginal mucosa by secreting recruiting cytokines and they are the main producers of IFN-γ, a cytokine with a direct antiviral effect (Figure 2B). This cytokine is pleiotropic and exert its powerful effect by several mechanisms: it inhibits viral replication by hindering the transactivation-induced transcription of HSV early genes [37]; it enhances NK cell activity and proliferation; it up-regulates adhesion molecules, which facilitate migration of other immune cells to the infection site [38]; it up-regulates MHC-I and II molecules on target cells, favoring the recognition of virus-infected cells by cytotoxic T cells [35]. On the other hand, CD8$^+$ cytotoxic effector T cells recognize virus infected cells through peptide-bound MHC class I molecules expressed on their surface, inducing apoptosis through perforin- and granzyme-mediated cytolysis or inducing apoptosis infected cells Fas-ligand [39]. Although their key role has been demonstrated in different studies, there is also evidence that patients with HSV-2 recurrent lesions have a great number of non-exhausted cytotoxic CD8$^+$ T cells, suggesting that they are important but still not enough to control infection completely [40]. Thus, a CD4$^+$ and CD8$^+$ balance is needed for controlling HSV-2 infection, being CD4$^+$ T cells more important at the earlier stages of infection and CD8$^+$ T cells becoming more important later [19,32]. The importance of an appropriate cooperation between CD4$^+$ and CD8$^+$ T cells for the protection against viral infections in the FGT was also suggested by the demonstration that the crucial role of CD8$^+$ T cells in controlling chronically infected viruses including HIV relies on functionality of CD4$^+$ T cells [41].

More recently, it has been described, that Th17 are present at mucosal surfaces and are thought to play a role in maintenance of immune homeostasis discriminating autochthonous microbiota from pathogens [42]. They have been involved in responses to fungi and bacteria at mucosal sites, and only very recently they have been

studied in the context of viral infections. Although little is known about their function at the FGT, there is a preferential loss of Th17 in HIV infected patients, which is correlated with the long term progression of the disease [43,44]. The mechanisms by which Th17 numbers are associated to long term non progression, are object of current investigation. Contrasting the protective role of Th17 in HIV infections, they seem to contribute to pathogenesis in HSV-2 infections. Kim et al. reported that Th17 -/- mice survive lethal challenge with HSV-2 and suggested a role of this Th cell population in the detrimental effects observed during HSV-2 infection. Nevertheless, the mechanisms have not been characterized yet [45].

Establishment of Long-Term Memory

In a mouse model of HSV-2 infection, it has been shown that after immunization, there is a formation of vaginal-associated lymphoid tissue (VALT) cluster-like structures [46]. These aggregates in the vaginal tissue contain both HSV-specific memory $CD4^+$ and $CD8^+$ T cells, which are critically for controlling HSV-2 secondary infection. The stimulation of these populations requires different cell types. Whereas $CD4^+$ T cells are dependent of DCs or B cells and exert their protective mechanisms by secreting IFN-γ, $CD8^+$ T cells depends on DCs and $CD4^+$ T cells [46]. During HSV-2 reactivation events, IFN-γ is rapidly produced and constitutes the most important antiviral mechanism. Recent investigations have shown that the microenvironment of the genital mucosa is enough to generate memory responses against HSV-2 [46-48]. Furthermore, evidence has been presented that immunization in the absence of secondary lymphoid organs is still effective in generating effector memory responses strong enough to provide protection against viral exposures [49].

Modulation of Mucosal Antiviral Responses by TLR Ligands

There is a great amount of evidence, that TLRs initiate the inflammatory responses in the FGT and that their stimulation offers a link to the activation of adaptive immunity. Therefore the activation of innate immunity to enhance antiviral mechanisms at early stages may be an alternative strategy to develop new vaccination strategies. The wide-spread expression of TLRs in the FGT offers the possibility to use them as targets to modulate genital infections. Thus, there is a relatively new area of research focused on the use of TLR-ligands to control infections at this and other mucosal sites. A decade ago, two independent groups have shown that synthetic CpG ODN applied topically to genital mucosa was able to protect 50 % of mice from a lethal HSV-2 challenge [34,50-52]. The use of CpG ODN as a therapeutical agent in a model of HSV-2 reactivation in guinea pigs was also successfully tested in reducing the magnitude of virus shedding but not the frequency of the reactivation episodes [34,50-52]. Harandi et al. reported that the single vaginal administration of CpG ODN induces Th1-associated cytokines: IFN-γ, IL-12 and IL-18; being IFN-γ the crucial cytokine for protection from HSV-2 infection [34]. The topical use of the TLR7/8 agonist resiquimod is more controversial because while one study showed reduced HSV-2 sheddings in patients with recurrent activations of genital herpes [53], another study could not reproduce these results [54].

TLRs pathways are targeted by HPV oncoproteins resulting in an aberrant expression pattern which contributes to the virus tenacity and carcinogenic potential [55]. The tumorigenic E6 and E7 genes in HPV 16 are responsible for the down-regulation of TLR9, which is known to respond to DNA threats and evoke an innate immune reply. Moreover, an increasing trend in TLR3 expression is observed in dysplastic epithelium [55]. It has been suggested that mucosal application of TLR ligands might substantially increase the effectiveness of parenterally administered vaccines. In this regard, it was recently reported that that intra-vaginal instillation of CpG-ODN or poly-(I:C) after subcutaneous E7 vaccination increased 5 fold the number of vaccine-specific IFN-γ-secreting $CD8^+$ T cells in the genital mucosa of mice [56]. The selective recruitment of $CD8^+$ T cells induced by CpG-ODN or poly-(I:C) was mediated by TLR9 and TLR3/MDA-5 signaling pathways, respectively. Most interestingly, intra-vaginal CpG-ODN following vaccination led to complete regression of large genital HPV tumors in 75% of mice, instead of 20% with vaccination alone [56].

Effect of Natural FGT Microbiota on Antiviral Immunity and Possible Role of Immunobiotic LAB

The normal human female genital microbiota is complex, dynamic (as affected by the menstrual cycle and age) and rich in *Lactobacillus* spp. A recent epidemiological study in USA showed that the main species found in North-American women were *Lactobacillus iners, L. crispatus, L. gasseri* and *L. jensenii* [57]. On the contrary, in the most frequent clinical gynecological condition called bacterial vaginosis (BV), the number of lactobacilli decreases and there is an enrichment of the anaerobic polymicrobial population, especially *G. vaginalis, Prevotella, Porphyromonas*, and peptostreptococci. Epidemiological evidence attributes to BV (whether symptomatic or not) an increased risk to acquire sexual transmitted diseases (including HPV, HSV-2 and HIV) and it also negatively influences reproductive health. Supporting these observations, Hummelen et al. [58] showed that the main species found in HIV^+ African women were *L. iners* and *G. vaginalis*. Thus, it has been hypothesized that the vaginal microbiota plays an important role in preventing infections of viral, bacterial, fungal or parasitic ethiology. This is mainly attributed to lactic acid bacteria (LAB) and the general mechanisms proposed are lactic acid production, which lowers the vaginal pH, the production of bacteriostatic and bactericidal compounds and competition for adhesion sites and nutrients. But although it is though that they also enhance innate immunity, there are very few reports on the immunomodulatory mechanisms of indigenous lactobacilli and even less on exogenous lactobacilli. Jiang et al. [59] reported a positive correlation between three species of *Lactobacillus* and defensins (HBD-2 and HBD-3) in healthy controls, which were not observed in HIV^+ patients. To our knowledge, only Mastromarino et al. [60,61] have reported antiviral activity of *Lactobacillus* spp. against HSV-2 and have shown that the cell wall alone is responsible for antiviral activity. Nevertheless, the studies did not elucidate any mechanisms and the authors did not explore innate immunity pathways, which may have been activated by these bacterial components. A recent report has shown that a strain of *Bifidobacterium adolescentis* down-regulates HPV E6 and E7 expression at mRNA and protein level [62] but the mechanisms still need to be investigated. Another recent study reported that two *Enterococcus spp*. strains produce pediocin-like bacteriocine with antiviral activity against HSV-1 [63,64]. This may be one of the first evidences that LAB are able to produce antiviral peptides and may contribute to the innate protection observed in women with a healthy genital microbiota rich in LAB. Even though these few preliminary reports are based on *in vitro* assays and do not give an insight into mechanisms, are still important as they reflect the unexplored antiviral potential of LAB in the genital tract.

Recombinant LAB as Mucosal Vaccines for the Prevention of FGT Viral Infections

It is well established that the main site of entry and persistence of viral pathogens are the mucosae of the whole body. In spite of this, most vaccination strategies are systemic and they do not confer the necessary protection to fully control some viral infections at mucosal sites (e.g.: HIV, RSV, HSV and others). Indeed, the lack of proper mucosal delivery vehicles may be one of the reasons why therapeutic and prophylactic vaccine development is still hindered. Some studies have pointed out the importance of locally inducing $CD8^+$ cytotoxic lymphocytes as well as secretory IgA apart from immune responses at systemic level in order to control viral infection at early stages before dissemination [33,34,65]. Thus, the importance of vaccination at mucosal sites relies not only on the local response obtained but also on the possibility to trigger immune responses on distal mucosal effector sites. Some advantages of mucosal vs. systemic vaccines consist on their simplicity of administration, which does not need the use of needles, syringes, and trained personnel, being both less invasive and more economical. Last but not least, mucosal vaccines provoke both systemic and mucosal immune responses [66-69]. The direct use of antigens or subunits at mucosal sites is usually inefficient due to the presence of tolerance mechanisms on the one hand and in the case of the intestinal mucosa, the antigen may remain immunogenic after the harsh gastric-duodenal passage. A relatively new alternative to generate mucosal vaccines relies in the use of recombinant LAB. Besides being long-used organisms belonging to the GRAS group (Generally Regarded As Safe), some strains may be good candidates for oral vaccine formulations as they resist the gastro-intestinal conditions, they lack an outer membrane typical of Gram negative bacteria, which allows them to display heterologous biologically active proteins at their surface or to secrete them. In addition to these properties some LAB strains possess intrinsic adjuvant properties which could significantly improve the capacity of the vaccine to generate protective immunity [68,70-73]. Most recombinant LAB tested until now as mucosal vaccines have been developed for bacterial antigens, whereas an extensive literature search revealed only a few using viral antigens, most of them for animal pathogens. This promising field has not been yet extensively exploited and the immunological aspects of vaccination for viral antigens using recombinant LAB have been mostly investigated at the humoral response level and without the respective pathogen challenges. Thus, there is a need for more comprehensive studies concerning the cellular immune response elicited by these experimental vaccines. Moreover, LAB-based antiviral vaccines should be effectively proved in challenge-infection experiments in order to clearly demonstrate their protective activity.

Our experience in using probiotic LAB as mucosal adjuvants and recombinant LAB as antigen delivery vehicles for bacterial mucosal vaccines, taught us that the effect of these bacteria are multifactorial and concern all arms of the immune response, i.e. the innate and adaptive immunity including both cellular and humoral responses [73-80]. The possible administration routes explored until now for recombinant LAB as mucosa vaccines have been the nasal, the oral, the gastric and the genital ones. According to experimental evidence gathered in the past years, the nasal administration route seems to be the most effective one and it also provides protection at distant sites such as the intestinal and the genital mucosae [72,73,81-83]. Bermudez-Humarán's group has been working over a decade on recombinant lactococci and later lactobacilli and proved in a very elegant study, the benefits of administration of a recombinant lactococci strains producing cell wall anchored HPV-16 E7 antigen and secreting IL-12 over oral administration of the same strain [71]. Their results clearly showed that the elicited immune response to the nasally administered vaccine comprised both E7 specific antibodies, and IFN-γ producing lymphocytes [71,72]. Results from our group concerning a recombinant *Lactococcus lactis* expressing the pneumococcal protective protein A (PppA), also showed that the nasal administration route is superior as the oral one regarding immune responses at the respiratory tract although oral administration was successful in triggering specific immunity at the distal respiratory mucosa [73,78,82,84]. Other research groups working with parasite proteins expressed by LAB had better results with the oral route [85]. These apparent discrepancies may be related to the nature of the antigen and the main mucosal target of the pathogen. In any case, it is important to test the optimal administration route for each recombinant vaccine as well as to adjust the scheme of immunization as the line between inducing immunity and tolerance is delicate.

It has been shown in several reports that the ability to induce maturation of myeloid DCs is strain dependent; therefore, the process of pre-selection of an adjuvant strain for vaccine developing strategies should be done thoroughly. The stimulation differences observed between strains even of the same species has been correlated to the differential expression (especially density) of microbial associated molecular patterns (MAMPs). To date, LAB are reported to be recognized as whole bacteria via their LTA by TLR-2 and TLR-2/6 complexes and their DNA can simulate TLR-9 [86]. Recently, Kajikawa et al. [87] tested a novel approach by expressing not only the HIV protein Gag in a *L. acidophilus* strain, but also by including a second TLR ligand not present in lactobacilli: TLR5-ligand, protein FliC, the main constituent of flagella. They proved *in vitro* in reporter cell lines, that recombinant bacteria expressing both antigens were more efficient in activating innate immunity pathways. Furthermore, they also have a greater influence on maturation of human myeloid DC co-cultured with bacteria displaying both antigens as shown by their increased expression of co-stimulatory markers (CD80, CD83, CD86, and CD40). They also followed the adaptive immune response in intragastric inoculated mice and observed development of virus specific secretory IgA and detected increased numbers of IFN-γ producing lymphocytes not only in PPs, but even more interesting in the FGT [87].

The heterologous proteins can be expressed at three sites: extracellular, intracellular, and cell-wall anchored. Extracellular expression has the advantage that it allows to produce higher amounts of antigens in conditions which would be toxic if expressed intracellular or membrane-anchored and the protein can be directly released into its target site. The second location offers protection to the proteinaceous antigen to the harsh conditions typical of mucosal sites, especially of the gastrointestinal passage, but at the same time has the disadvantage that it requires bacterial cell lysis to deliver the antigen. Last, the protein can be anchored to the protein by constructing fusion proteins to know domains anchored at the membrane such the cell wall covalent binding protein LPXTG motif. The latter have been shown to be the most effective mucosal vaccines as they are somehow protected from the environment but do not require cell lysis to contact the target cells at the mucosa.

From the LAB tested up to date, *Lactococcus lactis* is more versatile for the cloning and expression procedures whereas Lactobacilli (especially *L. casei*) have shown to have stronger adjuvant properties.

Cloning procedures and protein expression strategies are out of the scope of this book chapter. However, it should be mentioned that the most used expression vectors are based on pCYT, pSEC, and pCWA backbones, and have been modified to have constitutive or inducible gene expression [68,70,88-92]. These vectors have been tested in *L. lactis* but can also be used in other genera of LAB such as *Lactobacillus* spp., *Streptococcus* spp., and *Enterococcus* spp. and have been reviewed somewhere else.

The result of these strategies is a genetically modified organism (GMO), which may be difficult to have acceptance by the people and market of several countries, including members of the EU due to their strict health and environmental safety regulatory issues. Nevertheless, there is a recent approach to use LAB as mucosal vehicles without generating GMOs. This strategy consist on generating fusion proteins fused to the peptidoglycan-binding domain of the major autolysin AcmA from *Lactococcus lactis* and the endolysin Lyb5 of *Lactobacillus fermentum* bacteriophage FYB5 which contained three LysM repeats in their C-terminus. After over-expression in *E. coli* or in a LAB, the purified protein is able to attach to the peptidoglycan of acid pre-treated non-viable LAB in a stable form without losing antigenicity, and therefore, the antigens can be displayed in a similar way as in cell-anchored proteins.

Another possibility that offer recombinant LAB is the generation of therapeutic vaccines. LAB may be transformed to produce cytokines, growing factors, vitamins, antimicrobial factors and other proteins, which may exert their effects directly at the target site. IL-10 expressing lactobacilli have proven in mice to be useful for ameliorating a model of murine colitis [92-94]; IL-12 secreting lactococci co-expressing viral antigens in their surface have shown to have better immunogenic properties than the bacteria only displaying the antigen [71]. A very recent study reported the feasibility of using antiviral expression lactobacilli as possible approach for treatment and prevention of sexual transmitted diseases such as HIV. Liu et al. [95] constructed a genetically stable recombinant *L. jensenii* expressing a potent microbicide [96-100], which has proven antiviral activity against HSV and CCR5- and CXCR4-tropic HIV. This bacterium has also colonized mice vagina, and has been thought to be administered as a vaginal suppository to be used on regular basis in order to assure that the antiviral peptides amounts produced reach the needed concentrations and are delivered directly at the main port of entry of HIV, the cervico vaginal mucosa [95]. Local application microbicides are a very interesting approach to treat or protect from viral infections. They should not irritate mucosa nor affect the vaginal microbiota, as was the case of nonoxynol-9 [101,102]. Therefore, the use of colonizing LAB or non-resident LAB which could be applied regularly may represent best candidates to exert protective effects on the genital mucosa, being at the same time of low cost and high safety.

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