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**ROLE OF THE MUCOSAE-ASSOCIATED EPITHELIAL CHEMOKINE  
(MEC/CCL28) IN THE MODULATION OF THE IMMUNE RESPONSE  
AGAINST VIRAL INFECTIONS**

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*Alla mia famiglia e a Simone*

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## **List of abbreviations**

ADCC: Antibody-Dependent Cell-mediated Cytotoxicity  
APOBEC3G: APOlipoprotein B mRNA-editing Enzyme Catalytic polypeptide-like editing complex 3G  
APCs: Antigen Presenting Cells  
BAL: BronchoAlveolar Lavage  
BAX: BCL-2-Associated X protein  
Bcl-2: B cell lymphoma gene-2  
CCL25: chemokine C-C motif Ligand 25  
CD: Cluster of Differentiation  
CFA: Complete Freund's Adjuvant  
CIN: Cervical Intraepithelial Neoplasia  
CNS: Central Nervous System  
CT: Cholera Toxin  
CTL: Cytotoxic T Lymphocyte  
DC: Dendritic Cell  
DNA: DeoxyriboNucleic Acid  
DT: Diphtheria-Tetanus  
E6-AP: E6-Associated Protein  
ER: Endoplasmic Reticulum  
GTP: Guanosine TriPhosphate  
HIV: Human Immunodeficiency Virus  
FcRn: neonatal Fc Receptor  
FDC: Follicular Dendritic Cell  
GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor  
Gp120: GlycoProtein 120  
HA: Hemagglutinin  
HAV: Hepatitis A Virus  
HCV: Hepatitis C Virus  
HEK 293T: Human Embryonic Kidney cell-line 293T  
HEVs: High Endothelial Venules  
IC50: half maximal Inhibitory Concentration  
IL: Interleukin  
IFA: Incomplete Freund's Adjuvant  
IFN- $\gamma$ : InterFeron- $\gamma$   
LC: Langerhans Cell  
LCR: Long Control Region  
LFA-1: Leukocyte Function Associated molecule-1  
LN: Lymph Node  
LP: Lamina Propria  
LT $\alpha_1\beta_2$ : LymphoToxin  $\alpha_1\beta_2$   
LTR: Long Terminal Repeat  
mAbs: monoclonal Antibodies  
MadCAM-1: Mucosal ADdressin Cellular Adhesion Molecule-1  
MDP: Muramyl DiPeptide  
MHC: Major Histocompatibility Complex  
MIP-3- $\beta$ : Macrophage Inflammatory Protein-3-beta  
M2: Matrix 2  
mRNA: messengerRNA  
NA: NeurAminidase

NF- $\kappa$ B: Nuclear Factor-kappa B  
NK: Natural Killer  
NP: NucleoProtein  
ORF: Open Reading Frame  
PAMPs: Pathogen-Associated Molecular Patterns  
PC: Plasma Cell  
pIgA: polymeric Immunoglobulin A  
pIgR: polymeric Immunoglobulin Receptor  
pIg: polymeric Immunoglobulin  
PP: Peyer's Patch  
PRRs: Pathogen-Recognition Receptors  
PTX: Pertussis ToXic  
RBC: Red Blood Cell  
RNA: Ribonucleic Acid  
SIgA: Secretory IgA  
SIgM: Secretory IgM  
TGF- $\beta$ : Transforming Growth Factor- $\beta$   
VCAM-1: Vascular Cell Adhesion Molecule-1  
VLP: Virus-Like Particle  
RNA: RiboNucleic Acid  
vRNA: viralRNA

***Abstract***



## RIASSUNTO

### Introduzione

L'immunità umorale è la principale linea di difesa contro le infezioni a trasmissione mucosale poiché fornisce un meccanismo di blocco del patogeno mediato dalle IgA secretorie a livello della superficie mucosale. CCL28 è una chemochina che lega CCR3 e CCR10 e attrae le plasmacellule IgA-secerenti (IgA-ASCs) nella lamina propria delle mucose. Le Virus-like Particles (VLPs) rappresentano una nuova strategia vaccinale basata sull'utilizzo di particelle non patogeniche che mimano la struttura di autentiche particelle virali. L'immunogenicità dei vaccini può essere migliorata con l'utilizzo di adiuvanti costituiti da chemochine. In questa tesi verranno descritti gli approcci sperimentali per la valutazione degli effetti immunomodulatori di CCL28 in tre modelli animali immunizzati con VLPs di HIV-1<sub>III<sub>B</sub></sub>, Influenza Virus di tipo A (H7N1) e HPV-16, per un futuro utilizzo della chemochina come adiuvante nello sviluppo di vaccini preventivi contro infezioni a trasmissione mucosale.

### Materiali e Metodi

VLPs di HIV-1<sub>III<sub>B</sub></sub>, H7N1 e HPV-16 sono state sintetizzate in collaborazione con l'Università Ruhr di Bochum, l'Università di Montpellier, ed il Centro Tedesco di Ricerca sul Cancro di Heidelberg. Topi femmina Balb/c di 6-8 settimane sono stati randomizzati in diversi gruppi di trattamento a ricevere le VLPs di HIV-1<sub>III<sub>B</sub></sub>, o H7N1 o HPV-16 in combinazione o meno con il plasmide codificante il CCL28 ai giorni 0 e 14. Il sacrificio degli animali è stato compiuto al giorno 28. Le VLPs di HIV-1<sub>III<sub>B</sub></sub> e di HPV-16 e i vettori d'espressione delle chemochine CCL28 e CCL19 sono stati somministrati per via intramuscolare; le VLPs di H7N1 sono state somministrate per via intraperitoneale. I campioni ematici e i lavaggi vaginali sono stati raccolti secondo le metodiche standard ai giorni 0, 14 e 28. I campioni di saliva dei topi immunizzati con le VLPs di H7N1 sono stati ottenuti ai giorni 0, 14 e 28 in seguito ad iniezione intraperitoneale di carbacholo ed induzione di ipersalivazione. I lavaggi broncoalveolari sono stati ottenuti al giorno 28 tramite inserimento in trachea di una sonda per alimentazione forzata e ripetuta iniezione ed aspirazione di PBS. In ultimo, la milza e i campioni di colon, polmone e cervice uterina sono stati prelevati al giorno 28 per l'isolamento dei linfomonociti tissutali e per l'analisi immunoistochimica dell'infiltrato infiammatorio. L'espressione dei recettori CCR3 e CCR10 è stata valutata sugli splenociti circolanti mediante citometria a flusso. La produzione di citochine di tipo Th1 e Th2 è stata valutata tramite tecnica ELISA in colture di splenociti e di linfociti tissutali re-stimolati ex vivo o con la proteina HIV-1<sub>III<sub>B</sub></sub> gp120, o con l'emoagglutinina (HA) del virus H7N1 o con la proteina L1 di HPV-16. Il dosaggio degli anticorpi IgG e IgA antigene-specifici nel plasma e nei campioni mucosali è stato eseguito tramite una tecnica ELISA basata sull'utilizzo delle proteine ricombinanti HIV-1<sub>III<sub>B</sub></sub> gp120, H7N1 HA e HPV-16 L1. Il ruolo protettivo degli anticorpi sierici e mucosali è stato valutato mediante saggi di neutralizzazione in vitro. Infine, la migrazione delle IgA-ASCs a livello mucosale indotta da CCL28 è stata valutata mediante analisi immunoistochimiche dei tessuti derivanti dal tessuto linfoide associato alle mucose (MALT).

### Risultati

I risultati ottenuti nei tre modelli animali hanno mostrato un incremento statisticamente significativo nella percentuale di splenociti CD19<sup>+</sup> esprimenti CCR3 e/o CCR10 e nell'intensità media di fluorescenza (MFI) dei relativi recettori nei topi immunizzati con VLPs in combinazione con CCL28 rispetto ai topi immunizzati unicamente con l'immunogeno. La produzione HIV-1<sub>III<sub>B</sub></sub>, H7N1- e HPV-16-specifica di citochine sia di tipo I (IFN- $\gamma$ ) che di tipo II (IL-4 e IL-5) è risultata aumentata nei topi immunizzati con VLPs in combinazione con CCL28 rispetto ai topi immunizzati con il solo immunogeno. L'analisi delle risposte umorali mucosali ha mostrato un marcato incremento, in termini di variazione dal valore basale, dei livelli di IgA totali al giorno 28 nei lavaggi vaginali dei topi immunizzati con HIV-1<sub>III<sub>B</sub></sub>-VLPs e HPV-16-VLPs e nella saliva, nei lavaggi broncoalveolari e negli omogenati di tessuto polmonare dei topi immunizzati con H7N1-VLPs in presenza di CCL28. Risultati simili sono stati ottenuti nell'analisi dei livelli di anticorpi IgA antigene-specifici nelle secrezioni vaginali dei topi immunizzati con HIV-1<sub>III<sub>B</sub></sub>-VLPs e HPV-16-VLPs e nel tessuto polmonare dei topi immunizzati con H7N1-VLPs in combinazione con CCL28. In ultimo, i livelli sierici di anticorpi IgG e IgA antigene-specifici sono risultati aumentati, in tutti e tre i modelli animali, nei topi immunizzati con VLPs in combinazione con CCL28 rispetto ai gruppi controllo. Tali dati sono stati confermati dall'osservazione di un aumento dell'attività neutralizzante dei campioni sierici e mucosali, e di un massivo reclutamento di IgA-ASCs a livello del colon, della mucosa cervicale e del tratto respiratorio nei topi immunizzati con VLPs in presenza del plasmide codificante la chemochina.

### Conclusioni

CCL28 è in grado di regolare le risposte immunitarie a livello mucosale; l'effetto è riproducibile utilizzando la chemochina come adiuvante in modelli animali immunizzati con VLPs di virus a trasmissione mucosale e suggerisce un possibile utilizzo di CCL28 come adiuvante nella preparazione di vaccini finalizzati alla prevenzione dell'infezione da HIV-1, Influenza virus A e da HPV. Le prospettive future sono rivolte all'analisi dei correlati della risposta immune mucosale alla vaccinazione con VLPs e chemochine.

## ABSTRACT

### Introduction

Secretory immunity is the main line of defence against mucosal infections as it can provide pathogen-blocking secretory IgA at the mucosal surface. CCL28 is a chemokine that binds to CCR3 and CCR10 and potently recruits IgA-secreting plasma cells (IgA-ASCs) in the mucosal lamina propria. Virus-like particles (VLPs) are a novel vaccine approach based on non-pathogenic particles that mimic the structure of authentic virus particles. Immunogenicity of vaccines can be improved by the use of chemokine molecular adjuvants. Here we propose experimental strategies for evaluating CCL28 immunomodulatory effects in mice after vaccination with VLPs of HIV-1<sub>III<sub>B</sub></sub>, Influenza A virus (H7N1) and HPV-16 for potential future use of the chemokine as an adjuvant in the development of preventive vaccines against mucosal infections.

### Materials and Methods

HIV1<sub>III<sub>B</sub></sub>-, H7N1- and HPV16-VLPs were produced in collaboration with the Ruhr-Bochum University, the University of Montpellier and the German Research Cancer Centre of Heidelberg, respectively. Inbred female Balb/c mice were randomized to receive HIV1<sub>III<sub>B</sub></sub>-VLPs or H7N1-VLPs or HPV16-VLPs in the presence or absence of the murine CCL28-expressing plasmid on days 0 and 14. Mice were euthanized on day 28. The murine CCL19 expression vector was used as negative control, as this chemokine binds to CCR7 receptor. HIV1<sub>III<sub>B</sub></sub>-VLPs, HPV16-VLPs and CCL28- and CCL19-expressing plasmids were administered intramuscularly; H7N1-VLPs were administered intraperitoneally. Blood samples and vaginal secretions were collected by standard methods on days 0, 14 and 28. Saliva samples of H7N1-VLPs treated mice were collected on days 0, 14 and 28 after intraperitoneal injection of carbachol to induce mice drooling. Bronchoalveolar lavages were collected on day 28 by insertion into the trachea of a blunt animal-feeding needle and repeated cycles of injection and aspiration of PBS. Tissues obtained from the spleen, colon, lungs and the uterine cervix were collected on day 28 to obtain the lymphocyte-enriched cell population for cell culture assays and to evaluate IgA-plasma cell distribution at the mucosal level by immunohistochemistry analyses. CCR3 and CCR10 surface receptors were evaluated on circulating splenocytes by flow cytometry. Th1- and Th2-type cytokine production was evaluated in the supernatants from cultivated splenocytes and mucosal T-cells after ex vivo re-stimulation with recombinant HIV-1<sub>III<sub>B</sub></sub> gp120 or hemagglutinin (HA) from Influenza virus A H7N1 or HPV-1 L1 protein. Antigen-specific IgG and IgA were measured in sera and mucosal secretions by an ELISA method based on recombinant HIV-1<sub>III<sub>B</sub></sub> gp120, HA from Influenza A virus H7N1 and HPV-16 L1 protein. Neutralizing activity of both systemic and mucosal antibodies was assessed as well. Finally, CCL28-mediated recruitment of IgA-ASCs at mucosal sites was evaluated by immunohistochemistry analyses of tissues obtained from the mucosa-associated lymphoid tissue (MALT).

### Results

Results presented herein show a significant increase in the percentage and in the mean fluorescence intensity (MFI) of CCR3 and CCR10 on CD19<sup>+</sup> splenocytes in VLP-CCL28 mice compared to VLP-CCL19 or VLP alone or CCL28 alone or CCL19 alone or saline mice. Antigen-specific production of Th1-type (IFN- $\gamma$ ) and Th2-type (IL-4 and IL-5) cytokines was significantly augmented in splenocytes and mucosal tissues of VLP-CCL28 mice compared to VLP-CCL19 or VLP alone mice. Total IgA levels were significantly increased compared to baseline values in vaginal secretions of HIV1<sub>III<sub>B</sub></sub>-VLP-CCL28 and HPV16-VLP-CCL28 mice, and in saliva samples, BALs and lung homogenates of H7N1-VLP-CCL28 mice on day 28. Antigen-specific IgA were similarly augmented in vaginal secretions of HIV1<sub>III<sub>B</sub></sub>-VLP-CCL28 and HPV16-VLP-CCL28 mice and in lungs of H7N1-VLP treated mice. Data were confirmed by the observation that the neutralization ability of both immune sera and mucosal secretions was significantly up-regulated in mice receiving CCL28. Finally, results presented here indicate that immunization on the presence of the CCL28-expressing plasmid significantly increases the quantity of mucosal IgA-ASCs in the rectum of HIV1<sub>III<sub>B</sub></sub>-VLP-receiving mice, in the lungs of H7N1-VLP-receiving mice and in the uterine cervix of HPV16-VLP-receiving mice.

### Conclusion

CCL28 mediates mucosal immunity; the effect is reproducible using this chemokine as an adjuvant in mice immunized with VLPs of mucosally transmitted viruses. CCL28-containing adjuvants should be considered in the development of vaccines against HIV-1, Influenza A virus and HPV to prevent infection of mucosal sites via modulation of mucosal IgA. Nevertheless, the efficacy evaluation of CCL28 adjuvanticity requires further experiments to define immune correlates of mucosal responses to vaccination with VLPs in combination with chemokine adjuvants.

***Introduction***

## 1. MUCOSAL IMMUNITY

A large number of mucosal infections continue to represent a challenge for the development of both prophylactic and therapeutic vaccines [1]. The vast majority of infections involve the mucosae with regard to initial microbial colonization and/or entry into the body.

Epithelial cells line the insides of the lungs, the gastrointestinal tract, the reproductive and urinary tracts, the outer surface of the cornea and make up the exocrine and endocrine glands. Mucosal epithelia comprise an extensive vulnerable barrier which is reinforced by numerous innate defence mechanisms cooperating intimately with adaptive immunity [1,2,3].

Innate immunological defence mechanisms consist of anti-microbial peptides, complement, polymorphonuclear cells and macrophages which ingest and kill invading pathogens.

The adaptive immune system of mucosal surfaces, together with the local LNs, forms the mucosa-associated lymphoid tissue (MALT), which is quantitatively the most important organ of adaptive humoral immunity.

The mucosal immune system is highly compartmentalized. Although gut-associated lymphoid tissues (GALT), including PPs in the distal ileum, the appendix and numerous isolated lymphoid follicles, constitutes the major part of MALT, induction of mucosal immune responses can take place also in the palatine tonsils and other lymphoepithelial structures of Waldeyer's pharyngeal ring, including nasopharynx-associated lymphoid tissue (NALT) such as the adenoids and bronchus-associated lymphoid tissue (BALT). However, the latter type of MALT is not present in normal lungs of adult humans [4].

### 1.1 Mucosal lymphoid organization

Mucosae-associated lymphoid tissues (MALTs) are organized lymphoid tissues in close relationship with surface and glandular epithelia to constitute both inductive and effector sites of mucosal immune responses (Fig. 1) [1].

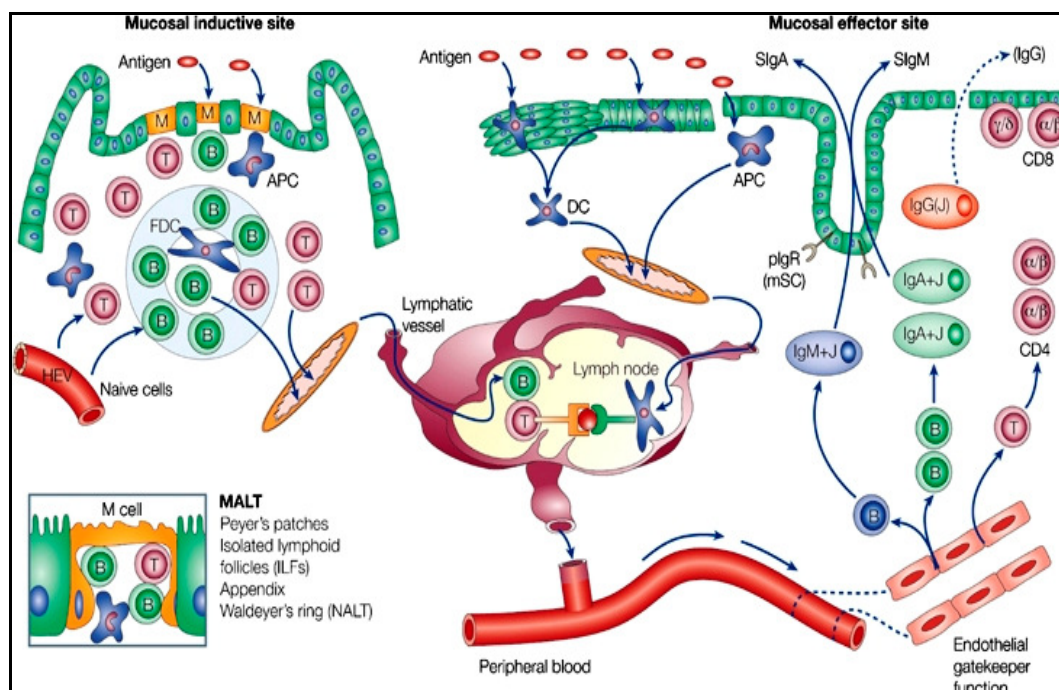


Figure 1: depiction of the human mucosal immune system.

Inductive sites (tonsils, adenoids as NALT, BALT, and PPs and appendix/colonic-rectal solitary follicles as GALT) are characterized by several follicles where B-cells are preferentially found and contain, after antigenic stimulation, secondary germinal centres. These follicles are surrounded by more diffuse lymphoid tissue (extra-follicular area or T-cell zone) and their luminal side (often called "dome") is covered by the so-called follicle-associated epithelium (FAE), which contains microfold cells (M-cells) sampling the antigenic luminal content.

The FAE expresses certain chemokines (e.g. CCL20) that may explain a dense accumulation of professional APCs in the PP domes. Numerous putative APCs also occur immediately underneath the FAE, while the M-cell pockets are dominated by memory T and B lymphocytes in approximately equal proportions. M-cells differentiate from enterocytes under the influence of membrane-bound  $LT\alpha_1\beta_2$  that is present on local lymphoid cells, mainly B-cells.

MALTs lack afferent lymphatics, which are replaced by specialized high endothelial venules, therefore microbial stimuli must come directly from the epithelial surfaces, mainly via the M-cells but also aided by DCs which may penetrate the surface epithelium with their processes.

Induction of mucosal immunity hence takes place primarily in MALTs and in the local draining LNs. In addition, intra- or subepithelial DCs may capture antigens and migrate via draining LNs to regional LNs where they become active APCs, which stimulate T-cells for productive or suppressing immune responses depending on associated danger signals.

Conversely, effector sites are represented by the diffuse lamina propria of the different mucosa and exocrine glands, also in striking relationship with the epithelium.

Although mucosal and systemic immune systems do not appear totally segregated, MALT has some specific features such as a large predominance of IgA-producing immunocytes.

Several characteristics of the MALT were demonstrated in studies mainly related to the gut, although most of them probably also apply to the respiratory tract. However, a major difference between the airway and digestive mucosa is that MALT and M-cells are virtually absent from the normal respiratory tract [5]. Thus, the organization of the MALT, including M-cell epithelial differentiation is probably induced only when airway and lung tissues are exposed to an increased antigenic load.

Although the genital tract is considered to be a component of the mucosal immune system, it displays several distinct features not shared by other mucosal tissues or the systemic compartment [6].

Mucosal lymphoid nodules and M-cells for antigen uptake overlaying organized lymphoid tissue like in the intestinal tract are missing in the reproductive tract. There are lymphoid aggregates in the basal layers of the uterus but their presence and distribution strongly varies under the hormonal influence. Besides lymphoid aggregates, the genital tract mucosa contains APCs such as macrophages and DCs as well as epithelial cells. Furthermore, particularly the mucosal immune system of the female genital tract is under strong hormonal control that regulates the transport of Igs, the levels of cytokines, the distribution of various cell populations and antigen presentation in the genital tissues during the reproductive cycle.

## **1.2 Stimulation of mucosal immunity**

Naïve B-cells enter MALT through high endothelial venules by a multistep process of extravasation into inductive sites. There, they are primed in extra-follicular areas by local CD4<sup>+</sup> T-cells, which are activated by interdigitating APCs that have processed a luminal antigen. In addition, luminal peptides may be taken up and presented by B-cells and epithelial cells to subsets of intra- and subepithelial T lymphocytes [7].

Both professional mucosal APCs, B-cells and epithelial cells, as well as FAE except for the M-cells, express MHC class I and II molecules. Interestingly, MHC class II-expressing naïve and memory B-cells aggregate together with T-cells in the M-cell pockets, which thus represent the first contact site between immune cells and luminal antigens. Other types of professional APCs are mainly located below the FAE and between the follicles.

Primed B-cells migrate specifically in the germinal centres where they are induced to lead to memory B-cells or initiate isotype switching of their heavy chain constant region (CH) gene from C<sub>μ</sub> to downstream isotypes. This isotype switching constitutes the terminal phase of B-cell maturation into Ig-producing immunocytes. CD4<sup>+</sup> helper T-cells activated in MALT are known to release cytokines such as TGF-β and IL-10 which drive the class switch and differentiation of mucosal B-cells to predominantly IgA-committed plasmablasts, although their detailed regulation still remains unclear [8]. However, the precise reason why IgA-producing immunocytes represent the predominant mucosal mature plasma cell remains obscure.

Thus antigen specific B-cells rapidly recirculate from MALT through lymph and blood to become seeded by preferential homing mechanisms into distant mucosal effector sites, where they finally differentiate to PCs. This terminal differentiation is modulated by “second signals” from local antigen-sampling DCs, CD4<sup>+</sup> T-cells and available cytokines.

## **1.3 Secretory immunity**

The secretory antibody basis for antigen exclusion and/or virus and toxin neutralization within epithelial cells is locally provided by the mucosae and associated exocrine glands which harbour most of the body's activated B cells, terminally differentiated to Ig-producing plasmablasts and PCs.

The molecular heterogeneity of IgA in serum and secretions is mainly related to its polymerization state. Serum IgA in humans and primates consists mostly of monomeric IgA (88%), which is produced by bone marrow plasma cells, and its concentration in serum is about five-fold lower than that of IgG. By contrast, mucosal plasma cells produce mostly pIgA (80%, mainly dimeric), which is the predominant form of IgA in secretions.

SIgA antibodies are remarkable stable hybride molecules, mainly consisting of PC-derived IgA dimers with one or more “joining” (J) chain(s) and an epithelial portion called bound secretory component (SC) which is disulfide linked to one of the IgA subunits (Fig. 2).

Most mucosal PCs (70-90%) do normally produce dimers and some trimers of IgA which, together with J chain-containing pentameric IgM, are exported by secretory epithelial cells to provide SIgA and SIgM antibodies. The J chain is a 15 kDa peptide that is essential for correct polymerization of pIgA and pentameric IgM and their subsequent binding to pIgR.

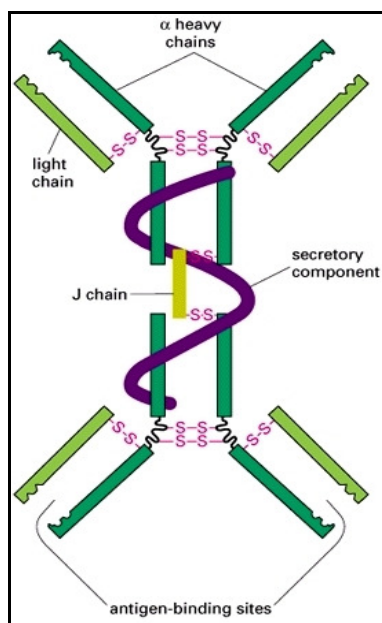


Figure 2: secretory IgA antibody structure.

The pIgR is expressed on the basolateral pole of epithelial cells, mainly of the serous type in the gut, whereas mucous and ciliated cells also express this surface receptor in bronchi, although to a lesser extent than the serous phenotype [5].

The human pIgR/SC consists in a 100 kDa protein of 693 amino acids which comprises an N-terminal signal peptide, five Ig-like domains D1-D5 and a sixth extracellular domain, followed by a membrane-spanning segment and a highly conserved cytoplasmic tail.

The binding of pIgA to pIgR is initiated by a noncovalent interaction between a loop region of the third constant domain of IgA (C $\alpha$ 3) and a conserved sequence in D1 of the pIgR. The pIgR complexed with a pIg is endocytosed and delivered to basal early endosomes. The ligand-receptor complexes are transcytosed in microtubular structures and trapped in apical vesicles to reach the apical membrane. There, the pIgR is released as the SC by a proteolytic cleavage just upstream from the membrane-spanning segment. The cleavage releases J-chain-containing pIgA, covalently linked to SC to generate SIgA, whereas IgM is noncovalently complexed to SC to form SIgM (Fig. 3).

Cleaved unoccupied pIgR is released in variable amounts to the secretions as so called free SC. Association with SC was shown to protect SIgA from proteolytic degradation, although the latter is much less pronounced in the respiratory tract than in the large bowel.

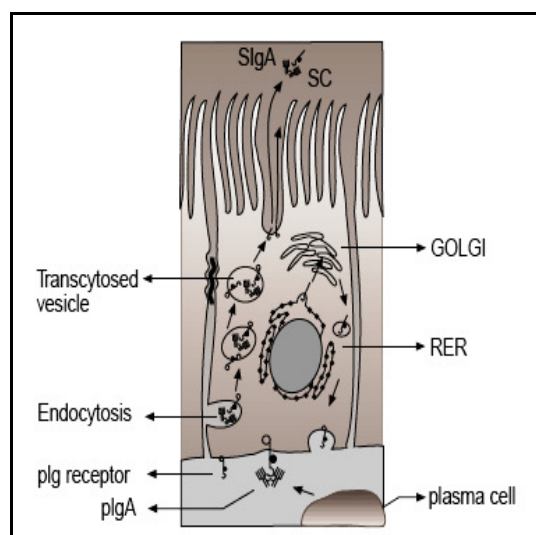


Figure 3: transcytosis of pIgA mediated by the pIgR.

In addition, nonsecretory Igs such as monomeric IgA, IgG, IgD or IgE can also reach secretions, mostly by passive diffusion through the endothelial and epithelial tight junctions from submucosal blood capillaries, or from locally infiltrating plasma cells producing these monomeric Igs.

Moreover, these monomeric Igs might be cotransported *via* the pIgR, concomitantly with an immune complex involving at least one pIg.

Although the membrane SC/pIgR expression is constitutively regulated, it can be enhanced at the transcriptional level by the immunoregulatory cytokines IFN- $\gamma$  and IL-4, as well as by the proinflammatory cytokines TNF- $\alpha$  and IL-1.

#### **1.4 Function of secretory IgA, IgM and local IgG antibodies**

In cooperation with a variety of innate mucosal defence mechanisms, the function of secretory antibodies is to perform immune exclusion of exogenous antigen.

Because of its remarkable stability, SIgA can retain its antibody activity for prolonged periods in hostile environments such as the gut lumen [9] and oral cavity.

The antibody function of SIgA is most likely enhanced significantly by the high level of cross-reacting activity as also detected in human secretions [10]. These natural antibodies are apparently designed for urgent protection before an adaptive specific immune response is elicited; they are therefore reminiscent of innate immunity.

In the lumen, SIgA will coat commensal bacteria, apparently without stopping their growth, most likely reflecting cross-reactive natural antibodies, although microbial binding via the Fc portion of IgA cannot be excluded. Regardless of mechanism, such coating reduces bacterial access to the epithelial surface and protect against bacterial overgrowth and invasion, containing the indigenous microbiota in the lumen to maintain the host-parasite mutualism.

It's been traditionally believed that SIgA can also efficiently inhibit epithelial colonization and invasion of pathogens.

The agglutinating and microbial enzyme- as well as virus-neutralization effect of pIgA and SIgA is superior compared with monomeric antibodies and SIgA can effectively block epithelial penetration in experiments with HIV [11]. Also individuals negative for HIV who live together with HIV-positive partners for several years often appear to be protected by specific IgA antibodies in their genital tract [12].

IgA antibodies can enhance the striking of bacteria or other antigens to mucus because of the mucophilic properties of its bound SC, thereby promoting clearance of immune complexes by respiratory ciliary movement and intestinal peristalsis.

Animal and culture experiments have suggested that secretory antibodies can perform immune exclusion not only on the epithelial surface. During the pIgR-mediated transport of pIgA and pentameric IgM, such antibodies may even inactivate viruses (e.g. Influenza virus and HIV) inside of secretory epithelial cells and carry the pathogens and their products back to the lumen, thus avoiding cytolytic damage to the epithelium [13,14].

It has also been demonstrated that pIgA antibodies can remove antigens from the lamina propria [15] and neutralize bacterial LPS within intestinal epithelial cells [16], suggesting novel anti-inflammatory and non-cytotoxic roles for this antibody class during its export to the lumen.

Thus, IgA appears to be able to neutralize infectious agents or antigens at the three levels of the mucosal tissues: into the lumen ("exclusion" of bacteria), inside the epithelial cell ("neutralization" of viruses), and in the lamina propria ("excretion" of immune complexes) (Fig. 4).

Potentially important additional defence function is the suggested ability of SIgA antibodies to promote biofilm formation [17], induce loss of bacterial plasmids that code for adherence-associated molecules and resistance to antibiotics [18], interfere with growth factor (e.g. iron) and enzymes necessary for pathogenic bacteria and parasites [19], and promote the bacteriostatic effects of lactoferrin [20] and peroxidase system [21].

Whereas IgA1 represents the predominant isotype, a relative increase of IgA2 expression characterizes mucosal (especially in the large bowel, but also in the bronchi) as compared to systemic Ig-producing cells. IgA2 lacks most of the hinge region and therefore appears less susceptible to degradation by bacterial IgA-proteases.

Therefore, because of the intrinsic resistance of IgA2 to IgA1 proteases of many pathogenic bacteria, the increased proportions of IgA2 may provide functional advantage to certain specific antibodies.

Alternatively, for IgG-producing mucosal cells (representing about 3 and 20% of the Ig-producing cells in the gut and bronchi, respectively), the predominant isotype is IgG1.

Moreover, IgG3<sup>+</sup> cells are more frequent than IgG2<sup>+</sup> cells in the upper airways, in contrast with the distal gut. IgE-producing plasma cells are virtually absent, except in the mucosa from some allergic patients.

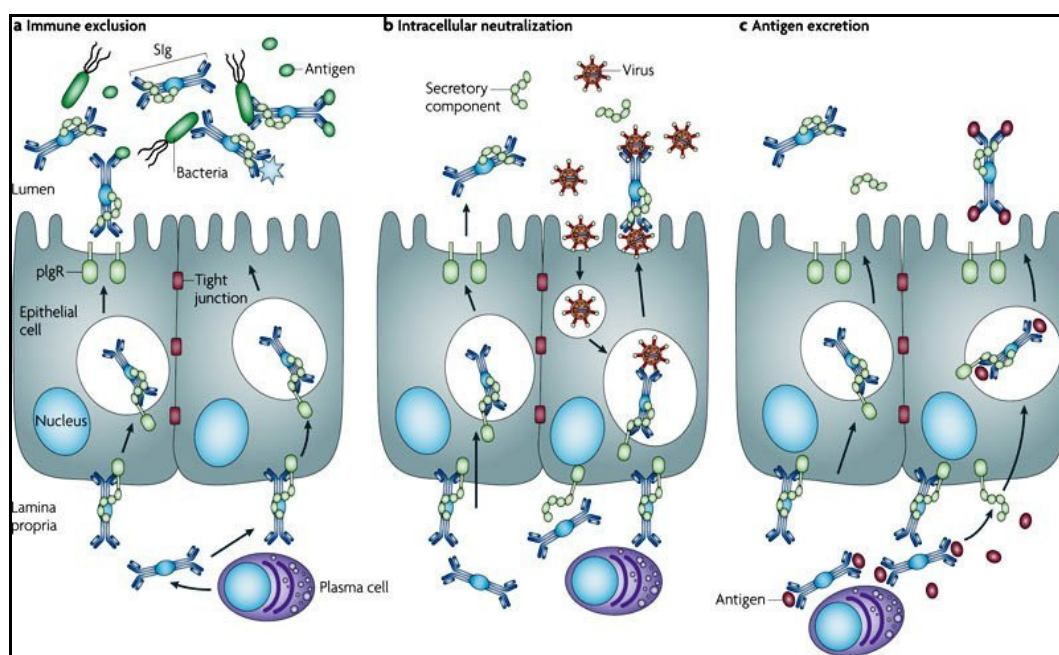


Figure 4: schematic representation of three levels at which SIgA may provide mucosal immune protection.

Antigen-specific IgA, IgG and IgM antibodies and SCs can be detected in the external secretions of the human genital tracts.

As far as the female genital tract is concerned, IgG levels in the lower genital tract secretions seem to equal or even exceed the levels of S-IgA whereas the dominant isotype of other mucosal secretions is S-IgA.

In contrast to other mucosal secretions in which 80% of IgA is polymeric, cervical mucus contains about 70% pIgA and vaginal secretions contain almost equal proportions of polymeric and monomeric IgA, clearly demonstrating that the genital tract relies heavily on antibodies derived from serum and systemic immunity. Of note, both the rectal and vaginal mucosa are drained by the iliac LNs and there is indirect evidence that IgA-ASCs in the vaginal mucosa originate from the solitary lymphoid nodules of the rectum [22], which resemble PPs of the small intestine in their cellular structure and phenotype.

IgG occurring in genital secretions is deemed to be mainly serum-derived, but a significant enrichment of the IgG1 subclass suggest also some local influence. However, the mechanisms involved in the appearance of IgG in cervical secretions remain unclear [6].

Although SIgA is the chief effector of immune exclusion, SIgM also contributes, particularly in the newborn period and in IgA deficiency.

In addition, there may be a significant contribution to immune exclusion by serum-derived or locally produced IgG antibodies transferred passively to the lumen by paracellular leakage or exported actively by the FcRn expressed particularly on the gut epithelium.

IgG and monomeric IgA antibodies, when cross-linked via antigen with pIgA of the same specificity, might contribute to pIgR-mediated epithelial excretion of foreign material from the intestinal LP [1].

Notably, however, because IgG is complement-activating, its contribution to surface defence is potentially proinflammatory, which could jeopardize the epithelial barrier function. Such deterioration of local homeostasis is counteracted by competition with anti-inflammatory IgA and by a variety of complement regulatory factors produced by mucosal epithelia.

Studies in mucosally vaccinated wild-type mice challenged with Influenza virus intranasally have suggested that while SIgA antibodies are essential to control virus replication locally, serum IgG antibodies protect against clinical illness [23]. However, although systemic immunity may be considered a life-saving layer of defence, it operates at the risk of causing inflammation and tissue damage.

Thus it has been experimentally documented that SIgA antibodies prevent virally induced pathology in the upper airways, whereas IgG antibodies neutralize newly replicated virus after the initiation of infection [24]. Therefore, in the face of most infections, it is important that vaccination induces both secretory and serum antibodies.

### 1.5 Evaluation of the protective effect of secretory immunity

It is difficult to evaluate the protective effect of SIgA and SIgM antibodies during mucosal infection due to concurrent induction of systemic immunity.

As alluded to above, a protective effect of serum antibodies (mainly IgG) may contribute to immune exclusion, particularly in the respiratory tract, in addition to the general importance of inhibiting further spread



of the infectious agent by performing antibody-dependent neutralization and immune elimination within the lamina propria [25].

An essential protective role of SIgA has been questioned by observations in IgA knockout (IgA<sup>-/-</sup>) mice [26]. These mice remain healthy under ordinary laboratory conditions and when challenged with Influenza virus, they show similar pulmonary virus levels and mortality as wild-type (IgA<sup>+/+</sup>) mice. In addition, the IgA<sup>-/-</sup> mice usually show a compensatory SIgM response and, compared with wild-type mice, they are less prone to inflammation-induced pathology, apparently because of reduced antigen-presenting ability [27].

Leakage of serum IgG antibodies through an irritated mucosal surface epithelium may play a significant protective role in the airways [28].

However, the controversy of IgA- versus IgG-mediated protection of the respiratory tract has been recently resolved by the observation that both antibodies are important, with plasma IgG serving as the back-up for SIgA-mediated protection in the nasal compartment, and IgA being the dominant antibody in protection of the lung [29].

The fact that humans with selective IgA deficiency do not suffer significantly more than others from intestinal virus infections, may largely be ascribed to their consistently enhanced SIgM and IgG1 responses in the gut, as well as increased numbers of intraepithelial lymphocytes [30].

The notion that secretory antibodies are essential for an optimal mucosal barrier function is supported by the systemic IgG antibody response against *E. coli* observed in plgR<sup>-/-</sup> mice, reflecting excessive penetration of antigens from the gut microbiota [31]. Such knockout mice have also been used to test the role of SIgA antibodies in the defence against influenza virus [32]. After intranasal immunization, wild-type mice showed complete protection or partial cross-protection when challenged with live A/PR8 Influenza virus. Lack of SIgA, and SIgM, in the airways of plgR<sup>-/-</sup> mice caused reduced protection and cross-protection, despite leakage of IgG and some IgA from serum into nasal secretions. This result emphasizes the importance of SIgA antibodies in mucosal virus defence.

Moreover, induction of SIgA responses has been shown to interfere significantly with mucosal uptake of macromolecules in experimental animals without causing immunopathology, in contrast to the adverse effects on the barrier function caused by serum IgG antibodies [15].

In keeping with these early observations, SIgA antibodies are essential in protection against CT, as recently demonstrated in knockout mice lacking J chain [33] or plgR, two central components of the secretory immune system. The same is probably true for heat-labile *E. coli* enterotoxin, which binds with high affinity to the same ganglioside receptor as CT.

The initial epithelial colonization of bacterial pathogens is apparently also largely controlled by SIgA antibodies, even in naïve animals.

Most importantly, the natural horizontal faecal-oral spread of gut pathogens is significantly diminished by secretory antibodies [34], apparently because the SIgA coating of bacteria reduces both their mucosal colonization and faecal shedding.

IgAs have been implied in the neutralization of viruses, as recently demonstrated in HIV-exposed but uninfected individuals (ESNs).

In an important cross-sectional study, made of sex professionals in Kenya, it was observed that 75% of the women who were immunologically resistant to HIV had specific SIgA against the virus in the genital tract, while only 26% of the women infected by this virus had this antibody [35,36]. It was found, in further studies, those in uninfected individuals who are continuously exposed to HIV, that specific secretory IgA not only neutralizes the virus in secretions, but also induces transcytosis, in which this antibody blocks the action of the virus, interfering with its synthesis, besides neutralizing intracellular viruses [37,38].

Induction of secretory immunity has been speculated also as a possible correlate of protection against HPV infections, in contrast to authors who explain the natural history of HPV infections with the dichotomous model (Th1/Th2) of immune response, in which cell-mediated immunity determines the evolution of HPV-induced lesions [39].

Furthermore, recent studies have shown that patients with genital HPV presented lower salivary IgAs levels than the HPV-negative group [40]. The genital-HPV-positive women were more predisposed to present HPV in the oral cavity. In this same study, it was also observed that patients who presented concomitant oral and genital HPV, had still lower levels of IgAs than those who had only genital HPV. It appears that these individuals had a secretory IgA deficiency, either due to intrinsic causes (systemic or local), or some mechanism induced by the virus presence. It must be considered that some immunologically-competent women may present specific and transitory immunological deficiencies for particular infectious agents in the mucosa. Perhaps higher secretory IgA levels affect the initiation and progress of HPV infection.

## 2. HOMING OF ANTIBODY-SECRETING CELLS (ASCs)

Tissue-resident PCs derive from antigen stimulation of naïve or memory B-cells in secondary lymphoid tissues such as the spleen, LNs or MALT.

In response to ligation with their surface-immunoglobulin, naïve cells undergo clonal expansion and differentiation, developing either into memory cells or APCs.

The first APCs to arise mainly secrete IgM and initially produce a low affinity humoral response. Other activated B-cells receive co-stimulation by specialized CD4<sup>+</sup> T helper cells and form germinal centres, in which class switching, affinity maturation and somatic hypermutation occur. The result is the generation of memory B-cells and ASC precursor (plasmablasts) that express high affinity antibodies for their cognate antigen.

Plasmablasts can differentiate locally into sessile PCs that reside in the secondary lymphoid tissue of origin, a fate that seems particularly common for IgM ASCs, or they can traffic back through the efferent lymph to the blood to populate distant sites. Furthermore, most ASCs leave lymphoid tissues and travel to the bone marrow, where a small fraction becomes long-lived PCs.

Recent studies indicate that chemoattractant cytokines (chemokines), together with tissue-specific adhesion molecules, coordinate the migration of ASCs [41,42,43] from their sites of antigen-driven differentiation in lymphoid tissues to target effector sites.

Developing ASCs downregulate the expression of receptors for lymphoid tissue chemokines and selectively up-regulate the expression of chemokine receptors that might target the migration of IgA ASCs to mucosal surfaces, IgG ASCs to sites of tissue inflammation and both types of ASCs to the bone marrow, an important site for serum antibody production.

By directing PC homing, chemokines might help to determine the character and efficiency of mucosal, inflammatory and systemic antibody responses.

### 2.1 Chemokines and plasma-cell homing

Chemokines are small chemoattractive proteins found in mammals, birds and fish [44].

Four subtypes have been identified, named after the number and spacing of cysteine residues in the amino-terminal region as C, CC, CXC or CX<sub>3</sub>C. All are secreted molecules except the single defined CX<sub>3</sub>C chemokine, fractalkine, which has transmembrane and cytoplasmic domains (Fig. 5).

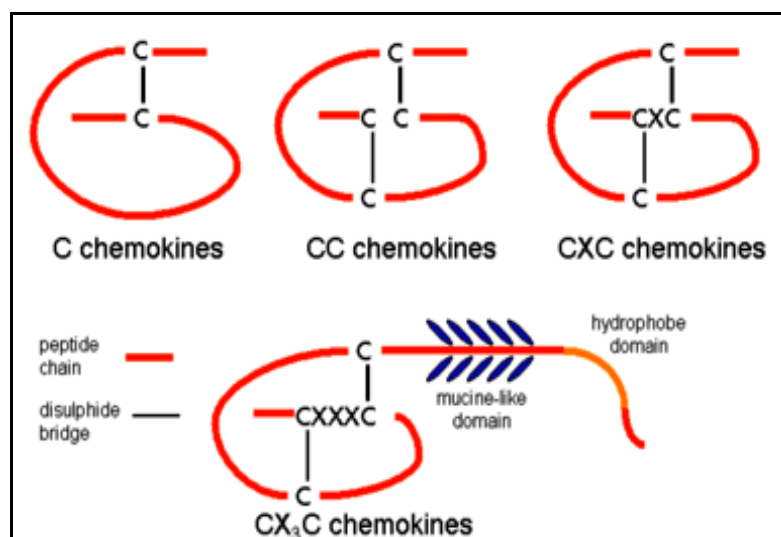


Figure 5: structure of chemokine classes.

Chemokines are highly basic proteins and this property may help mediate stable gradient formation by promoting interactions with sulfated proteins and proteoglycans.

Chemokines signal through transmembrane receptors that cross the cell membrane seven times and couple to heterotrimeric GTP-binding proteins (G proteins). Induction of migration up a chemokine gradient (chemotaxis) requires activation of PTX-sensitive G proteins, also known as G<sub>i</sub>.

The first chemokines were identified as molecules induced at sites of inflammation, where they function to recruit granulocytes, monocytes, immature DCs and activated T-cells. Some also activate cells for the respiratory burst or induce release of inflammatory mediators.

Different tissues produce different repertoires of inflammatory chemokines and this helps to attract appropriate subsets of effector cells into the tissue to respond to the particular pathogen or type of tissue damage.

More recently, chemokines have been identified that are expressed constitutively within lymphoid organs and that attract naïve as well as activated lymphocytes [45]. These “lymphoid chemokines” and their receptors appear to be critical for the homeostatic trafficking of leukocytes into subcompartments of lymphoid organs. These homeostatic chemokines include CXCL13/BCL, CCL19/ELC, CCL21/SLC and CXCL12/SDF1.

CXCL13 is made by stromal cells within the B-cell zones (lymphoid organs) in spleen, LNs, PPs and tonsil, and both CXCL13 and its receptor, CXCR5, are required for B-cell migration into follicles.

CXCL13 is also made by cells within the body cavities and it is required for normal accumulation of body cavity B-cells. B-cells up-regulate CXCR5 during their development, achieving maximal expression levels as mature B-cells.

In contrast with CXCL13, CCL19 and CCL21 are produced by T zone stromal cells, and CCL21 is also made by HEVs, the specialized vessels by which most lymphocytes enter LNs and PPs, and by lymphatic vessels within non-lymphoid tissues.

The CCL19 and CCL21 receptor, CCR7, is required for T-cell and DC migration to lymphoid organ T zones [46]. B-cells also express CCR7 and this chemokine contributes to B-cell attachment to HEVs and entry into LNs and PPs.

Within the lymphoid tissue, CXCL13 appears to be the dominant chemokine for naïve B-cell, guiding the cells into follicles. However, within a few hours of antigen receptor engagement, B-cells increase their expression of CCR7, and the balance of chemokine responsiveness shifts such that the cells localize at the boundary of follicles and T-cell zones. It is typically in this location that the first plasmablasts emerge.

CXCL12 is the unique identified natural ligand of the receptor CXCR4 and participates in ASC homing within secondary lymphoid organs and to the bone marrow. CXCL12 is constitutively displayed by epithelial cells in the bone marrow and secondary lymphoid organs, where it seems to be produced by nearby osteoblasts, stromal cells, or tonsil epithelium.

Current findings suggest that ASCs may be directed out of the lymphoid areas of spleen and LNs at least in part due to the loss of their ability to respond to the lymphoid chemokines CXCL13, CCL19 and CCL21. Having reached the perimeter of the lymphoid compartments, the cells enter areas rich in CXCL12 and this chemokine then influences their migration.

In addition, CXCR4 is required for plasmablasts to migrate from the marginal zone bridging or junction zones into the red pulp of the spleen during terminal differentiation into PCs.

Furthermore, CXCL12 plays dual roles in ASC homing to the bone marrow, helping to promote adhesion and transmigration of immature APCs across the bone marrow endothelium and subsequently helping to retain mature ASCs within the bone marrow, in close contact with stromal cells.

## **2.2 Trafficking patterns of IgA and IgG ASCs**

The site of antigen presentation and ASC differentiation, together with the nature of the stimulating antigen, determines the main immunoglobulin isotype that is expressed by the induced ASCs, and both the site of induction and the isotype expressed correlate with the homing potential and final tissue distribution of the resulting ASCs [24].

The majority of IgA PCs has a half-life of 5 days, as shown in the mouse gut; therefore a continuous supply must be guaranteed by a daily migration and maturation of B-cells into mucosal tissues.

Repopulation studies have clearly indicated that mucosal effector immunocytes are largely derived from B-cells initially induced in MALTs. Moreover, a regional specificity characterizes this mucosal homing, since primed B-cells migrate preferentially into effector tissues corresponding to the inductive site where they have been initially stimulated (Fig. 6a).

Conversely, IgG ASC precursors, whether from peripheral or gut-associated LNs, or the spleen, are largely unable to access mucosal tissues and instead preferentially populate the lymphoid tissues and the non-mucosal sites of chronic inflammation (Fig. 6b).

Several studies established that this specific mucosal homing is supported by specific cell-to-cell interactions between B-cells and endothelial cells in venules of the parafollicular areas (inductive sites) or of the lamina propria (effector sites).

The interaction between  $\alpha_4\beta_7$  integrin expressed by mucosal B- and T-cells and MadCAM-1, constitutively expressed by mucosal endothelial cells from HEVs, has been shown to support in the gut both the attraction of naïve B-cells in inductive sites and emigration of primed B-cells in effector tissues.

More specifically, the former is characterized by the interaction between  $\alpha_4\beta_7$  integrin associated with L-selectin (CD62 ligand) and a MadCAM-1 molecule with a modified O-glycosylation pattern, while the latter involves the interaction between  $\alpha_4\beta_7$  integrin, without L-selectin, with unmodified MadCAM-1.

The molecular interactions underlining the specificity of B-cell migration to the airway and lung mucosa remain unprecised, since  $\alpha_4\beta_7$  is well expressed in the airways, but MadCAM-1 is only very weakly expressed by the bronchial endothelium.

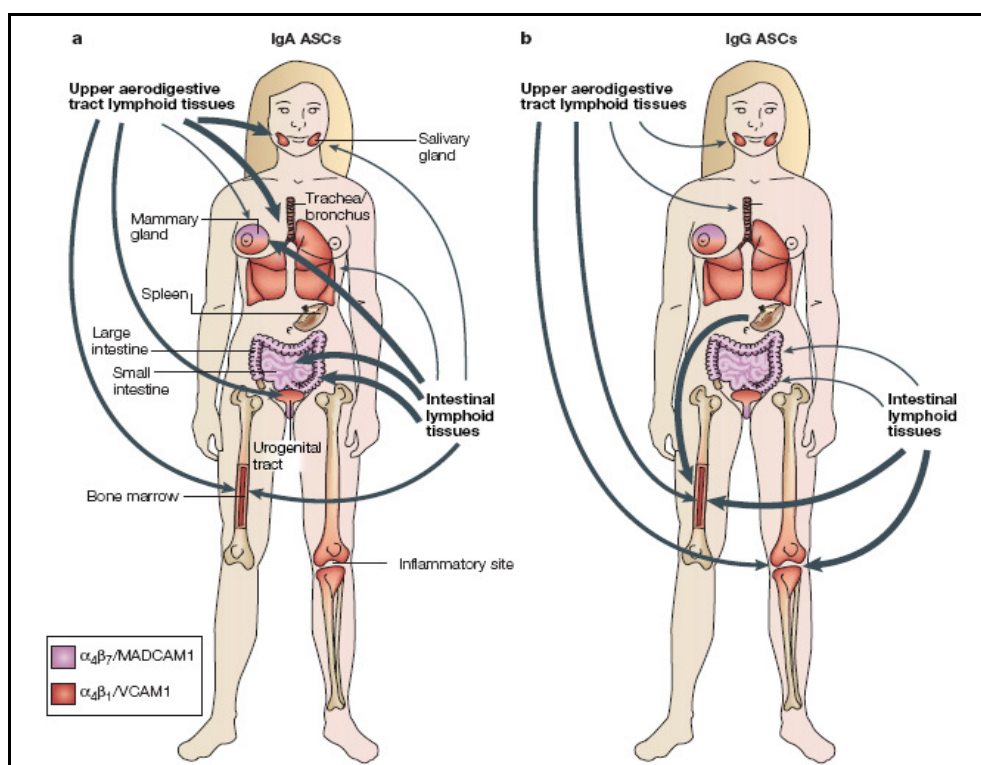


Figure 6: IgA and IgG distinctive ASC trafficking pattern.

Most post-capillary venules in non-intestinal and non-mucosal sites lack expression of MadCAM-1 and instead express the  $\alpha_4\beta_1$  ligand VCAM-1, although the mammary glands, the urogenital tract and the placenta contain both MadCAM-1 and VCAM-1 expressing vessels.

IgG ASCs induced systemically can express  $\alpha_4\beta_1$ , however it is less clear what level of  $\alpha_4\beta_1$  is expressed by  $\alpha_4\beta_7$ -positive intestinally derived ASCs.

The differential interactions of ASCs with VCAM-1 versus MadCAM-1 are likely to be an important determinant of selective trafficking of IgA versus IgG ASCs, but there is no clear data on the expression of other tissue-specific adhesion molecules such as L-selectin that might also contribute to the dissemination of IgA or IgG ASCs.

However, the shared vascular expression of VCAM-1 by the tissues of the upper aerodigestive tract, in which IgA ASCs predominate, and by the systemic tissues that are selectively populated by IgG ASCs indicates the existence of additional mechanisms of homing selectivity.

### 2.3 IgA ASC trafficking to mucosal tissues

Although the IgA immune system has historically been considered a “common mucosal immune system” [47], more recent data suggest that the IgA immune system has both common and regional characteristics. Small intestine-derived IgA plasmablasts appear to be more likely to migrate to both the intestines and non-intestinal tissues, such as the upper airways, lacrimal, salivary and mammary glands [48], suggesting some commonality.

However, the restricted transfer of antigen-specific IgA to the upper airways, lacrimal gland, nasal cavity, and genital tract after nasal immunization, suggests regionalization of the upper aero-digestive and genital tract. IgA ASCs in mucosal lymphoid tissues can up-regulate the expression of two chemokine receptors, CCR9 and CCR10, which are associated with the mucosal tissue trafficking patterns of these cells (Fig. 7).

CCL25 is a small cytokine belonging to the CC chemokine family that is also known as TECK (Thymus-Expressed ChemoKine). It is chemotactic for thymocytes, macrophages and DCs, eliciting its effects by binding the chemokine receptor CCR9.

Responsiveness to the CCR9 ligand CCL25 is a characteristic of mouse IgA ASCs in PPs and MLNs, but not those in lymphoid tissues of the upper aerodigestive tract [49,50], indicating that the expression of CCR9 is induced during the development of IgA ASCs in the lymphoid tissues of the intestinal mucosa. Similarly, in humans, CCR9-expressing IgA ASCs are abundant in the appendix, but are rarer in the tonsil [51].

Moreover, CCR9 expression seems to be associated specifically with cells that are induced by antigens presented in the small intestine. Infection with the small intestinal pathogen rotavirus, for example, leads to the induction of virus-specific IgA ASCs that migrate in response to CCL25 [31].

Most IgA ASCs in the LP of the small intestine are CCR9 positive, whereas expression of CCR9 by IgA ASCs from other segments of the gut is rare and CCR9 is not expressed by IgA ASCs in the lungs.

Consistent with this restricted receptor expression, the CCR9 ligand CCL25 is expressed at high levels by crypt epithelial cells [52] and endothelial cells [53] in the small intestine, but shows low levels of or no expression at other mucosal sites.

So, CCR9 and CCL25, which also mediate the homing of a specialized subset of memory T-cells to the small intestine [54], seem ideally suited to focus the immune response to antigens in the small intestine.

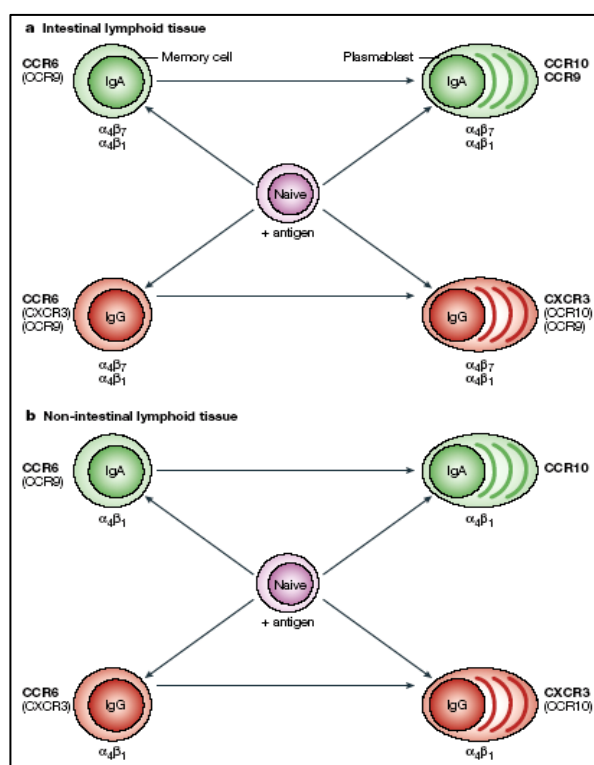


Figure 7: changes in the expression of chemokine receptors during B-cell differentiation in intestinal and non-intestinal lymphoid tissues.

By contrast, the CCR10 ligand CCL28 is produced by epithelial cells in various mucosal tissues (the large intestine, stomach, trachea and bronchi, mammary glands and salivary glands) [55,56,57], indicating a broad role for this chemokine in mucosal immunity (Fig. 8).

CCL28, also known as mucosae-associated epithelial chemokine (MEC), is a chemokine that regulates the chemotaxis of cells that express the chemokine receptors CCR3 and CCR10.

Human CCL28 is encoded by an RNA transcript of 373 nucleotides and a gene with four exons. The gene codes for a 127-amino acid CCL28 protein with a 22-amino acid N-terminal signal peptide. It shares 76% nucleic acid identity and 83% amino acid similarity to the equivalent molecule in mouse.

Almost all IgA ASCs, whether they are from intestinal or non-intestinal lymphoid tissues or mucosal effector tissues, express CCR10 and respond to CCL28 (Fig. 7). Surprisingly, even IgA ASCs in the small intestine express CCR10.

In mice, approximately half of the IgA ASCs in the gut wall can be derived from B1 lineage B-cell precursors, although the pathway of migration and sites of maturation of these ASCs are uncertain and controversial [58,59].

It is worth noting that the roles of MEC and TECK in IgA ASC biology are well conserved between human and mouse: in mice, CCL28 is a highly efficacious chemoattractant for IgA (but not IgG or IgM) ASC from multiple mucosal tissues (small and large intestines, lung and mammary gland) and the LNs that drain those tissues, while CCL25 responsiveness is relatively limited to IgA ASCs within the small intestine and its associated lymphoid tissues.

The essentially universal expression of CCR10 by IgA ASCs of the lamina propria argues that B1-derived IgA-ASCs, as well as the conventional B2-derived IgA-ASCs, express this shared mucosal trafficking receptor. By contrast, few IgG or IgM ASCs are CCR10 positive, re-emphasizing the selective association of this receptor with IgA ASCs.

The expression of the CCR10 ligand CCL28 by various mucosal epithelial tissues is probably the missing link that allows intestinal IgA ASCs to populate various mucosal sites, a hallmark of the common mucosal IgA immune system.

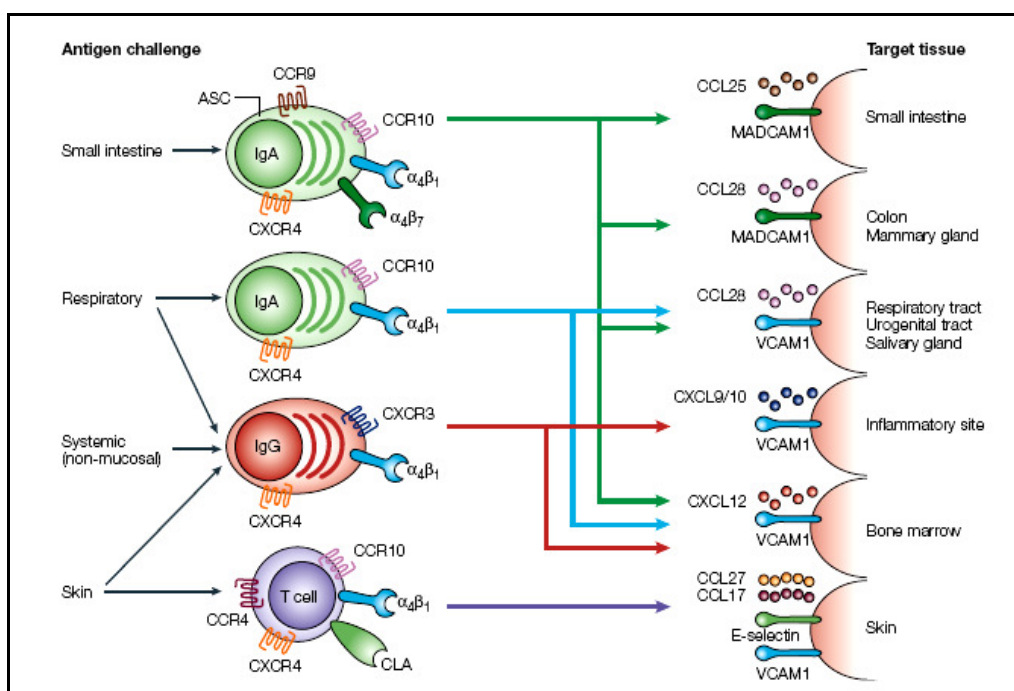


Figure 8: model of trafficking of IgA and IgG ASCs mediated by specific adhesion molecules and chemokine receptors.

The unique patterns of expression of CCR9 and CCR10 might also help to explain the differences in dissemination of antigen-specific IgA after nasal or oral immunization, which is likely to be a result of differential migration properties. In fact, CCR9 and CCR10 expression patterns appear to depend on both the site of antigen exposure and differentiation state.

In both humans and mice, oral immunization leads to the presence of antigen-specific IgA in both intestinal and non-intestinal mucosal tissues, such as the salivary glands, the respiratory tract and the mammary glands [60]. Conversely, nasal immunization leads to a more restricted distribution of antigen-specific IgA in the upper aerodigestive tract and the urogenital tract [61].

The up-regulation of expression of CCR10, but not CCR9, and the expression of  $\alpha 4\beta 1$ , but not  $\alpha 4\beta 7$ , by IgA ASCs induced in the lymphoid tissues of the upper aerodigestive tract after intranasal immunization would allow their trafficking to tissue sites that express VCAM-1 and the CCR10 ligand CCL28. So, nasally induced IgA ASCs would traffic efficiently to the salivary glands, respiratory tract and urogenital tract, but not to the intestine (especially the small intestine) (Fig. 8).

By contrast, the induction of expression of CCR9 as well as CCR10, and the expression of  $\alpha 4\beta 7$  in addition to  $\alpha 4\beta 1$  by intestinally derived IgA ASCs after oral immunization should allow trafficking to sites that express CCL28 or CCL25 together with either MAdCAM-1 or VCAM-1 that is to essentially all mucosal tissues, including the small intestine (Fig. 8).

It is possible that selective antigen challenge in the colon could induce IgA-secreting plasma blasts that express CCR10 and  $\alpha 4\beta 7$  without expression of CCR9, and these cells would be expected to traffic preferentially back to the colon.

While neither CCR9 nor CCR10 appear to be expressed on resting naïve B cells, we cannot rule out that they are expressed at very low levels.

CCR9 is expressed on approximately 20% of circulating IgA<sup>+</sup> B-cells with a memory phenotype. In contrast, CCR10 is absent from memory B cells but is expressed on IgA class-switched plasmablasts and PCs. Therefore, the expression of both receptors may be regulated by cytokines such as TGF- $\beta$  that are involved in IgA class switching.

The absence of CCR10 on memory B-cells, however, suggests that CCR10 expression may be more closely linked to PC differentiation, particularly in mucosal sites.

The expression of CCR9 on a subset of memory B cells may mean that these B-cells were originally activated in small-intestinal-associated lymphoid tissues, but the specific signals involved in CCR9 induction (e.g., TGF- $\beta$  and/or MAdCAM-1<sup>+</sup> DCs) remain to be elucidated.

Up to 50% of small intestinal PCs express both CCR9 and CCR10. This may mean that these double positive cells arose from IgA<sup>+</sup> memory B-cells expressing CCR9 or naïve B-cells activated in small intestinal lymphoid tissues and acquired CCR10 upon PC differentiation. Expression of both CCR9 and CCR10, in conjunction with  $\alpha 4\beta 7$  integrin, would allow these plasmablasts to enter mucosal tissues throughout the body.

Conversely, IgA<sup>+</sup> memory B-cells that do not express CCR9 may therefore represent B-cells that class-switched in lymphoid tissues outside of the small intestine, for instance in large-intestinal follicles or the tonsil, where only 5% of the PCs express CCR9. These CCR9-IgA<sup>+</sup> memory cells would become CCR10<sup>+</sup> upon PC differentiation and traffic into mucosal tissues expressing CCL28, including the small intestine if they also co-express  $\alpha 4\beta 7$  integrin (a likely scenario for PCs induced in colon follicles but not in the tonsil) [34].

Up to 15% of circulating IgG ASCs express low levels of CCR10, which might help to mediate their localization to mucosal epithelial tissues, in particular to tissues of the upper respiratory tract. However, if these same IgG ASCs also lack high levels of  $\alpha 4\beta 7$  expression, their relatively low level of expression of CCR10 could also help to explain the low frequency of IgG ASCs that are normally present in intestinal mucosal tissues.

At the same time, low levels of CCR10 expression, together with CXCR3 expression and CLA expression, could facilitate the recruitment of IgG ASCs to the skin, where the other CCR10 ligand CCL27 is expressed, during chronic inflammation.

It is intriguing that the two chemokines that attract IgA ASCs CCL28 and CCL25 have marked homology and belong to a subfamily of chemokines that are selectively expressed by epithelial cells.

Other members of this subfamily include the keratinocyte-expressed chemokine CCL27, also known as cutaneous T-cell attracting chemokine (CTACK), which is a second ligand for CCR10. CCL27 is of interest because, even though it is a ligand for CCR10, its role seems to be limited to the recruitment of cutaneous memory T-cells [62,63]. IgA ASCs respond to CCL27 *in vitro*, but probably lack expression of CLA, a homing receptor for E-selectin, which is required for efficient recruitment of CCR10-expressing T-cells to the skin.

Thus there is an interesting bifurcation in the evolutionary development of tissue-specific T and B cell trafficking.

CCR9 appears to be rather specifically associated with both small-intestinal IgA ASCs and small-intestinal T-cells, while it is largely absent from both T and PCs in other mucosal sites. On the other hand, even though the CCR10 ligand CCL28 is expressed in a variety of mucosal tissues, few if any T-cells in these tissues express CCR10. Thus, in contrast to the small intestine where CCR9 appears to be involved in both T and B cell biology, CCR10 is associated with mucosal PC trafficking and cutaneous T cell trafficking.

Additionally, CCL28 is expressed by bone marrow stromal cells, suggesting that the interaction of this chemokine with CCR10<sup>+</sup>/CCR3<sup>+</sup> B-cells may contribute to the integration between the mucosal and the systemic immune responses [40].

## **2.4 Role of MEC/CCL28 in mucosal immunity**

The ability of the CCL28-CCR3/CCR10 circuit to chemoattract ASCs into multiple mucosal sites has been a founding element of the concept of a common mucosal immune system, and very recent results indicate that CCL28 has a fundamental role in the modulation of mucosal humoral responses against virus transmitted via mucosal surfaces, such as HIV [64].

High concentrations of HIV-specific mucosal IgA have been described in both HIV-infected [65] and HIV-exposed but uninfected individuals (ESNs) [12,66]. HIV-specific IgA seen in these two groups have nevertheless shown to be qualitatively different: IgA of ESN recognize epitopes different from those seen in HIV-infected individuals [67], are capable of inhibiting virus transcytosis through epithelial layers *in vitro* and have a potent neutralizing activity.

The CCL28-dependent, but not the CCL25-dependent chemotactic circuit is up-regulated in HIV exposure and infection, therefore justifying the augmented mucosal concentrations of IgA seen in these conditions.

However CCL28 seems to have dual functions in mucosal immunity as a chemokine attracting cells expressing CCR10 and/or CCR3 and also as an apically secreted molecule with a potent antimicrobial activity against a broad spectrum of microbes [40].

Antimicrobial peptides are the evolutionary ancient weapons of multicellular organisms against a wide range of microbes, including bacteria, fungi, protozoa, and enveloped viruses.

Structurally, antimicrobial peptides are grouped roughly into four classes, which comprise anti-parallel-sheet peptides stabilized by two to three disulfide bridges, amphipathic-helical peptides, proline-rich coiled peptides, and looped or cyclic peptides.

The fundamental structural principal underlying all classes of antimicrobial peptides seems to be the amphipathic design, in which clusters of hydrophobic and cationic amino acids are organized in discrete sectors.

The basis of the selectivity of these peptides against microbes is considered to be due to the relative abundance of negative charges in the microbial cell membranes compared with those of the host's cells. Thus these peptides are selectively attracted to the microbial plasma membrane by the mechanism of electrostatic interactions, which explains why most antimicrobial peptides are ineffective at high salt

concentrations. Subsequently, most peptides are considered to be spontaneously inserted into the membrane and to generate physical holes that cause cellular contents to leak out [68].

CCL28 is produced by epithelial cells of human and mouse salivary glands and is secreted into human saliva and milk at high concentrations.

However, the very high levels of expression of CCL28 in salivary glands may suggest that CCL28 also has a role in salivary glands that is different from cell recruitment.

Given the particularly strong and selective expression of CCL28 in certain mucosal tissues such as salivary glands, mammary glands, trachea and large intestine, which commonly secrete low-salt fluids because of re-absorption of sodium ions by the epithelial cells expressing the epithelial sodium channel, one important function of CCL28 in these tissues may be that it is apically secreted as an antimicrobial protein and protects the mucosal surfaces against colonizing microbes.

Chemokines are well known to bind to heparan sulphate, an abundant component of epithelial cell surface and extracellular matrix. This ability is considered to keep chemokines locally concentrated in the vicinity of producing cells and to form a gradient within the tissue [69]. The same ability is also likely to help secreted CCL28 to be highly concentrated on the mucosal surfaces to form a barrier shield against colonizing microbes.

Thus, even though the concentrations of CCL28 found free in saliva and milk are relatively low compared with its IC<sub>50</sub> for various microbes, its high surface concentrations on the mucosal surfaces together with synergistic effects with other antimicrobial factors rich in mucosal secretions [70] may provide a highly effective barrier protection against a wide spectrum of microbes.

The mature protein of CCL28, which constitutes 127 amino acids with six cysteine residues instead of the standard four, has an extended C terminus. The 28-amino acids C-terminal segment after the conserved 4th cysteine residue contains as many as eight histidine residues and has 53% sequence similarity with human histatin-5, a histidine-rich peptide secreted into saliva that has a potent antimicrobial activity against *C. albicans* [71].

However CCL28 shows a much broader spectrum of antimicrobial activity than does histatin-5, which is mainly effective against *Candida* species. Like most other antimicrobial peptides, CCL28 apparently exerts its antimicrobial activity by spontaneous membrane insertion and pore formation in target microbes.

Thus, CCL28 may represent the first chemokine that not only attracts cells expressing its receptors CCR10 and CCR3 into certain mucosal tissues but also is constitutively secreted by epithelial cells in such tissues and functions as a potent antimicrobial factor in physiological settings.



### 3. MUCOSAL INFECTIONS

Mucosal surfaces provide portals of entry for pathogens. Increased sanitation and hygiene, the use of antibiotics and childhood vaccination have enormously decreased the death rate from infectious diseases over the last century.

Thus, infectious agents not controlled by antibiotics and improved sanitation and hygiene measures would most likely be prevalent under current conditions [72]. Pathogens in this category include respiratory viruses (e.g. Influenza virus). Another group includes sexually transmitted disease (STD) pathogens.

The fact that Influenza virus and HIV infection were listed as the leading causes of death by infectious agents in the United States in 1997 confirms this notion [55].

However, other viral STDs are of major concern. In the United States, it is estimated that approximately 18.9 million new cases of STD infections occurred in the year 2000, of which 9.1 million (48%) were among young persons aged between 15 and 24. Among them, HPV was the most commonly acquired [73].

Sexually transmitted diseases (STDs) are caused by a group of infectious microorganisms that are transmitted mainly through sexual activity. Bacterial STDs include those caused by *Chlamydia trachomatis* (chlamydia), *Neisseria gonorrhoeae* (gonorrhea), *Treponema pallidum* (syphilis), *Haemophilus ducreyi* (chancroid). *Trichomonas vaginalis* (trichomoniasis) is a common protozoal STD and *Candida* species (candidiasis) are sexually transmitted yeasts. Ultimately, viral STDs include the viral infections caused by HIV (HIV infection), *Herpes simplex virus type-2* (genital herpes) and *Human Papillomavirus* (HPV infection). Bacterial and protozoan infections are curable with antimicrobial therapy, while viral infections are treatable but not curable in the classic sense and appear to be lifelong infections.

STDs represent an important global health priority because of their devastating impact on women and infants and their inter-relationships with HIV/AIDS.

STDs and HIV are linked by biological interactions and because both infections occur in the same populations. Infection with certain STDs can increase the risk of HIV acquisition and transmission as well as alter the course of disease progression.

In addition, STDs can cause long-term health problems, including pelvic inflammatory disease, infertility, tubal or ectopic pregnancy, cervical cancer in women, and perinatal or congenital infections in infants born to infected mothers.

#### 3.2 Influenza virus

Influenza viruses are among the most common causes of human respiratory infections [74], and among the most significant because they cause high morbidity and mortality. In addition to annual winter outbreaks, pandemic influenza viruses occasionally emerge, as they have every 8 to 41 years for at least several centuries.

Up to 50% of the population can be infected in a single pandemic year, and the number of deaths caused by influenza can dramatically exceed what is normally expected [75].

Influenza viruses, of the family *Orthomyxoviridae*, are enveloped negative-strand RNA viruses with segmented genomes containing seven to eight gene segments [76] (Fig. 9).

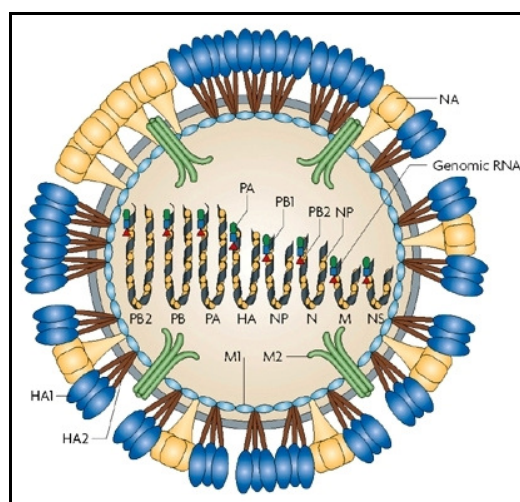


Figure 9: structure of the Influenza virion.

One genus includes influenza A and B viruses, and the other comprises influenza C viruses. The three virus types differ in host range and pathogenicity.

Type B and C influenza viruses are isolated almost exclusively from humans, although influenza B viruses have been isolated from seals and influenza C viruses have been isolated from pigs and dogs. Influenza A viruses, however, infect a wide variety of warm-blooded animals, including birds, swine, horses, humans, and other mammals. Avian influenza viruses in aquatic birds serve as the natural reservoir for all known subtypes of influenza A virus.

Influenza A and B viruses have a similar structure, whereas influenza C is more divergent. The virus particle is 80–120 nm in diameter and usually roughly spherical, although filamentous forms can occur. These filamentous forms are more common in influenza C, which can form cordlike structures up to 500 micrometres long on the surfaces of infected cells.

A and B type viruses contain eight discrete gene segments, each of them coding for at least one protein, and are covered with projections of three proteins: HA, NA and M2 (Fig. 10). For example, the influenza A genome contains 11 genes on eight pieces of RNA, encoding for 11 proteins: HA, NA, NP, M1, M2, NS1, NS2, PA, PB1, PB1-F2 and PB2. Influenza C viruses have seven segments and only one surface glycoprotein (Fig. 10).

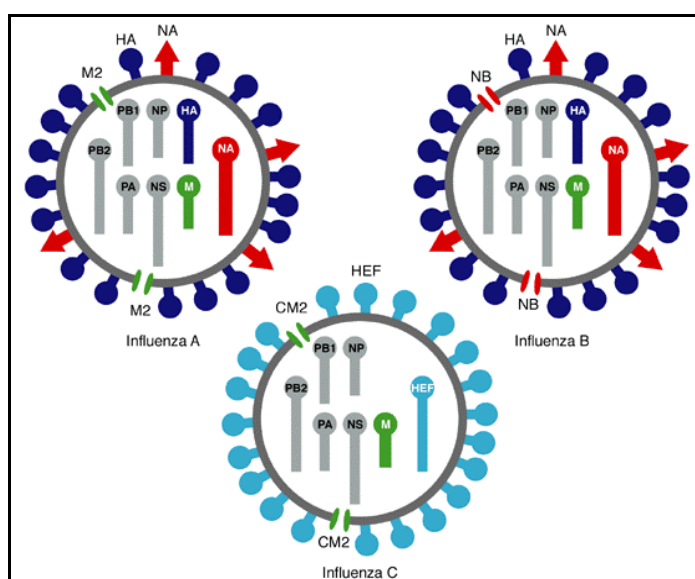


Figure 10: structure and genome organization of Influenza viruses.

HA and NA are the two large glycoproteins on the outside of the viral particles which are antigens to which antibodies can be raised. Furthermore, they are targets for antiviral drugs.

HA is a homotrimeric integral membrane glycoprotein. It is shaped like a cylinder and is approximately 13.5 nm long. The three identical monomers that constitute HA are constructed into a central  $\alpha$ -helix coil; three spherical heads contain the sialic acid binding sites.

HA monomers are synthesized as precursors that are then glycosylated and cleaved into two smaller polypeptides: the HA1 and HA2 subunits. Each HA monomer consists of a long, helical chain anchored in the membrane by HA2 and topped by a large HA1 globule.

HA has two functions. Firstly, it allows the recognition of target vertebrate cells, accomplished through the binding of these cells' sialic acid-containing receptors. Secondly, once bound, it facilitates the entry of the viral genome into the target cells by causing the fusion of host endosomal membrane with the viral membrane [77] (Fig. 11).

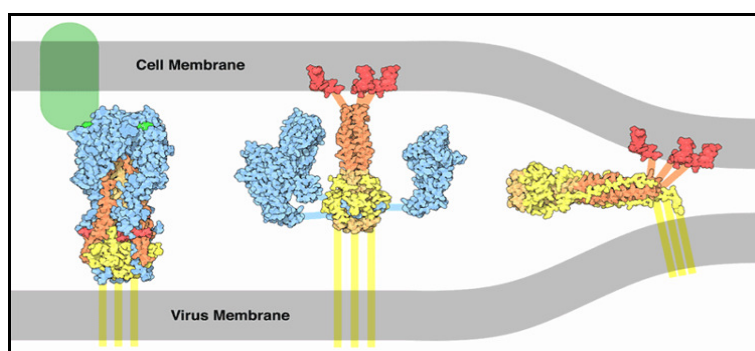


Figure 11: mechanism of action of Influenza hemagglutinin.

NA exists as a mushroom-shape projection on the surface of the influenza virus. It has a head consisting of four co-planar and roughly spherical subunits, and a hydrophobic region that is embedded within the interior of the virus' membrane. It comprises a single polypeptide chain that is oriented in the opposite direction to the HA antigen. The composition of the polypeptide is a single chain of six conserved polar amino acids, followed by hydrophilic, variable amino acids.

NA catalyzes the hydrolysis of terminal sialic acid residues from the newly formed virions and from the host cell receptors.

Viruses can only replicate in living cells. Influenza infection and replication is a multi-step process [78]. Influenza viruses bind through HA onto sialic acid sugars on the surfaces of epithelial cells, typically in the nose, throat and lungs of mammals and intestines of birds. HA binds to the monosaccharide sialic acid which is present on the surface of its target cells, which causes the viral particles to stick to the cell's surface.

The cell membrane then engulfs the virus and the portion of the membrane that encloses it pinches off to form a new membrane-bound compartment within the cell called an endosome, which contains the engulfed virus.

The cell then attempts to begin digesting the contents of the endosome by acidifying its interior and transforming it into a lysosome. However, as soon as the pH within the endosome drops to about 6.0, the original folded structure of the HA molecule becomes unstable, causing it to partially unfold and releasing a hydrophobic portion of its peptide chain that was previously hidden within the protein. This so-called "fusion peptide" acts like a molecular grappling hook by inserting itself into the endosomal membrane and locking on.

Then, when the rest of the HA molecule refolds into a new structure, which is more stable at the lower pH, it "retracts the grappling hook" and pulls the endosomal membrane right up next to the virus particle's own membrane, causing the two to fuse together.

Finally the M2 ion channel allows protons to move through the viral envelope and acidify the core of the virus, which causes the core to disassemble and release the viral RNA molecules, accessory proteins and RNA-dependent RNA polymerase into the cytoplasm (Fig. 12).

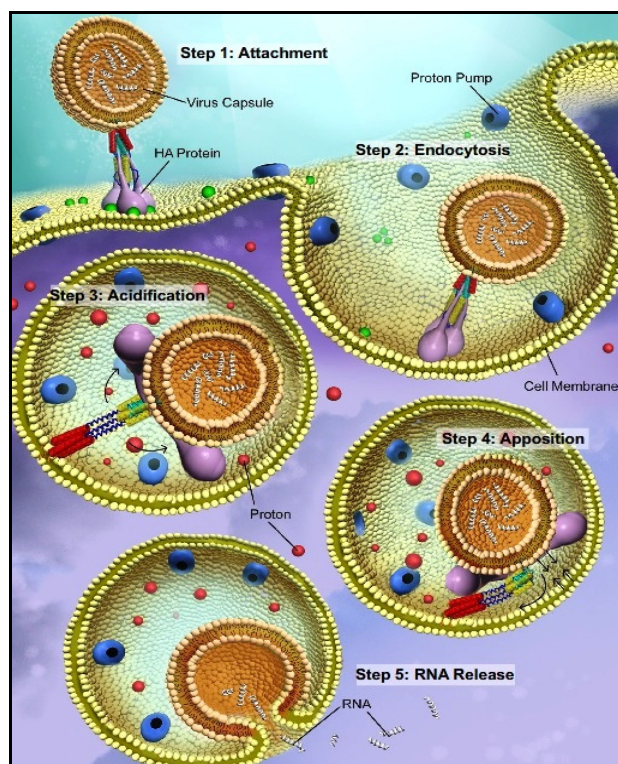


Figure 12: Influenza virus entry into target cells.

These core proteins and viral RNA are transported into the cell nucleus, where the RNA-dependent RNA polymerase begins transcribing complementary positive-sense viral RNA. The viral RNA is either exported into the cytoplasm, and translated, or remains in the nucleus.

Newly synthesised viral proteins are either secreted through the Golgi apparatus onto the cell surface or transported back into the nucleus to bind viral RNA and form new viral genome particles. Other viral proteins have multiple actions in the host cell, including degrading cellular mRNA and using the released nucleotides for viral RNA synthesis and also inhibiting translation of host-cell mRNAs.

Negative-sense viral RNAs that form the genomes of future viruses, RNA-dependent RNA polymerase, and other viral proteins are assembled into a virion.

HA and NA molecules cluster into a bulge in the cell membrane. The viral RNA and viral core proteins leave the nucleus and enter this membrane protrusion. The mature virus buds off from the cell in a sphere of host phospholipid membrane, acquiring HA and NA with this membrane coat.

NA promotes the release of progeny viruses and the spread of the virus from the host cell to uninfected surrounding cells. It also cleaves sialic acid residues from viral proteins, preventing aggregation of viruses. Thus its activities include assistance in the mobility of virus particles through the respiratory tract mucus and in the elution of virion progeny from the infected cells (Fig. 13).

After the release of new influenza viruses, the host cell dies.

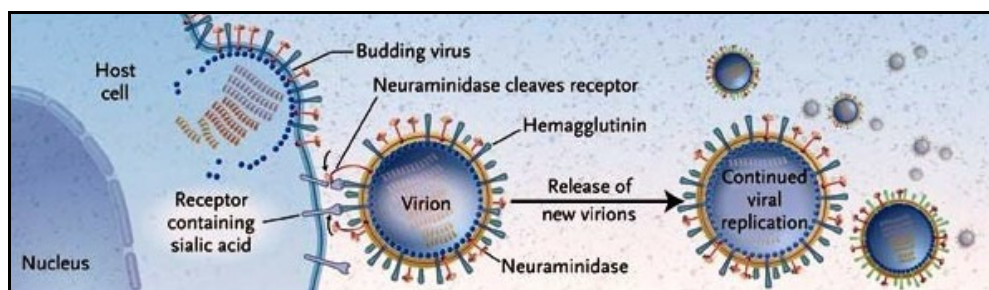


Figure 13: mechanism of action of Influenza neuraminidase.

Influenza viruses accumulate point mutations during replication because their RNA polymerase complex has no proofreading activity. Their genes have high mutation rates (ranging from approximately  $1 \times 10^3$  to  $8 \times 10^3$  substitutions per site per year). Mutations that change amino acids in the antigenic portions of surface glycoproteins may produce selective advantages for viral strains by allowing them to evade pre-existing immunity.

The HA molecule initiates infection by binding to receptors on specific host cells. Antibodies against the HA protein prevent receptor binding and are effective at preventing re-infection with the same strain.

The HA and NA can evade previously acquired immunity by either antigenic drift, in which mutations limit or prevent antibody binding, or antigenic shift, in which the virus acquires HA of a new subtype by re-assortment between two influenza A viruses [56].

Antigenic drift occurs in all types of influenza including influenza virus A, influenza B and influenza C. Antigenic shift, however, occurs only in influenza virus A because it infects more than just humans. Affected species include other mammals and birds, giving influenza A the opportunity for a major reorganization of surface antigens. Influenza B and C principally infect humans, minimizing the chance that a re-assortment will change its phenotype drastically.

Although an antigenically novel HA subtype is a likely requirement for the emergence of an influenza pandemic, human infections with animal-adapted influenza virus of novel HA subtype have been observed in which the virus is not transmitted efficiently from person to person, suggesting that stable adaptation to humans by re-assortment or whole genome adaptation is required for the emergence of a pandemic strain.

Typically, influenza is transmitted through the air by coughs or sneezes, creating aerosols containing the virus. Influenza can also be transmitted by direct contact with bird droppings or nasal secretions, or through contact with contaminated surfaces.

Influenza is an acute respiratory disease characterized in its full form by the sudden onset of high fever, nasal congestion, cough, headache, prostration, malaise and inflammation of the upper respiratory tree and trachea. In most cases, pneumonic involvement is not clinically prominent. Acute symptoms and fever often persist for 7 to 10 days. Weakness and fatigue may linger for weeks.

People with chronic pulmonary or cardiac disease, or diabetes mellitus, are at high risk of developing severe complications from influenza A viruses, which may include hemorrhagic bronchitis, pneumonia (primary viral or secondary bacterial) and death. Fulminant fatal influenza viral pneumonia occasionally occurs; dyspnea, cyanosis, hemoptysis, pulmonary edema and death may proceed in as little as 48 hours after the onset of symptoms.

The mechanisms by which influenza infection causes symptoms in humans have been studied intensively. Knowing which genes are carried by a particular strain can help predict how well it will infect humans and how severe this infection will be [79]. For instance, part of the process that allows influenza viruses to invade cells is the cleavage of the viral HA protein by any one of several human proteases.

In mild and avirulent viruses, the HA can only be cleaved by proteases found in the throat and lungs, so these viruses cannot infect other tissues. However, in highly virulent strains, such as H5N1, the HA can be cleaved by a wide variety of proteases, allowing the virus to spread throughout the body.

The viral HA protein is responsible for determining both which species a strain can infect and where in the human respiratory tract a strain of influenza will bind. Strains that are easily transmitted between people

have HA proteins that bind to receptors in the upper part of the respiratory tract, such as in the nose, throat and mouth. In contrast, the highly lethal H5N1 strain binds to receptors that are mostly found deep in the lungs. This difference in the site of infection may be part of the reason why the H5N1 strain causes severe viral pneumonia in the lungs, but is not easily transmitted by people coughing and sneezing.

Common symptoms of influenza such as fever, headaches, and fatigue are the result of the huge amounts of proinflammatory cytokines and chemokines (such as IFN- $\gamma$  and TNF- $\alpha$ ) produced from influenza-infected cells [80]. Influenza does cause tissue damage, so symptoms are due to either a massive immune response that produce a life-threatening cytokine storm or the massive levels of viral replication inside the infected cells.

Effective measures against influenza A and B diseases include prevention of infection by either vaccination with inactivated or live attenuated vaccines or administration of antiviral drugs prophylactically or therapeutically.

Chemically inactivated influenza vaccines consist of detergent-split virion subunits composed of HA and lesser amounts of other virion proteins including NA. This inactivated, split vaccine is not completely effective, particularly in the elderly. The efficacy of the vaccine is significantly compromised when circulating viruses do not have a good match with vaccine strains due to antigenic drift or inaccurate epidemiological predictions. A live, attenuated influenza virus vaccine (FluMist®) is also licensed for seasonal influenza and is intended for intranasal administration to people 6 months to 49 years of age. Both types of licensed influenza vaccines are produced using fertilized chicken eggs as substrates.

Current influenza vaccines normally protect only for a matter of months; in any case, continuous viral antigenic drift of influenza A viruses makes once effective vaccines ineffective after a few years' time. Furthermore, they do not generate broadly neutralizing antibodies to multiple strains of influenza and must be re-formulated annually to match the predominant virus strains circulating each year.

Due to limitations in vaccine supply from the conventional egg-based manufacturing system and its susceptibility to a pandemic outbreak that could threaten egg-substrate availability, it is important to develop alternative influenza vaccines not relying on egg substrates. Several recent studies have focused on developing non-replicating VLPs as alternative influenza vaccines [81].

Antiviral drugs can have both therapeutic and prophylactic effects, but to prevent disease they must be administered continuously at times of high influenza activity. Matrix 2 ion channel blockers are effective against influenza A viruses, but resistant viral strains develop rapidly and have been recognized in approximately one-third of treated patients. The more recently developed NA inhibitors are effective against both influenza A and B viruses. Both classes of drugs are effective in preventing influenza when administered prophylactically [82]. However, the importance of predicting the emergence of new circulating influenza strains for subsequent annual vaccine development cannot be underestimated.

### 3.1 Human immunodeficiency virus (HIV)

HIV is a member of the family *Lentiviridae*. Two closely related types of HIV, designated HIV-1 and HIV-2, have been identified. HIV-1 is by far the most common cause of AIDS, but HIV-2, which differs in genomic structure and antigenicity, causes a similar clinical syndrome [83].

An infectious HIV particle consists of two identical strands of RNA packaged within a core of viral proteins and surrounded by a phospholipid bilayer envelope derived from the host cell membrane but including virally encoded membrane proteins (Fig. 14).

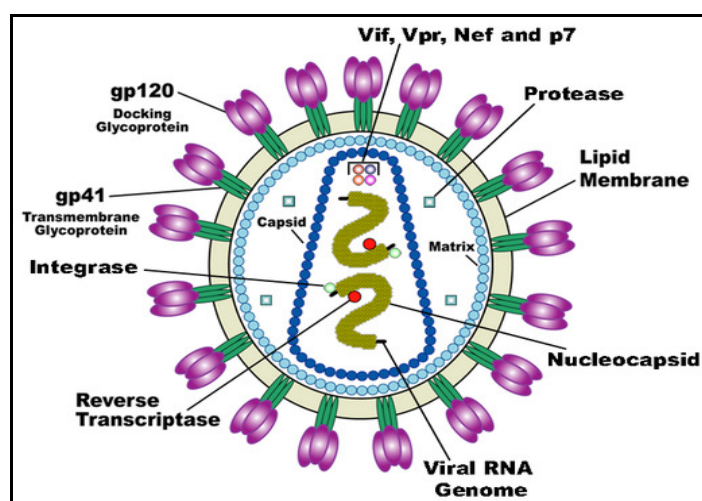


Figure 14: structure of Human Immunodeficiency Virus type-1.

The RNA genome of HIV is approximately 9.2 kb long and has the basic arrangement of nucleic acid sequences characteristic of all known retroviruses.

LTRs at each end of the genome regulate viral gene expression, viral integration into the host genome, and viral replication. The *gag* sequences encode core structural proteins. The *env* sequences encode the envelope glycoproteins gp120 and gp41, which are required for infection of cells. The *pol* sequences encode reverse transcriptase, integrase, and viral protease enzymes required for viral replication. In addition to these typical retrovirus genes, HIV-1 also includes six other regulatory genes, namely, the *tat*, *rev*, *vif*, *nef*, *vpr*, and *vpu* genes, whose products regulate viral reproduction in various ways (Fig. 15).

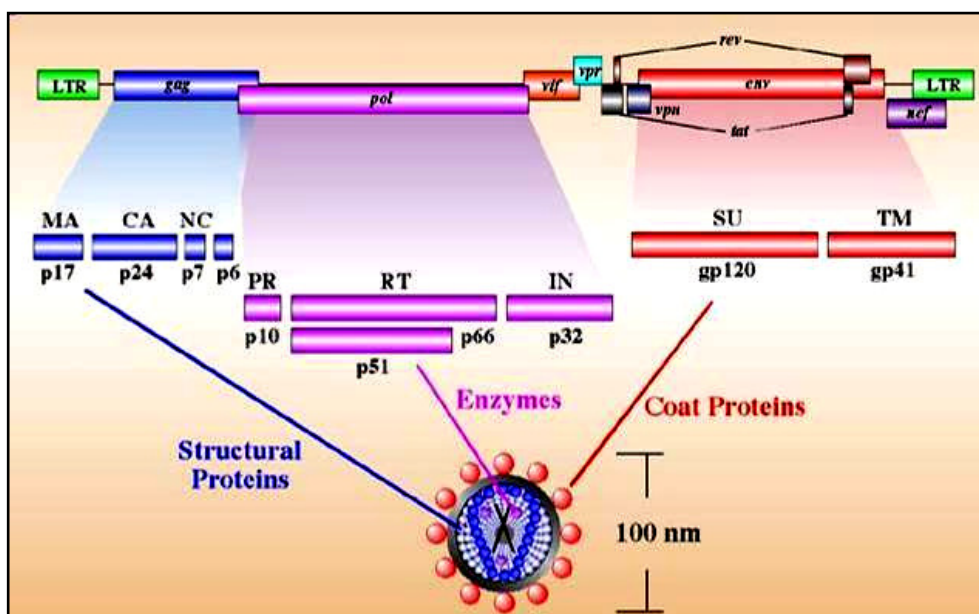


Figure 15: HIV genome organization.

HIV infection of cells begins when the envelope glycoprotein (Env) of a viral particle binds to both CD4 and a coreceptor that is a member of the chemokine receptor family.

The viral particles that initiate infection are usually in the blood, semen, or other body fluids of one individual and are introduced into another individual by sexual contact, needle stick, or transplacental passage.

Env is a complex composed of a transmembrane gp41 subunit and an external, noncovalently associated gp120 subunit. The Env complex is expressed as a trimeric structure of three gp120/gp41 pairs. This complex mediates a multistep process of fusion of the viral envelope with the membrane of the target cell (Fig. 16).

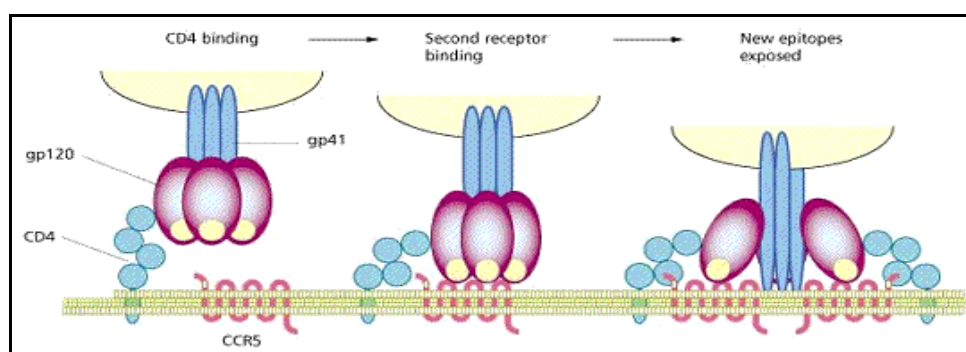


Figure 16: HIV entry mechanism in the host cell.

The first step of this process is the binding of gp120 subunits to CD4 molecules, which induces a conformational change that promotes secondary gp120 binding to a chemokine coreceptor. Coreceptor binding induces a conformational change in gp41 that exposes a hydrophobic region, called the fusion peptide, which inserts into the cell membrane and enables the viral membrane to fuse with the target cell membrane.

Once an HIV virion enters a cell, the nucleoprotein core of the virus becomes disrupted, the RNA genome of HIV is transcribed into a double-stranded DNA form by viral reverse transcriptase and the viral DNA enters the nucleus.

The viral integrase also enters the nucleus and catalyzes the integration of viral DNA into the host cell genome. The integrated HIV DNA is called the provirus and may remain transcriptionally inactive for months or years, with little or no production of new viral proteins or virions, and in this way HIV infection of an individual cell can be latent.

Transcription of the genes of the provirus is regulated by the LTRs upstream of the viral structural genes, and cytokines or other physiologic stimuli to T-cells and macrophages enhance viral gene transcription. The LTRs contain polyadenylation signal sequences, the TATA box promoter sequence and binding sites for two host cell transcription factors, NF- $\kappa$ B and SP1. Initiation of HIV gene transcription in T-cells is linked to activation of the T-cells by antigen or cytokines.

The Tat protein is required for HIV gene expression and acts by enhancing the production of complete viral mRNA transcripts. In fact, few if any HIV mRNA molecules are actually synthesized because transcription of HIV genes by mammalian RNA polymerase is inefficient and the polymerase complex usually stops before the mRNA is completed. Tat protein binds to the nascent mRNA and increases the "processivity" of RNA polymerase by several hundred-fold, which allows transcription to be completed to produce a functional viral mRNA.

The mRNAs encoding the various HIV proteins are derived from a single full-genome-length transcript by differential splicing events.

The Rev, Tat, and Nef proteins are early gene products encoded by fully spliced mRNAs that are exported from the nucleus and translated into proteins in the cytoplasm soon after infection of a cell.

Late genes include *env*, *gag*, and *pol*, which encode the structural components of the virus and are translated from singly spliced or un-spliced RNA. The Rev protein initiates the switch from early to late gene expression by promoting the export of these incompletely spliced late gene RNAs out of the nucleus.

The *pol* gene product is a precursor protein that is sequentially cleaved to form reverse transcriptase, protease, ribonuclease, and integrase enzymes. The *gag* gene encodes a 55-kD protein that is proteolytically cleaved by the viral protease into p24, p17 and p15 polypeptides, which are the core proteins required for assembly of infectious viral particles. The primary product of the *env* gene is a 160-kD glycoprotein (gp160) that is cleaved by cellular proteases within the endoplasmic reticulum into the gp120 and gp41 proteins required for HIV binding to cells.

Assembly of infectious viral particles then begins by packaging full-length RNA transcripts of the proviral genome within a nucleoprotein complex that includes the *gag* core proteins and the *pol*-encoded enzymes required for the next cycle of integration. This nucleoprotein complex is then enclosed within a membrane envelope and released from the cell by a process of budding from the plasma membrane.

The rate of virus production can reach sufficiently high levels to cause cell death. In addition, gp120 and gp41, which are expressed on the plasma membrane of infected cells before virus is released, can mediate cell-cell fusion with an uninfected cell that expresses CD4 and coreceptors, and HIV genomes can then be passed between the fused cells directly.

HIV is transmitted via mucosal surfaces. Major groups at risk for the development of AIDS include homosexual or bisexual persons, intravenous drug abusers, heterosexual partners of members of other risk groups and babies born of infected mothers. Health care workers have a small increased risk for infection.

Different isolates of HIV have distinct tropisms for different cell populations that are related to the specificity of gp120 variants for different chemokine receptors. All HIV strains can infect and replicate in freshly isolated human CD4<sup>+</sup> T-cells that are activated in vitro.

In contrast, some strains will infect primary cultures of human macrophages but not continuous T-cell lines (macrophage-tropic, or M-tropic, virus), whereas other strains will infect T-cell lines but not macrophages (T-tropic virus). Some virus strains also infect both T-cell lines and macrophages (dual-tropic virus). Macrophage-tropic virus isolates express a gp120 that binds to CCR5, which is expressed on macrophages (and some memory T-cells), whereas T cell-tropic viruses bind to CXCR4, which is expressed on T-cell lines. In many HIV-infected individuals, there is a change from the production of virus that uses CCR5 and is predominantly macrophage tropic early in the disease to virus that binds to CXCR4 and is T-cell line tropic late in the disease.

The T-tropic strains tend to be more virulent, presumably because they infect and deplete T-cells more than do M-tropic strains.

HIV disease begins with acute infection, which is only partly controlled by the adaptive immune response, and advances to chronic progressive infection of peripheral lymphoid tissues (Fig. 17).

Acute infection is characterized by infection of memory CD4<sup>+</sup>/CCR5<sup>+</sup> T-cells in mucosal lymphoid tissues, and death of many infected cells. Because the mucosal tissues are the largest reservoir of T cells in the body, and the major site of residence of memory T-cells, this local loss is reflected in considerable depletion of lymphocytes.

The transition from the acute phase to a chronic phase of infection is characterized by dissemination of the virus, viremia and the development of host immune responses.

DCs in epithelia at sites of virus entry capture the virus and then migrate into the LNs. DCs express a protein with a mannose-binding lectin domain that may be particularly important in binding the HIV envelope and transporting the virus.

Once in lymphoid tissues, DCs may pass HIV on to CD4<sup>+</sup> T-cells through direct cell-cell contact. Within days after the first exposure to HIV, viral replication can be detected in the LNs.

This replication leads to viremia, during which large numbers of HIV particles are present in the patient's blood, accompanied by an acute HIV syndrome that includes a variety of nonspecific signs and symptoms typical of many viral diseases. The viremia allows the virus to disseminate throughout the body and to infect helper T-cells, macrophages and DCs in peripheral lymphoid tissues.

As the HIV infection spreads, the adaptive immune system mounts both humoral and cell-mediated immune responses directed at viral antigens. These immune responses partially control the infection and viral production, and such control is reflected by a drop in viremia to low but detectable levels by approximately 12 weeks after the primary exposure.

In the next, chronic phase of the disease, LNs and the spleen are sites of continuous HIV replication and cell destruction. Although the majority of peripheral blood T-cells does not harbor the virus, destruction of CD4<sup>+</sup> T-cells within lymphoid tissues steadily progresses during the latent period, and the number of circulating blood CD4<sup>+</sup> T-cells steadily declines.

As the disease progresses, patients become susceptible to other infections, and immune responses to these infections may stimulate HIV production and accelerate the destruction of lymphoid tissues.

HIV disease progresses to the final lethal phase, called AIDS, when the blood CD4<sup>+</sup> T-cell count drops below 200 cells/mm<sup>3</sup>. HIV viremia may climb dramatically as viral replication in other reservoirs accelerates unchecked.

Patients with AIDS suffer from combinations of opportunistic infections, neoplasms, cachexia (HIV wasting syndrome), kidney failure (HIV nephropathy), and CNS degeneration (AIDS encephalopathy).

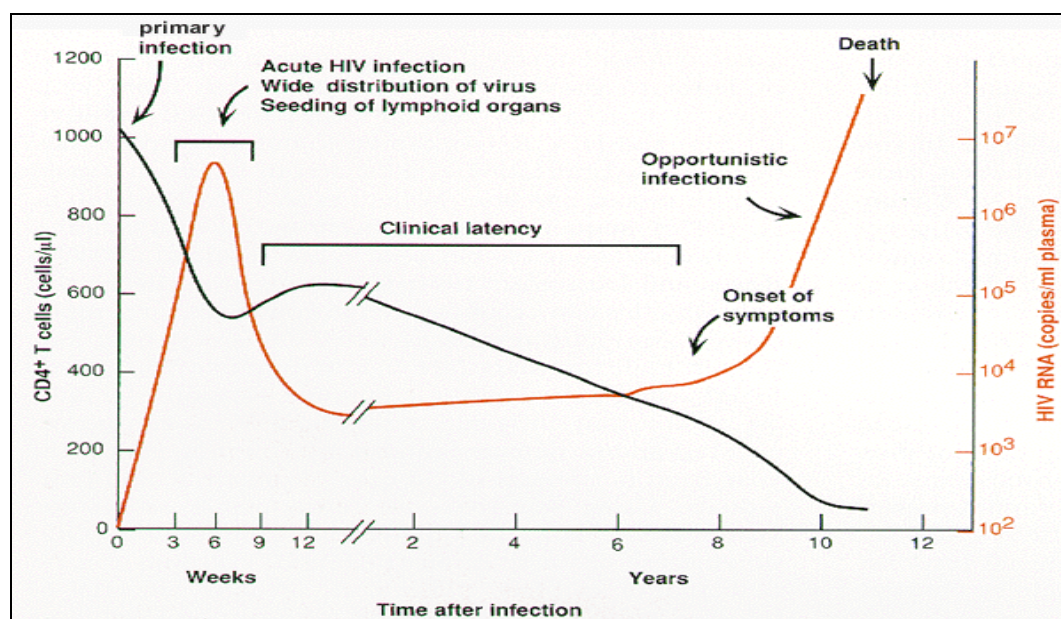


Figure 17: typical course of HIV infection.

Naïve T-cells are resistant to HIV infection because these cells contain the active form of the enzyme APOBEC3G, a cytidine deaminase that introduces cytosine to uracil mutations in the viral DNA produced by reverse transcription, inhibiting retroviral replication.

Activation of T-cells converts cellular APOBEC3G into an inactive complex. HIV has evolved to counteract this cellular defence mechanism through the viral protein Vif, which binds to APOBEC3G and promotes its degradation by cellular proteases

Macrophages express much lower levels of CD4 than helper T-cells do, but they do express CCR5 coreceptors and are susceptible to HIV infection. However, because macrophages can be infected but are relatively resistant to the cytopathic effects of HIV, they may become a reservoir for the virus.

DCs can also be infected by HIV but are not directly injured by HIV infection. However, these cells form intimate contact with naïve T-cells during the course of antigen presentation and may thus be an important pathway for T-cell injury.

FDCs in the germinal centres of LNs and the spleen trap large amounts of HIV on their surfaces, in part by Fc receptor-mediated binding of antibody-coated virus, and can infect macrophages and CD4<sup>+</sup> T-cells in the LNs.



HIV infection results in impaired function of both the adaptive and innate immune systems.

An important cause of the loss of CD4<sup>+</sup> T-cells in HIV-infected people is the direct cytopathic effect of infection of these cells by HIV. Mechanisms in addition to direct lysis of infected CD4<sup>+</sup> T-cells by virus have been proposed for the depletion and loss of function of these cells in HIV-infected individuals.

Chronic activation of the T-cells may predispose the cells to apoptosis and apoptotic death of activated lymphocytes may account for the observation that the loss of T-cells greatly exceeds the numbers of HIV-infected cells.

HIV-specific CTLs are present in many patients with AIDS, and these cells can kill infected CD4<sup>+</sup> T-cells.

In addition, antibodies against HIV envelope proteins may bind to HIV-infected CD4<sup>+</sup> T-cells and target the cells for ADCC.

Binding of gp120 to newly synthesized intracellular CD4 may interfere with normal protein processing in the endoplasmic reticulum and block cell surface expression of CD4, making the cells incapable of responding to antigenic stimulation.

HIV-specific humoral and cell-mediated immune responses develop following infection but generally provide limited protection, primarily because of the depletion and functional inhibition of the CD4<sup>+</sup> T-cells.

The initial adaptive immune response to HIV infection is characterized by expansion of CD8<sup>+</sup> T-cells specific for HIV peptides, which nevertheless prove ineffective because of the emergence of viral escape mutants (variants with mutated antigens).

Antibody responses to a variety of HIV antigens are detectable within 6 to 9 weeks after infection. The most immunogenic HIV molecules that elicit antibody responses appear to be the envelope glycoproteins, and high titers of anti-gp120 and anti-gp41 antibodies are present in most HIV-infected individuals. Other anti-HIV antibodies found frequently in patients' sera include antibodies to p24, reverse transcriptase, and *gag* and *pol* products. The early antibodies are not neutralizing, and are generally poor inhibitors of viral infectivity or cytopathic effects. Neutralizing antibodies against gp120 develop 2 to 3 months after primary infection, but even these antibodies cannot cope with a virus that is able to rapidly change the most immunodominant epitopes of its envelope glycoproteins.

The failure of cell-mediated and humoral immune responses to eradicate HIV infection is probably due to several factors. Notably HIV has an extremely high mutation rate because of error-prone reverse transcription, and in this way it may evade detection by antibodies or T-cells generated in response to viral proteins.

Active research efforts have been aimed at developing reagents that interfere with the viral life cycle. Treatment of HIV infection and AIDS now includes the administration of three classes of antiviral drugs, used in combination, that target viral molecules for which no human homologues exist.

The first type of drug to be widely used consists of nucleoside analogues that inhibit reverse-transcriptase activity. These drugs include deoxythymidine nucleoside analogues such as 3'-azido-3'-deoxythymidine (AZT), deoxycytidine nucleoside analogues, and deoxyadenosine analogues. When these drugs are used alone, they are often effective in significantly reducing plasma HIV RNA levels for several months to years. They usually do not halt progression of HIV-induced disease, largely because of the evolution of virus with mutated forms of reverse transcriptase that are resistant to the drugs.

More recently, viral protease inhibitors have been developed that block the processing of precursor proteins into mature viral capsid and core proteins. When these protease inhibitors are used alone, mutant viruses resistant to their effects rapidly emerge.

However, protease inhibitors are now being used in combination with two different reverse-transcriptase inhibitors. This new triple-drug therapy, commonly referred to as HAART (highly active antiretroviral therapy), has proved to be remarkably effective in reducing plasma viral RNA to undetectable levels in most treated patients for up to 3 years. An integrase inhibitor is also now used as part of anti-viral therapy.

Although anti-retroviral therapy has reduced viral titers to below detection for up to 10 years in some patients, it is unlikely that such treatment can eliminate the virus from all reservoirs, especially long-lived infected cells, and resistance to the drugs may ultimately develop. Other problems associated with these new drug therapies, which will impair their effective use in many parts of the world, include high expense, complicated administration schedules and serious side effects.

Although there have been numerous approaches for generating HIV vaccines in the past 20 years, to date there is no approved vaccine on the market.

Several vaccine strategies are currently in various validation stages in clinical trials, including recombinant DNA plasmids [84], DNA prime and recombinant modified vaccinia Ankara (MVA) boost vaccines [85], recombinant viral vectors such as the canarypox immunogens (ALVAC) [86], replication-incompetent adenovirus vectors [87], smaller synthetic peptide-based vaccines [88] and recombinant Tat protein vaccines [89].

Whole killed virus vaccines (Remune) have been tested with some success in mice [90]. Live-attenuated virus candidates have not been considered seriously for human trials because of safety concerns, especially for immunocompromised patients.

Until recently, the most prominent approach has been the introduction of exogenous HIV antigens of the viral envelope region to HIV-infected individuals to induce renewed virus-specific effector T-cell and neutralizing antibody responses.

However, the results from phase III trials have been disappointing [91]. Purified recombinant gp12 failed to protect against HIV-1 infection and also failed to generate neutralizing antibodies to diverse primary isolates, since any antibodies produced were highly isolate-specific. The high rates of viral replications, persistent expression and presentation of new HIV-1 antigens to the immune system (high variability of Env protein through rapid genetic mutations), T-cell hyperactivation and exhaustion, and clonal T-cell anergy are all attributed to these failures.

Given the overall failure to date of all subunit approaches, presenting the envelope in its native form on a virus-like platform appears to be intuitively more sensible.

There are several advantages of using VLPs as a HIV vaccine. First, the immune system responds well to particulate antigens that are the size of viruses. Second, the self-assembling core structures of many different viruses can be adapted by recombinant technology to contain or display one or more antigens of HIV (or SIV) or other immunostimulatory molecules. Third, these particles neither replicate nor contain the HIV genome, they cannot produce progeny viruses and thus avoid the formidable safety concerns associated with whole-inactivated and live-attenuated virus vaccines. Finally, less amount of vaccine can be administered compared with other subunit vaccines without compromising immune responses, thus reducing the cost. The use of VLPs, therefore, is an attractive approach for viral vaccine development.

### 3.3 Human papillomavirus (HPV)

HPVs are a very large family comprising more than 130 genotypes that have been cloned from various clinical lesions. These viruses are not classified as serotypes but as genotypes on the basis of DNA sequence [92]; because in vitro culture of these viruses is problematic, HPV infection is determined by detecting HPV-DNA.

The viruses have a predilection for either cutaneous or mucosal epithelial surfaces and fall into two groups, low-risk types that predominantly cause benign warts or high-risk types associated with malignant disease. This risk stratification is shown clearly in the genital tract where about 30 to 40 HPVs regularly or sporadically infect the mucosal epithelium of men and women.

The two most common low-risk viruses that cause warts on the anogenital mucosae are HPV-6 and 11. There are about 15 oncogenic or high-risk HPVs that infect the genital tract, but the two major players are HPV-16 and 18, which are responsible for 70% of cervical carcinomas worldwide [93].

The HPV genome can be divided into a coding and a non-coding region. The non-coding region, the LCR, contains the origin of viral DNA replication and the enhancer/promoter elements regulating the viral transcription. The coding region consists of ORFs and encodes the early (E) and late (L) proteins (Fig. 18).

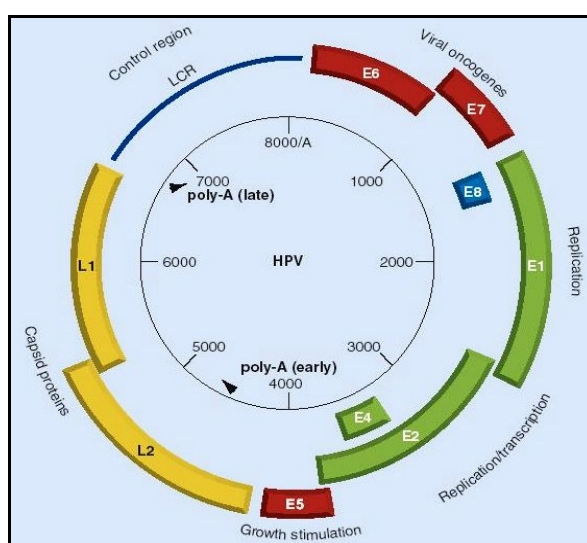


Figure 18: genomic organization of HPV.

The late genes encode structural proteins, the viral capsid proteins (L1 and L2) which self-assemble into the viral capsid interacting with a receptor of the target cell facilitating entry of the viral DNA (Fig. 19). The early proteins E1 and E2 are involved in regulation of viral transcription and DNA replication.

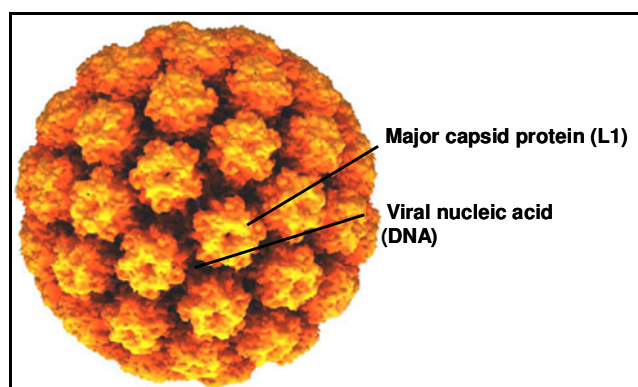


Figure 19: structure of Human Papillomavirus.

The E6 and E7 genes of high-risk HPV types encode for oncoproteins that can immortalize human keratinocytes [94]. This potential appears to be limited to high-risk types, because E6 and E7 from HPV-6 or HPV-11 are non-transforming.

E6 will bind, inactivate, and degrade the host's oncosuppressor protein p53, which results in loss of p53-induced apoptosis and G1 arrest of the cell cycle [95].

The p53 protein prevents cell growth and stimulates apoptosis in the presence of DNA damage. It causes BAX protein up-regulation, which blocks the anti-apoptotic effects of the mitochondrial BCL-2 protein. In addition, p53 also up-regulates the p21 protein, which blocks the formation of the Cyclin D/Cdk4 complex, thereby preventing the phosphorylation of retinoblastoma gene product (RB) and, in turn, halting cell cycle progression by preventing the activation of E2F. In short, p53 is a tumour suppressor gene that arrests the cell cycle when there is DNA damage.

E6 has a close relationship with the cellular protein E6-AP. E6-AP is involved in the ubiquitin ligase pathway, a system which acts to degrade proteins. E6-AP binds ubiquitin to the p53 protein, thereby flagging it for proteosomal degradation.

The binding of E7 to RB will lead to the transcriptional deregulation of cell-cycle control and results in uncontrolled cell proliferation [96]. When viral DNA integrates into the host genome (malignant transformation), it will cause successively the disruption of the E2 ORF, loss of E2 protein expression, the overexpression of E6 and E7, uncontrolled cell proliferation and in the end oncogenic transformation of the cell [97].

HPV genome is usually present in an episomal (circular and non-integrated) configuration in CIN, whereas in invasive cervical cancer the genome is commonly integrated into the host DNA [98]. HPV DNA integration appears to be the critical event in the development of cervical neoplasia, since HPV E6 and E7 are conserved intact and show persistent and increased expression in carcinomas.

From the evolutionary biological standpoint, HPVs are very successful infectious agents, as they induce chronic infections that have no systemic sequelae and rarely kill the host but periodically shed large amounts of infectious virus for transmission to naïve individuals [99].

Infection and vegetative HPV growth are absolutely dependent upon a complete program of keratinocyte differentiation (Fig. 20).

HPV penetrates the suprabasal cells in the cervical epithelium and tightly maintains a program of viral transcriptional repression of its late genes L1 and L2, which are potentially the most powerful immunogens that HPV synthesizes [100]. This repression allows escape from immune surveillance and recognition.

Viral gene expression is confined to the keratinocyte; there is no evidence that viral genes are expressed in any cell other than keratinocytes, and there is a spatial and temporal pattern of HPV gene expression in the infected epithelium.

The virus infects a subset of primitive basal cells, probably stem cells, at low copy number. Sometime after infection, there is a round of viral DNA replication that appears to be independent of the cell cycle and amplifies the viral copy number to around 50 to 100 copies per cell.

The infected cell is thought to then leave this primitive stem cell-like compartment and enter the transit amplifying, proliferative compartment of the epithelium. As HPV progresses through the layers of the epithelium, the replicative program of its genes changes in an orderly fashion.

When the infected keratinocyte enters the differentiating compartment, exiting the cell cycle, there is a massive up-regulation of viral gene expression and viral DNA replication occurs; there is amplification of viral copy number to at least 1,000 copies per cell, abundant expression of the early genes E6 and E7, and expression of the late genes from the late promoter [101].

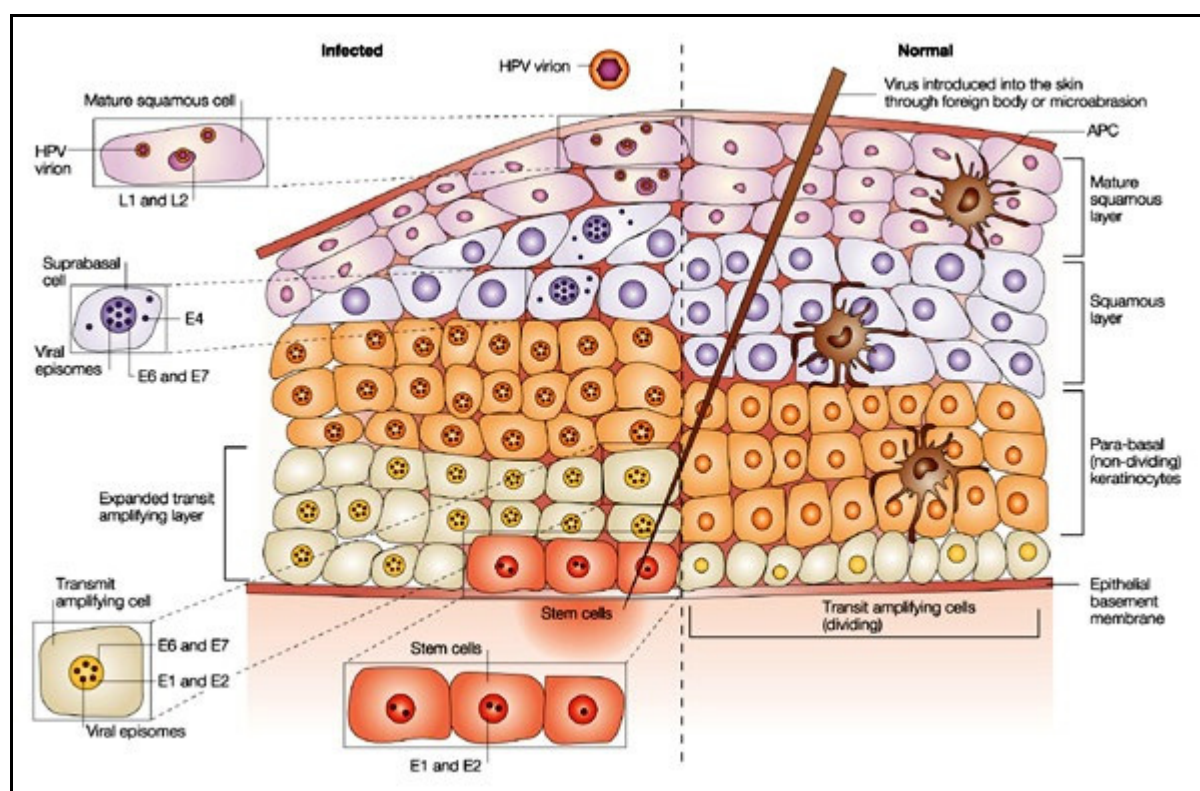


Figure 20: life cycle of Human papillomavirus.

It is important to recognize that these events occur in cells that are differentiating and have exited the cell cycle.

Papillomaviruses encode only one DNA replication enzyme, E1, and apart from this and the viral E2 protein, replication is totally dependent upon the cellular DNA synthetic machinery.

The challenge for the virus is that the cellular DNA polymerases and replication factors are only produced in mitotically active cells. To solve this problem, the viruses encode proteins that, in the context of the viral life cycle, reactivate cellular DNA synthesis in non-cycling cells, inhibit apoptosis, and delay the differentiation program of the infected keratinocyte, creating an environment permissive for viral DNA replication.

The precise details by which this is achieved are imperfectly understood, but the viral genes central to these functions are E6 and E7. Unfortunate but rare by-products of this role in high-risk HPV replication are the deregulation of growth control in the infected cell and the development of cancer [102].

In this infectious cycle, there is a replication strategy in which viral DNA replication and virus assembly occur in a cell that will terminally differentiate and die by natural causes. Thus, there is no viral-induced cytolysis or necrosis, and therefore no inflammation.

For most of the duration of the HPV infectious cycle, there is little or no release into the local milieu of proinflammatory cytokines, which is important for APC activation and migration. The central signals to kick start the immune responses in squamous epithelia are absent [103]. There is no blood-borne or viremic phase of the HPV life cycle, and only minimal amounts of replicating virus are exposed to immune defences; in effect, the virus is practically invisible to the host who remains ignorant of the pathogen for long periods of time.

HPV infections are exclusively intraepithelial. Theoretically, HPV attacks should be detected by the professional APCs of squamous epithelia, the LC, which is the intraepithelial DC. The activated LC should then migrate to the draining LN, processing HPV antigens and present antigen to naïve T-cells in the node. The T-cells should then differentiate into effector cells, migrate back to the infected site and destroy the infected keratinocytes.

Virus capsid entry is usually an activating signal for DCs, but there is evidence that LCs are not activated by the uptake of HPV capsids. LCs, when incubated with L1 VLPs of HPV-16, do not initiate epitope-specific immune responses against L1 derived antigens and, in effect, are tolerized by VLP uptake [104].

In contrast, stromal DCs are activated by VLPs and stimulate HPV-specific T-cells [105], but since the virus remains in the epithelium the probability of encountering stromal DCs is low, effectively disabling a key component of the immune response.

Even in the absence of viral-induced cytolysis and cell death, HPV infected keratinocytes should activate the production of type-1 interferons, a powerful, generic, antiviral and innate immune defence system. High-risk HPV infection downregulates IFN- $\alpha$  inducible gene expression and the HPV-16 E6 and E7 oncoproteins

directly interact with components of the interferon signalling pathways, therefore altering the expression of genes that enable host resistance to infection and immune function.

Despite the best efforts of the virus to evade host defences, most HPV infections resolve with time. Anogenital warts and low-grade intraepithelial lesions are cleared as a result of a successful cell-mediated immune response [106] directed against early HPV proteins, particularly E2 and E6.

In animal infections, this cell-mediated response is closely followed by seroconversion and antibodies to the major coat protein, L1 [107]. This is probably also true in humans [77]; however, the antibody concentrations achieved in animals and humans are low, and many women do not seroconvert [108].

This observation should be tempered by the recognition that the current methods of measuring antibody concentration are relatively insensitive with a low signal to noise ratio.

There is no viremia in natural infections. Furthermore, free virus particles are shed from the surface of squamous epithelia with poor access to vascular and lymphatic channels and to LNs where immune responses are initiated (Fig. 21).

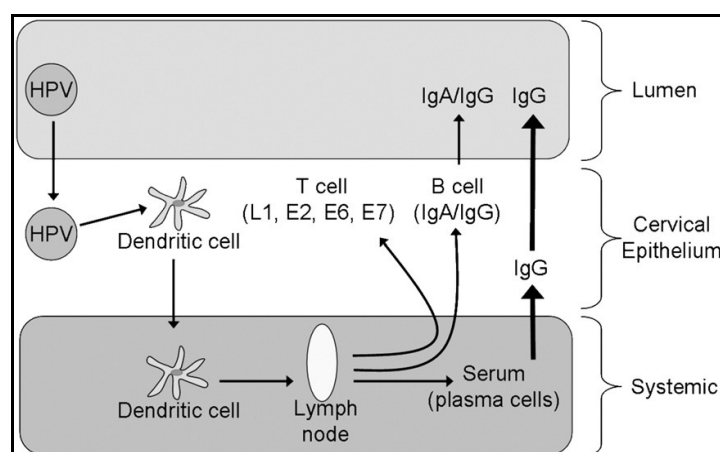


Figure 21: simplified scheme of immunological responses to HPV infection.

Although 80% to 90% of genital HPV infections resolve with time, about 10% to 20% of individuals do not become HPV-DNA negative and develop persistent infection. This group is at high risk for progression to high-grade cervical intraepithelial disease, CIN, a condition characterized by the expression of HPV E6 and E7 proteins in dividing cells, chromosomal instability and the progressive ability to resist both innate and adaptive antiviral immune defences.

Integration of HPV-DNA into the host chromosome is a well recognized event that has occurred in a high proportion of cervical carcinomas [71,72]. T-cell responses to E2 and E6 are lost or reduced in CIN and invasive carcinoma [109]. Even if HPV antigen-specific cytotoxic T-cells have been generated, regulatory T-cells increasingly dominate the lesions and abrogate the killer defence response [110].

The challenge for therapeutic vaccines for HPV-associated disease is in fact to reverse this immunologically suppressive microenvironment and allow the cytotoxic killers to access the infected and neoplastic cells.

Two HPV L1 VLP vaccines have been developed; Cervarix®, a bivalent HPV-16, -18 VLP vaccine from GlaxoSmithKline and Gardasil® also known as Silgard, a quadrivalent HPV-16/18/6/11 vaccine from Merck Vaccines.

These products are to be delivered in a 3 shot immunisation schedule and induce, at their peak after the third immunisation at 6 months, high concentrations of neutralizing antibodies to L1 and virtually all subjects in the vaccine trials have seroconverted [111,112]. These vaccines are delivered intramuscularly, resulting in rapid access of antigen to the local LNs, thus circumventing the immune avoidance strategies of the viral intraepithelial infectious cycle.

The currently available vaccines have been shown to be highly efficacious in the various Phase II and Phase III randomized control trials achieving over a 5 year period 100 per cent protection against HPV-16/18 caused high grade CIN in 15-26 year old women naïve for HPV-16 and/or -18 at trial entry [113,114].

Currently, the best assumption is that the mechanism of protection elicited by VLPs is serum antibodies. The most unequivocal evidence for this notion comes from experiments in rabbits and dogs [115,116] in which it was shown that naïve animals passively immunized with purified serum IgG from either VLP immunized or naturally infected animals were completely protected against high viral challenge.

The mechanism by which neutralizing antibodies to HPV prevent viral entry is at present speculative. However, new data on virus entry to cells suggest different stages at which neutralizing antibodies could be effective.

Recent studies have shown that HPV infection requires a micro-abrasion of the squamous epithelium that results in epithelial denudation but retention of the epithelial basement membrane [117]. HPV initially binds by a primary receptor to this exposed basement membrane before entering the keratinocyte, presumably as the keratinocyte migrates along the basement membrane to repair the small wound. This is a protracted process extending over 24 to 48 h, during which it is speculated the virus capsid undergoes conformational changes that expose the secondary receptor by which the virus binds to and enters the keratinocyte. Virus neutralizing antibodies could act therefore, by binding to the receptors or by binding to the capsid and preventing the conformational distortion essential for successful viral entry. Sera from naturally infected individuals or from vaccinated women show no or only a limited response to other HPV types. Thus, despite the good performance of the current vaccines, the development of second-generation products combined with very potent novel adjuvants is mandatory. Furthermore available vaccines do not exhibit any therapeutic activity. This means that women with a persisting infection are not protected against the development of the resulting lesions, although they may benefit in the long term from a possible novel infection with the same or of the other vaccine types. Therefore the development of a therapeutic vaccine is a high priority.

## 4. VIRUS-LIKE PARTICLES

### 4.1 General characteristic of VLPs

The structural proteins of many viruses have the ability to assemble into repeated arrays, or VLPs, following recombinant DNA expression in a variety of culture systems. Such VLPs fall in the general size range of viruses (22–150nm), with their exact size and morphology depending on the particular viral proteins incorporated, but VLPs are non-infectious because they assemble without incorporating genetic material [118].

In some cases, VLPs are similar to naturally occurring subviral particles (SVPs). For example, expression of the small envelope protein of HBV in yeast or mammalian cells leads to the formation of 22 nm VLPs that are essentially identical to the SVPs that are a natural product of HBV infection, found in patient blood at levels far greater than the virion itself. Notably, these plasma-derived SVPs provided the first-generation HBV vaccines.

In other cases, VLPs can be exploited as “platforms” for the presentation of foreign epitopes and/or targeting molecules on chimeric VLPs. This can be achieved through modification of the VLP gene sequence(s), such that fusion proteins of VLP proteins and foreign vaccine proteins are assembled into VLPs during *de novo* synthesis. Fusion of peptide sequences with the core gene (HBcAg) of hepatitis B virus provided an early example of this approach.

Alternatively, foreign vaccine proteins may be chemically conjugated to pre-formed VLPs. As one example, this approach has been used in the production of HBcAg VLPs containing the extracellular domain of the M2 ion channel protein of Influenza A virus.

Different viruses provide a variety of building blocks for the production of VLPs. For non-enveloped viruses (such as HPV), or core particles of enveloped viruses (such as HBcAg, Influenza virus and HIV), one or more chimeric capsid proteins are expressed for self-assembly. Multiple capsid proteins may be assembled either from expression and subsequent processing of a precursor protein or by co-expression of the capsid proteins from bicistronic or multicistronic vectors in the same cell.

For enveloped viruses, VLPs are assembled from envelope proteins with or without the matrix/capsid proteins that form the authentic viral particle. In either case, the envelope proteins bud from the usual cellular compartments, ER, plasma membrane or lipid rafts associated with the plasma membrane, and thus contain the cellular lipids that make up the viral lipoprotein envelope. In some instances the VLPs may also include host cell proteins, for example, CD55, CD59 and CD46 in HIV, which help evade complement lysis [119].

Among non-enveloped VLPs, HPV VLPs are most thoroughly studied, and the expression of the major capsid protein L1 leads to assembly of pentameric capsomeres, 72 of which form a VLP indistinguishable from authentic viral particles to the immune system [120].

VLPs produced in yeast or insect cells were highly immunogenic resulting in successful clinical trials for preventing HPV infection and FDA approval for human use [121,122,123,124].

Although clinical trials have demonstrated the potency of HPV VLPs in inducing immunity and in preventing persistent infection [38,39], due to the variability of the L1 major capsid protein, current HPV VLP vaccines can only offer genotype-specific protection against infection with papillomavirus in animals and humans. Nonetheless, the production of chimeric VLPs constructing by fusing HPV16 E7 sequences into the C-terminus of the viral L1 gene is going to constitute the first generation of both preventive and therapeutic vaccines currently under development [125].

VLPs from viruses with lipid envelopes represent more difficult challenges because of the complexity of the particle budding process for incorporating biologically active glycoproteins.

The first human vaccine manufactured using recombinant DNA technology was the enveloped HBV vaccine produced in yeast, which has been used for over a decade [126].

Enveloped influenza VLPs have been developed by several laboratories and were demonstrated to induce protective immunity in animal studies [81]. For influenza viruses, VLPs are assembled in producer cells and released into culture medium mimicking the viral budding process, which incorporates viral glycoproteins HA and NA on their surfaces in a native conformation, unmodified by fixatives or chemicals for inactivation.

Many recent studies have provided pre-clinical evidence that influenza VLPs can be developed as promising vaccines against both seasonal influenza and pandemic influenza viruses. In addition to the desirable safety features of non-replicating, non-infectious VLPs, influenza VLPs may provide a broader range of protection against antigenic variants of the virus. Also, they are likely to induce cellular immune responses known to play a contributing role in broadening protection, which will be especially beneficial in high-risk groups such as the elderly population [81].

HIV presents another example where substantial efforts have been made to recreate the virion envelope in a form that permits the efficient induction of broadly neutralising antibodies [127]. For HIV, VLPs are based on Gag precursor polypeptide (Gagpr55) and must contain Gagp24 for particle assembly, budding and release from the host cell. Each particle is enveloped by a lipid bilayer derived from the host cell. Multiple combinations of viral proteins may be used to generate HIV VLPs. Furthermore, VLPs can contain different

forms of the ENV protein, including unprocessed Env (Env<sub>gp160</sub>), processed Env, truncated Env or cleavage-defective Env.

HIV VLPs have been demonstrated to be safe to administer to animals and human patients as well as being potent and efficient stimulators of cellular and humoral immune responses [128]. Therefore, VLPs are being considered as possible HIV vaccines. Chimeric HIV-1 VLPs constructed with either HIV or SIV capsid protein plus HIV immune epitopes and immunostimulatory molecules have further improved on early VLP designs, leading to enhanced immune stimulation [118].

There are many expression systems for the production of VLPs and these include: various mammalian cell lines, either transiently or stably transfected or transduced with viral expression vectors, the baculovirus/insect cell system, various species of yeast including *Saccharomyces cerevisiae* and *Pichia pastoris*, and *Escherichia coli* and other bacteria.

Ease of expression, ability to scale-up and cost of production have made yeast a popular choice, however considerations such as appropriate protein glycosylation and correct folding and assembly as well as codon optimisation may dictate alternative production systems.

*E. coli* does not allow for glycosylation, while yeast and baculovirus are limited to high mannose glycoprotein modification, and this is sometimes inconsistent.

Baculovirus-driven expression of Influenza VLPs has provided the novel challenge of separating the progeny baculovirus vector particles from the Influenza VLPs, with both having a similar size range of 80–120nm. Mammalian cell culture systems are favoured for appropriate modifications and authentic assembly, but are a less controllable system and more costly for production.

Retroviruses in particular also tend to include unwanted host cell membrane proteins in their envelope during assembly.

Future directions in manufacturing may include approaches such as *in vitro* chemical self-assembly of VLPs based, in the first instance, on capsid components [129].

## **4.2 VLPs and the innate and adaptive immune responses**

VLPs are commonly more immunogenic than subunit or recombinant protein immunogens, and are able to stimulate both the humoral and cellular arms of the immune system.

VLPs provide the spatial structure for display of conformational epitopes and in doing so are most likely to mimic the native virus structure, thereby enhancing the production of neutralizing antibodies. This may be especially true for surface proteins of enveloped viruses, where enveloped VLPs allow the vaccine proteins to be presented in their natural state as membrane-bound proteins, rather than as the soluble ectodomains alone.

VLPs may therefore offer a safe and effective approach for the induction of antibody to surface proteins where soluble forms of the proteins have failed to be effective.

In some cases, VLPs do not appear to require the use of adjuvants to achieve potent immune stimulation. The self-adjuvanting effects of such VLPs are inherent in their tendency to be a suitable size for uptake by DCs for processing and presentation by MHC class II and for directly promoting DC maturation and migration, essential for stimulation of the innate immune response [130].

Exogenous VLPs can also be taken up and processed via the MHC class I pathway (cross-presentation) for activation of CD8<sup>+</sup> T-cells, which are essential for the clearance of intracellular pathogens such as viruses. The ability of VLPs to target DCs is an important advantage of VLP vaccines, as targeting of this cell type is now understood to be essential for activating the innate and adaptive immune responses.

Some VLPs that resemble infectious viruses and retain their receptor binding regions are able to target and enter cells via their normal receptor and are taken up by antigen presenting cells as exogenous antigens for class I presentation.

Stimulation of DCs to produce cytokines, such as IFN- $\alpha/\beta$ , does not require replication of the virus but rather an intact envelope of a VLP, as shown for Influenza virus and HIV [131], or an intact non-enveloped VLP, such as HPV [132]. In this context VLPs may have advantages over the cognate live viruses for immune activation, because several viruses that replicate in DCs are known to block activation and maturation of the cell through expression of particular viral proteins [109].

## **4.3 Tailoring VLPs for vaccine development**

VLPs have been produced from the envelope or capsid components of a wide variety of viruses for the purpose of studying viral assembly and for the development of vaccines.

While HBV- and HPV- VLPs have made successful vaccines, pathogens that directly affect immune cells and those that successfully evade the immune system, such as HIV-1 and HCV have proven to be more challenging.



It is clear that many individuals are exposed to large amounts of these authentic viral particles for years during chronic infection without developing a fully effective immune response, which suggests that VLPs may also prove to be ineffective even when they closely mimic the native viral structure.

Some candidates may thus require adaptations in VLP design (such as particle size, envelope structure), targeting in the host (DCs, mucosal surfaces) and route of administration to achieve the desired immune response.

Chimeric VLPs provide a means for the incorporation of heterologous antigens into VLPs, including antigens that are unable to self-assemble in a particulate form (such as CTL epitopes and fragments of envelope proteins), and antigens from viruses where the intact virus particle may not have optimal immunogenicity (such as HIV and HCV).

Chimeric VLPs may consist of a homologous VLP platform assembled with the fusion of epitopes from other or multiple strains of the virus or additional epitopes to the core VLP structure, such as fusion of the E6/E7 non-structural proteins to the L1 capsid protein of HPV [117]. Alternatively, chimeric VLPs may consist of a VLP platform for presentation of polyproteins or epitopes of a totally unrelated virus or pathogen (e.g. HBV core VLPs with Influenza M2) [133].

Tailoring of VLPs may also be influenced by whether the vaccine is to act as a prophylactic or therapeutic vaccine, with the latter thought to require the inclusion of specific T-cell epitopes for CTL based clearance or as in the case of HPV the oncogenic E6/E7 proteins [134].

Other ways of utilising VLPs as a delivery platform is through chemical cross-linking of peptide epitopes to reactive sites on the platform. This has been done by coupling peptides containing a free cysteine residue with lysines situated in the immunodominant exposed region on HBV core particles [135].

Chimeric VLPs offer enormous potential in selective, multi-epitope presentation but their success will be dependent on a clear understanding of the correlates of immune clearance or protection, including the selection of the most relevant epitopes for vaccine efficacy. Unfortunately, this knowledge is lacking for many diseases.

## 5. VACCINES FOR MUCOSAL IMMUNITY AGAINST INFECTIOUS DISEASES

### 5.1 Challenges in mucosal vaccine development

A large number of mucosal infections continue to represent a challenge for the development of vaccines that either can prevent the pathogen from colonizing the surface epithelium (non-invasive bacteria), penetrate the surface barrier and replicate within the body (invasive bacteria and viruses), or block the binding of microbial toxins and neutralize them. In most cases it would seem desirable to induce specific SIgA antibodies associated with immunological memory, in addition to systemic immunity [136].

Mucosal immune responses are most efficiently induced by the administration of vaccines onto mucosal surfaces, whereas injected vaccines are generally poor inducers of mucosal immunity and are therefore less effective against infection at mucosal surfaces.

Nevertheless, clinical vaccine research has been based largely on injection of antigens, and most vaccines in use today are administered intramuscularly or subcutaneously. This is understandable because an injection delivers a known quantity of antigen into the body and results in the generation of specific antibodies and lymphoid cells that are readily measured in blood samples.

By contrast, our understanding of mucosal immunity and development of mucosal vaccines has lagged behind, in part because administration of mucosal vaccines and measurement of mucosal immune responses are more complicated. The dose of mucosal vaccine that actually enters the body cannot be accurately measured because antibodies in mucosal secretions are difficult to capture and quantitate, and recovery and functional testing of mucosal T-cells is labour intensive and technically challenging.

As a result, only a few mucosal vaccines have been approved for human use in the United States or elsewhere. These include oral vaccines against poliovirus, *Salmonella typhi*, *V. cholerae* and rotavirus, and a nasal vaccine against influenza virus.

Mucosal vaccines that are given orally or deposited directly on mucosal surfaces face the same gauntlet of host defences as do microbial pathogens: they are diluted in mucosal secretions, captured in mucus gels, attacked by proteases and nucleases, and excluded by epithelial barriers. So, relatively large doses of vaccine are required and it is impossible to determine exactly what dose actually crosses the mucosa. Soluble, non-adherent antigens are taken up at low levels, if at all, and in the intestine, such antigens generally induce immune tolerance.

In general, mucosal vaccines are likely to be most effective when they mimic successful mucosal pathogens in key respects: they would ideally be multimeric and/or particulate, adhere to mucosal surfaces (or even better, adhere selectively to M cells), efficiently stimulate innate responses, and evoke adaptive immune responses that are appropriate for the target pathogen.

The effectiveness of live pathogens as mucosal vaccines and vaccine vectors is partly a result of their adaptation to survive in luminal environments and to efficiently invade organized mucosal lymphoid tissues. The efficiency of non-living mucosal vaccines is unlikely to be comparable to these successful invaders, but uptake into the mucosa need to be significantly increased.

Particulate vaccines have several theoretical advantages for mucosal delivery. M cells are particularly accessible to microparticles and actively transport them into PPs. Particulate vaccines that enter mucosal inductive sites have the additional advantage of being readily taken up by mucosal DCs and providing antigen depots.

Virus-like particles and small vesicles derived from bacterial outer-membrane components are particularly promising as mucosal vaccines because their size is appropriate for uptake by M cells and DCs, their surface structures mimic those of mucosal pathogens, and they can activate an innate immune response. In practice, however, large amounts are needed for mucosal immunization because microparticles tend to be trapped in mucus and only a small fraction of the administered dose is likely to enter mucosal inductive sites. Non-living macromolecules, protein-subunit antigens and non-microbial particles generally evoke weak or undetectable adaptive immune responses when applied mucosally.

To be distinguished from harmless substances and nutrients, mucosal vaccines must raise alarms in the mucosa by including substances that activate innate signaling pathways in epithelial cells and/or in the underlying APCs. Thus, the superiority of live attenuated pathogens as mucosal vaccines and vaccine vectors is due in part to their ability to activate multiple innate responses. Nevertheless, live vaccines present safety and acceptability issues that might reflect innate immune responses and inflammation.

Ideally, vaccination at a single site would provide both humoral and cell-mediated protection, not only at the relevant mucosal surface, but also throughout the body.

In this regard, nasal vaccination has shown particular potential. In mice, monkeys and humans, nasal administration of vaccines has induced specific mucosal IgA antibody responses in the salivary glands, upper and lower respiratory tracts, male and female genital tracts, and the small and large intestines [137,138,139]. The nasal route can also induce CTLs in distant mucosal tissues including the female genital tract [140]. In addition, nasal immunization studies in humans and mice produced greater systemic antibody

responses than other mucosal immunization routes [136], presumably because antigens or APCs were readily trafficked to draining lymph nodes from this site.

In mice and monkeys, nasal immunization with certain live viral vectors generated systemic antiviral CTLs and IgG at concentrations that were comparable to those induced by parenteral vaccination routes [141]. However, rectal immunization of mice with a non-living peptide-based vaccine was more effective than nasal or oral routes at inducing systemic CTLs [142].

Taken together, the evidence suggests that the choice of mucosal vaccination route requires consideration of the species, the nature of the vaccine and the expected site of challenge.

Although nasal immunization might be particularly effective for protection against respiratory pathogens, optimal protection of the gastrointestinal tract, the rectum and female genital tract might still require oral, rectal or vaginal vaccines. However, the response to vaginal vaccines might be affected by the stage of the menstrual cycle during which immunization is carried out.

For many pathogens, optimal protection is likely to require both mucosal and systemic immune effectors, and the most effective mucosal vaccine strategies might be prime–boost combinations that involve both mucosal and systemic delivery. In addition, mucosal adjuvants that target specific immune responses are required.

## 5.2 Adjuvants

Adjuvants are molecules, compounds or macromolecular complexes that boost the potency and longevity of specific immune response to antigens, but cause minimal toxicity or long lasting immune effects on their own [143]. The addition of adjuvants to vaccines enhances, sustains and directs the immunogenicity of antigens, effectively modulating appropriate immune responses, reducing the amount of antigen or number of immunizations required and improving the efficacy of vaccines in newborns, elderly or immunocompromised individuals. Adjuvants have limited or no efficacy unless properly formulated, therefore both adjuvant components and formulation are crucial for enhancing vaccine potency.

Traditional live vaccines based on attenuated pathogens typically do not require the addition of adjuvants. Likewise, vaccines based on inactivated viruses or bacteria are often sufficiently immunogenic without added adjuvants, although some of these (e.g. HAV or whole cell Pertussis) can be formulated with adjuvants to further enhance the immune responses.

By contrast, protein-based vaccines, although offering considerable advantages over traditional vaccines in terms of safety and cost of production, in most cases have limited immunogenicity and require the addition of adjuvants to induce a protective and long-lasting immune response.

Recent advances have begun to shed light on the cellular and molecular nature of innate immunity and adjuvant activity. The immune system recognizes PAMPs by means of PRRs, which include the TLRs [4], C-type lectin-like receptors, cytosolic nucleotide oligomerization domain-like receptors and retinoic acid inducible gene-based-I-like receptors. These receptors bind microbial ligands (including cell wall components, lipoproteins, proteins, lipopolysaccharides, DNA and RNA of bacteria, viruses, protozoa and fungi) to trigger different types of immune responses [144]. These PAMPs, specifically those binding the TLRs, are the basis of many adjuvants. In addition, cytokines, bacterial toxins and glycolipids that alter antigen processing are being used in adjuvants to elicit immune responses.

Effective adjuvants and adjuvant formulations utilize multiple compounds and mechanisms to achieve the desired immunological enhancement, including generation of long lasting antigen depots, increased immunological presentation of vaccine antigens by DCs activated through the engagement of PRR and induction of CTL responses and/or CD4<sup>+</sup> T-helper lymphocyte responses (Th1 or Th2).

Two classes of adjuvants commonly found in modern vaccines include, as shown in Figure 22:

- immunostimulants that directly act on the immune system to increase responses to antigens (TLR ligands, cytokines, saponins and bacterial exotoxins);
- vehicles that present vaccine antigens to the immune system in an optimal manner, including controlled release and depot delivery systems to increase the specific immune response to the antigen (mineral salts, emulsions, liposomes, virosomes, biodegradable polymer microspheres and so-called immune stimulating complexes, such as ISCOM and ISCOMATRIX<sup>TM</sup>).

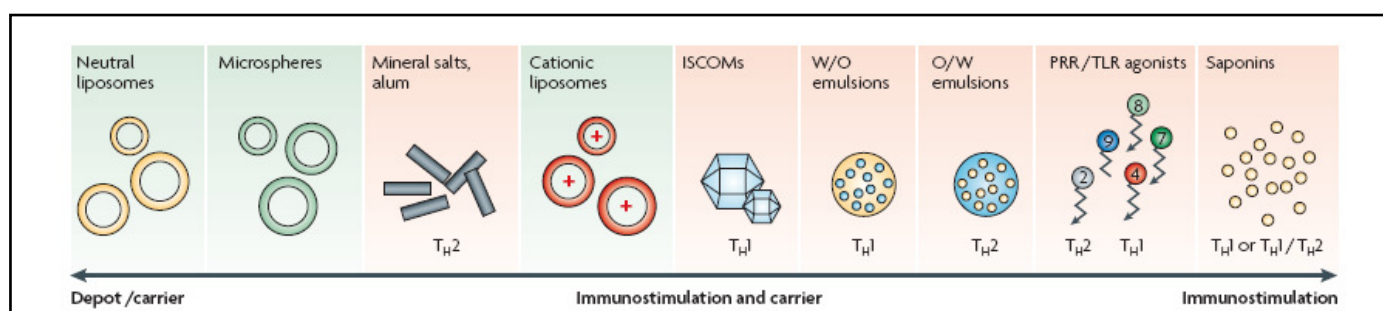


Figure 22: the main types of adjuvants with respect to their depot/carrier and immunostimulatory properties.

Adjuvants must be appropriately formulated for stability and maximum effect. Criteria involved in selecting the formulation for a given vaccine include the nature of the antigenic components, type of immune response desired, preferred route of delivery, avoidance of considerable adverse effects and stability of the vaccine. The optimally formulated adjuvant will be safe, stable before administration, readily biodegraded and eliminated, able to promote an antigen specific immune response and inexpensive to produce.

Furthermore, the ideally formulated adjuvant will be well-defined chemically and physically to facilitate quality control that will ensure reproducible manufacturing and activity.

Formulation should also be used to complement the inherent immunogenicity of vaccine antigens. For example, small soluble monomeric proteins, such as HIV gp120, tend to be poorly immunogenic compared to multimeric proteins that form VLPs. To enhance the immunogenicity of monomeric proteins, a formulation that renders it multimeric (e.g. incorporation into virosomes) might be most appropriate.

For multimeric proteins, virosome formulation might not be appropriate; instead adsorption of the protein to mineral salts might enhance its immunogenicity and stability [112]. What seems to be important is the size of the particles themselves (20-100 nm range) and the way they interact with and activate DCs. Hence, virus-sized particles could, by their size alone, act as a form of PAMP to stimulate both cellular and humoral immunity [145].

### 5.2.1 Adjuvants approved for human vaccines

Adjuvants in approved human vaccines include Alum, MF59TM (an oil-in-water emulsion), MPL1 (a glycolipid), VLP, Immunopotentiating Reconstituted Influenza Virosomes (IRIV) and CT.

Aluminum salt based adjuvants, referred to generically as 'Alum', are non-crystalline gels based on aluminum oxyhydroxide, aluminum hydroxyphosphate or various proprietary salts such as aluminum hydroxy-sulfate. These adjuvants are components of several licensed human vaccines, including Diphtheria-Pertussis-Tetanus, DT, DT combined with HBV, Haemophilus influenza B or inactivated polio virus, HAV, Streptococcus pneumonia, meningococcal and HPV. Formulation is achieved through adsorption of antigens onto highly charged aluminum particles. The mechanisms of action of the aluminum salts frequently cited include: depot formation facilitating continuous antigen release; particulate structure formation promoting antigen phagocytosis by APCs such as DCs, macrophages and B-cells and, induction of inflammation resulting in recruitment and activation of macrophages, and increased MHC class II expression and antigen presentation. The advantages of aluminum adjuvants include their safety record, induction of Th2 cell-responses, augmentation of antibody responses, antigen stabilization and relatively simple formulation for large-scale production. The major limitations of aluminum adjuvants include their inability to elicit cell-mediated Th1 or CTL responses that are required to control most intracellular pathogens.

MF59TM consists of an oil (squalene)-in-water nano-emulsion composed of <250 nm droplets [30] which is used in Europe as an adjuvant in influenza vaccines [146]. Overall, MF59TM has an acceptable safety profile, and with several antigens it generates higher antibody titers with more balanced IgG1: IgG2a responses than those obtained with Alum [147]. In the clinic, strong Th2 cell-responses were also observed as a result of vaccination [36]. MF59TM is believed to act through a depot effect and direct stimulation of cytokine and chemokine production by monocytes, macrophages and granulocytes. MF59TM does not induce increased CD4<sup>+</sup> Th1 immune responses, but because of its ability to increase the levels of functional haemagglutination inhibiting antibodies and CD8<sup>+</sup> T-cell responses, it has the potential for use in pandemic influenza vaccines [148].

MPL1 is a non-toxic derivative of the LPS of Salmonella minnesota and is a potent stimulator of Th1 responses. LPS consists of two basic structures: a hydrophilic polysaccharide portion and a hydrophobic lipid moiety (called lipid A) The lipid A portion is thought to be responsible for most of the endotoxic activity of LPS, whereas the polysaccharide portion enhances solubility. Lipid A from S. Minnesota is highly endotoxic but this can be reduced by defined structural modifications such as the removal of specific phosphate groups or varying the number and length of its acyl chains. AS04 is an aqueous formulation of MPL1 and Alum, resulting in higher levels of specific antibody and efficacy with fewer injections. AS04 is being assessed in clinical trials evaluating vaccines against HAV and HPV [149]. Furthermore, MPL1 is licensed in Europe for allergy treatment because of its ability to down-modulate Th2 responses to allergens.

IRIVs are proteoliposomes composed of phospholipids, influenza HA and a selected target antigen that are delivered to APCs that take up the Virosomes by HA receptor-mediated endocytosis. IRIV is registered as a component of the HAV vaccine in Europe, Asia and South America. Both types of particles are taken up by APCs by receptor-mediated endocytosis, and have been shown to stimulate cellular and humoral immune responses [150].

CTB is used to enhance mucosal immune responses of orally delivered vaccines. The naturally occurring CT belongs to the AB class of bacterial toxins. It consists of a pentameric B oligomer that binds to GM-1 receptors (e.g. on the surface of intestinal epithelial cells) and an enzymatically active A subunit that is responsible for the toxicity. The recombinant CTB (rCTB) consists only of the non-toxic B component of the cholera enterotoxin. The rCTB molecule consists of five identical monomers tightly linked into a trypsin-resistant pentameric ring-like structure. CTB can act as a mucosal adjuvant and enhance IgA levels to coadministered or coupled antigens intranasally [151]. CTB is used to enhance the immune response in a

licensed whole-cell orally delivered cholera vaccine. This vaccine has been shown to induce a high level of protection against cholera, but was short-lived [152,153].

### 5.2.2 Adjuvants in development

The development of additional adjuvants has been driven principally by the shortcomings of aluminum adjuvants (failure to stimulate T-cell responses, including CTL, loss of potency if frozen and causing granulomas at injection sites).

Montanides (ISA51 and ISA720) are water-in-oil emulsions containing mannide-mono-oleate as an emulsifier. Montanides have been developed in response to safety concerns with IFA in animal studies [154]. Montanides have been used in malaria, HIV and cancer vaccine trials [155]. They induce a strong immune response and are available without requiring a license or contractual agreement. A drawback of Montanides is that they are difficult to formulate because an extensive and costly emulsification procedure is required for each antigen. In several studies, they have produced unacceptable local reactions.

Saponins (Quil-A, ISCOM, QS-21) (also included in AS02 and AS01) are triterpene glycosides isolated from plants. The most widely used in adjuvant research is Quil-A and its derivatives, extracted from the bark of the *Quillaja saponaria* tree [58]. Quil-A is composed of a heterogeneous mixture of triterpene glycosides that vary in their adjuvant activity and toxicity. Saponins have been widely used as an adjuvant in veterinary vaccines.

Partially purified fractions of Quil-A have also been used in immunostimulating complexes (ISCOM) composed of antigen, phospholipids, cholesterol and Quil-A fractions. ISCOMs are 40 nm cage-like particles trapping the protein antigen through hydrophobic interactions, whereas ISCOMATRIX™ [59], pre-formed antigen-free particles, provides for more general applications by later accommodating non-hydrophobic antigens. Because of their particulate nature, ISCOMs are directly targeted to and more efficiently taken up by APC via endocytosis. Saponin-mediated targeting of DEC-205, a macrophage mannose receptor family of c-type lectin endocytic receptors, on the surface of DC might account for higher uptake and more efficient presentation of antigens to T-cells [156]. Antigen processing can occur in the endosome for both MHC class II and class I presentation, possibly by the recently described cross-presentation pathway [157]. ISCOMs have been shown to elicit high titer long-lasting antibodies and strong helper and CTL responses in different models [158].

QS-21 is a purified component of Quil-A that demonstrates low toxicity and maximum adjuvant activity. In a variety of animal models, QS-21 has augmented the immunogenicity of protein, glycoprotein and polysaccharide antigens [159]. QS-21 has been shown to stimulate both humoral and cell-mediated Th1 and CTL responses to subunit antigens [160]. Clinical trials are in progress with QS-21, alone or in combination with carriers and other immunostimulants for vaccines against infections including influenza, HSV, HIV, HBV and malaria, and cancers including melanoma, colon and B-cell lymphoma.

MPL1-SE is the result of MPL1 mixed with squalene oil, excipients (inactive substances used as carriers for the active ingredient) and water to produce a stable oil-in water emulsion. MPL1-SE is an excellent promoter of Th1 responses and is currently being evaluated in several clinical trials to treat and prevent leishmaniasis. The adjuvant system (AS) series of adjuvants are proprietary formulations, several of which contain MPL1. AS02 is an oil-in-water emulsion containing MPL1 and QS-21 that induces both strong humoral and Th1 responses. AS02 is being evaluated in vaccine clinical trials for HPV [161], HBV [162] and HIV [163].

AS01 is a liposomal formulation containing MPL1 and it induces potent humoral and cell-mediated responses including CTL responses. AS01 is being evaluated in clinical trials for malaria.

MDP is the minimal unit of the mycobacterial cell wall complex that generates the adjuvant activity of CFA. Several synthetic analogs of MDP, such as muramyl tripeptide phosphatidylethanolamine (MTP-PtdEtn), have been generated, and they exhibit a wide range of adjuvant potency and side effects. MTP-PtdEtn includes phospholipids that facilitate lipid interactions, whereas the muramyl peptide portion facilitates aqueous interactions. Thus, the MTP-PtdEtn itself is able to act as an emulsifying agent to generate stable oil-in-water emulsions.

Synthetic oligodeoxynucleotides, containing unmethylated CpG motifs, act through TLR-9 and induce activation of DC and secretion of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 and IL-6. TLR-9 activation also leads to secretion of the pro-inflammatory cytokines IFN- $\alpha$ , IFN- $\gamma$  and IL-12. CpGs are extremely efficient inducers of Th1 immunity and CTL responses [164] and induce protection against infectious disease, allergy and cancer in mice and primate models [165]. Ongoing clinical studies indicate that CpGs are relatively safe and well-tolerated in humans [166] but their use has been limited in most cases to therapy rather than prophylactic indications. These are being evaluated both in the absence of antigen, for certain types of cancer therapy, and with allergens. Because of the biological instability of CpG and their resulting short half-life, several approaches have been used to enhance their bioavailability. Stabilizing approaches involve complexing to cationic peptides or cationic carriers, conjugating to the vaccine antigen, or incorporating the CpG into nucleic acids that form double stranded hairpin loops.

Adjuvant activity and safety can be enhanced by combining formulations and immunostimulants [167]. However, finding the optimal association between adjuvants is difficult as antagonism or synergy can occur rather than synergy [168].

Co-formulating the antigen and immunostimulant might also be required, as shown by the need for TLRs and antigens to be in the same compartment for optimal antigen presentation [169]. Combining antigens in the same formulation also allows otherwise non-compatible antigens to be associated in multivalent vaccines [91] and can ensure that all antigens will be presented simultaneously. This can reduce the risks of immunodominance, should one antigen be presented before the others and take the lead [170].

Both antigen(s) and immunostimulant should retain their native conformation and ability to interact with their ligands. In this respect, the antigen-adjuvant couple can be crucial, as the antigen itself might increase or counteract adjuvant efficacy or affect the induced Th-cell bias. For instance, different antigens can induce Th1 cell or mixed Th1/Th2- cell responses even though they are present in identical formulations [171]. Finally, formulating immunostimulants in carriers such as liposomes or microparticles targeted to specific cells and organs might also increase the safety profile of these molecules, by limiting their distribution *in vivo*, thereby minimizing systemic toxicity.

Combined formulations have thus been developed, some from before TLRs were identified, and have reached clinical trials and market approval. Many are aimed at introducing a cellular Th1 response in addition to an antibody response to increase vaccine efficacy against targets such as certain viruses, for instance, through TLR4 or TLR9 agonists. For example, a subunit herpes vaccine adjuvanted with AS04 combining alum and the TLR4 agonist MPL showed partial efficacy in HSV1- and HSV2-seronegative women but a similar vaccine adjuvanted with an MF59 emulsion was not protective [172].

### 5.2.3 Cytokines and chemokines as adjuvants

The activity of several potent adjuvants, including IFA, CpG oligodeoxynucleotides and aluminium salts, has been shown to be due at least in part to the induction of a varying array of cytokines, including type I IFNs, IFN- $\gamma$ , IL-2, and IL-12, that play key roles in the innate immune response and the establishment of adaptive immunity [173].

Thus, although the action of adjuvants such as IFA or aluminium salts is complex involving carrier, depot, targeting and immunomodulatory functions, studies using mice lacking a functional type I IFN receptor have shown that the adjuvant activity of IFA and certain CpG oligodeoxynucleotides is due at least in part to their ability to induce the production of type I IFNs. It is not surprising therefore that cytokines should in their own exhibit potent adjuvant activity.

Rather than designing adjuvants that induce production of specific cytokines through activation of Th cells, specific cytokines may be used directly as adjuvants either alone or in combination with other immunopotentiating adjuvants and/or delivery systems.

Most cytokines have the ability to modify and redirect the immune response when given directly as protein or as DNA encoding a cytokine of interest. However, all of these molecules exhibit dose-related toxicity. The stability problems and relatively short half-life of recombinant homologues of cytokines has limited their use as vaccine adjuvants. These difficulties have been overcome by encapsulation into liposomes and the use of cytokine expression vectors co-administered with DNA vaccines.

Nevertheless, considerable progress has been made in the use of cytokines for the immunotherapy of cancer. Microparticles have been used as a delivery system for encapsulated cytokines, including GM-CSF and IL-12, mostly for use in oncology settings [174].

Although a number of cytokines including IFN- $\alpha$ , IL-2, IL-12, IL-15, IL-18, IL-21, GM-CSF and Flt-3 ligand have been shown to potentiate the immune response to vaccination in various experimental models [175,176,177,178], the full potential of cytokines as vaccine adjuvants remains to be established.

The development of effective vaccines against many targeted infectious diseases will most likely require adequate adjuvants that can drive the local and systemic immune responses towards pathogen eradication. Mucosal adjuvants will need to be able to survive in the harsh microenvironment of the mucosal tracts and break immunological tolerance. Once over the epithelial barrier, the adjuvants will need to act on cellular members of the innate and adaptive immune system.

Modulation of immune responses through the chemokine network is therefore an interesting concept for vaccine design, although the ability of mucosally expressed chemokines to act as adjuvants and elicit DNA vaccine-specific immune responses has yet to be shown.

***Aim***

A large number of mucosal infections continue to represent a challenge for the development of vaccines that can prevent the pathogen from colonizing the surface epithelium, penetrate the surface barrier and replicate within the body.

Mucosal epithelia comprise an extensive vulnerable barrier which is reinforced by numerous innate defence mechanisms cooperating intimately with adaptive immunity. Local secretion of SIgA constitutes the largest humoral immune system of the body. Secretory immunity is needful in the defence against mucosal infections because it can inhibit initial pathogen colonization by providing blocking SIgA antibody activity on the mucosal surface.

Migration of IgA-secreting plasma cells into the MLP was recently shown to be correlated with specific chemokines and chemokine receptors, including CCR3 and CCR10, that bind a CC chemokine named CCL28 (MEC, Mucosae-Associated Epithelial Chemokine). CCL28 is widely expressed in the epithelium of various mucosal sites, including the small intestine, colon, bronchi and mammary glands, and potently chemoattracts IgA-ASCs originating from diverse mucosal lymphoid organs, as well as from intestinal and extra intestinal tissues, in every studied mucosal effector site both in mice and humans.

Viruses that infect eukaryotic organisms have the unique characteristic of self-assembling into particles. Virus-like particles represent an antigen presenting and delivery system currently under investigation as potential vaccine for different human viruses. VLPs are non-replicating particles that are similar in conformation to intact virus and are able to stimulate both arms of the immune system by entering both MHC class I and II presentation pathways. A wide variety of VLPs has shown promising results in small animal models and may offer great potential for the development of vaccines against many mucosal infections.

One strategy that improves the immunogenicity of vaccines is the use of chemokine molecular adjuvants. Chemokines are inflammatory molecules that act primarily as chemoattractants and as activators of lymphocytes through the enhancement of  $T_H1$  and  $T_H2$  phenotypes. However the ability of mucosally expressed chemokines to act as adjuvants and elicit vaccine-induced antigen-specific immune responses has yet to be shown.

Furthermore, the aim of vaccines against mucosal infections is to induce specific SIgA antibodies associated with immunological memory, in addition to systemic immunity. Therefore the development of effective mucosal chemokine vaccine adjuvants has implications for prophylactic and therapeutic immunizations strategies that require both peripheral and mucosal physiologically relevant antigen-specific immunity.

The aim of this wide study is to evaluate the suitability of CCL28 as an adjuvant for induction of specific humoral immunity at mucosal sites in mouse models immunized with HIV1-VLPs, H7N1-VLPs and HPV16-VLPs, respectively.

With this intention, the influence of CCL28 upon the following elements has been studied:

- CCR3 and CCR10 receptor expression on  $CD3^+$ ,  $CD14^+$  and  $CD19^+$  splenocytes;
- systemic levels of antigen-specific IgG and IgA antibodies;
- mucosal levels of total and antigen-specific IgA antibodies;
- systemic and mucosal production of both  $Th1$ -type and  $Th2$ -type cytokines
- IgA-secreting plasma cell migration to the effector sites of the MALT



## ***Materials and Methods***

## 1. CYTOKINE-EXPRESSING PLASMIDS

The CCL28 murine chemokine gene was digested with *AgeI* from the pORF-mCCL28 plasmid (InvivoGen, San Diego, CA, USA) (Fig. 23) and a blunt end was produced with T4-DNA polymerase. Following digestion with *NheI*, the resulting 410 bp fragment was inserted into pCpGfree expression vector (InvivoGen), which had been previously digested with *Scal/NheI*.

The murine CCL19 gene inserted into a pCpGfree expression vector (InvivoGen) (Fig. 24) as described above was used as a negative control. CCL19 is a cytokine belonging to the CC chemokine family that is also known as MIP-3- $\beta$ . CCL19 is expressed abundantly in thymus and LNs, with moderate levels in trachea and colon and low levels in stomach, small intestine, lung, kidney and spleen. This chemokine elicits its effects on its target cells by binding to the chemokine receptor CCR7. It attracts certain cells of the immune system, including DCs and antigen-engaged B-cells, CCR7<sup>+</sup> effector-memory T-cells and is crucial for lymphoid cell trafficking and the structural organization of lymphoid tissues.

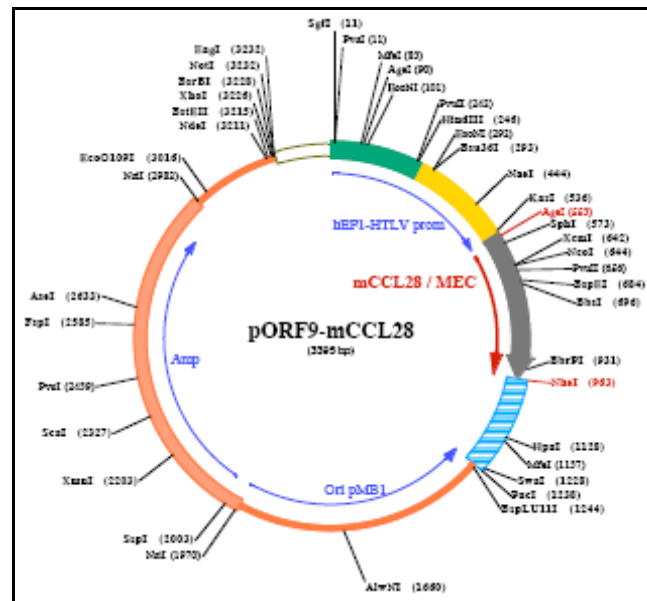


Figure 23: structure of the pORF-mCCL28 plasmid (InvivoGen).

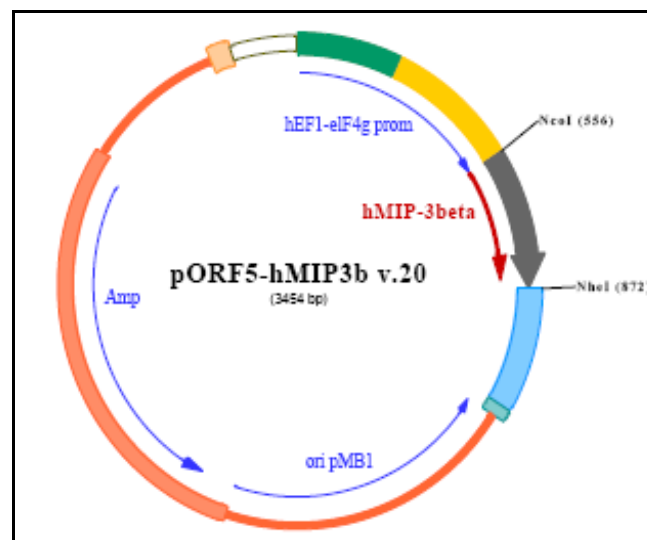


Figure 24: structure of the pORF-mCCL19 plasmid (InvivoGen).

## 2. HIV-1 VLPs-RECEIVING MICE

### 2.1 HIV-1<sub>III<sub>B</sub></sub> VLPs production

HIV-1<sub>III<sub>B</sub></sub> VLPs were provided as a part of collaboration with Dr. Klaus Überla, Department of Molecular and Medical Virology of the University of Ruhr, Bochum, Germany.

HIV-1 VLPs were produced by transient transfection of human embryo kidney carcinoma cell-line (293T-cells) with the plasmids pCD-Hgpsyn and pConBgp160-opt. pCD-Hgpsyn contains the codon-optimized gag-pol sequence of HIV-1 and was kindly provided by Geneart (Regensburg, Germany). pConBgp160-opt encodes a full-length subtype B consensus envelope of HIV-1 and was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIH, from Dr. Beatrice Hahn. The cells were plated into culture flasks (175 cm<sup>2</sup>) in DMEM medium supplemented with 10% FCS and Penicillin/Streptomycin. At a confluence of 80-90%, cells were transfected with 40 µg of each plasmid in DMEM medium supplemented with PEI [10 mg/ml], 5% FCS and Penicillin/Streptomycin. The transfection medium was changed 18 hours after transfection with DMEM medium supplemented with 2% FCS and Penicillin/Streptomycin and harvested 48 hr later. The harvested medium was centrifuged at 300 x g for 10 min and filtrated through a 0.45 µm filter to remove the cell debris. VLPs were further purified and concentrated from the conditioned medium by ultracentrifugation through a 20% sucrose gradient and a subsequent ultracentrifugation at 28,000 rpm for 2 hr a SW28 rotor. Supernatants were discarded and the pellets containing viral particles were resuspended in PBS. VLP production was confirmed by standard electron microscopy analysis.

The endotoxin level was measured by QCL-1000<sup>®</sup> Chromogenic LAL Endpoint Assay (Cambrex, Germany) according to the manufacturer's instructions, resulting in an endotoxin concentration of 1,3 ng/ml.

The concentration of viral proteins in the final VLP preparation was determined by an in-house ELISA and corresponded to 1500 ng/ml of HIV-1 Env and 550 ng/ml of HIV-1 Gag. The calibration curve was set by using recombinant HIV<sub>III<sub>B</sub></sub> Env gp120 (Baculo) (EVA607 supplied by the NIBSC centralized facility for AIDS Reagents supported by EU Programme EVA) and Gag<sup>p55</sup> (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH).

### 2.3 Immunization procedure

Adult inbred female Balb/c mice, 6-8 weeks old, were purchased from the Charles River Laboratories (Calco, Italy). Mouse colonies were maintained on a 12-h light-dark cycle in cages of 5 animals with water and food provided *ad libitum*.

Mice were randomized in six groups (5 mice/group) to receive VLPs (HIV-1<sub>III<sub>B</sub></sub> VLPs 150 ng Env/mouse) in combination with pCCL28 (50 µg/mouse) or VLPs (HIV-1<sub>III<sub>B</sub></sub> VLPs 150 ng Env/mouse) in combination with pCCL19 (50 µg/mouse) or VLPs alone (HIV-1<sub>III<sub>B</sub></sub> VLPs 150 ng Env/mouse) or pCCL28 alone (50 µg/mouse) or pCCL19 alone (50 µg/mouse). Control mice were treated with endotoxin-free phosphate-buffered saline (PBS, Organon Teknika Corp., Durham, NC).

HIV<sub>III<sub>B</sub></sub>-VLPs and pCCL28 or pCCL19 were separately administrated intramuscularly in the quadriceps muscle.

Mice were immunized on day 0, boosted on day 14 and sacrificed on day 28. Serum and vaginal secretions were collected on week 0, 2, and 4, whereas splenocytes and colon were collected on week 4.

### 2.4 Sample collection and processing

#### 2.4.1 Blood and vaginal secretion

50 µl of blood was collected by tail vein bleeding and added to 100 µl of phosphate-buffered saline (PBS, Organon Teknika Corp., Durham, NC) containing 100 IU of heparin (Hip-PBS). Serum was obtained after centrifugation at 800 x g for 10 min and stored at -80 °C.

Vaginal secretions were collected with 50 µl of pre-warmed PBS supplemented with 100X-concentrated protease inhibitor cocktail (1%, v/v) (O-complete, Roche Applied Science, Germany) by repeated aspiration until turbid. This usually took four to eight cycles of pipetting and required cutting the pipette tip back to a diameter of 1-2 mm. Material was collected and centrifuged at 12,000 x g for 10 min to separate the mucus from the PBS wash solution shortly after collection. The supernatant was transferred to sterile microcentrifuge tubes and frozen at -20 °C until use.

#### 2.4.2 Tissue harvesting

Mice were euthanized by an overdose of anesthetic (i.p. injection with 150 mg xylazine/kg body weight). Spleens were excised under sterile conditions in a laminar flow hood and put through a 100 µm plastic strainer (BD Falcon 2350, BD Biosciences, Bedford, MA) for cell recovery. Splenocytes were layered on a continuous 40-100% Percoll gradient (Sigma) and washed twice in PBS (Organon Teknika Corp., Durham, NC) to obtain the lymphocyte-rich cells. Cell viability was determined using trypan blue staining. Splenocytes

were resuspended in cell culture medium (RPMI 1640) (Organon Teknika Corp., Durham, NC) and used in cell culture assays.

PPs/colonic mononuclear cells were recovered from freshly obtained specimens. Colon specimens were first washed in HBSS (BioWhittaker Inc., Walkersville, MD) and cut into 0.5-cm pieces using scissors and tweezers. They were then incubated twice, each time for 15 min in HBSS containing EDTA (0.37 mg/ml) (Sigma) and dithiothreitol (DTT) (0.145 mg/ml) (Thermo Fisher Scientific, Rockford, IL) at 37°C. The tissues were further digested at 37°C for 10 min in Collagenase D (400 U/ml) and DNase I (0.01 mg/ml) (Sigma) supplemented RPMI. Tissue-released cells were resuspended in a continuous 100-40% Percoll gradient to obtain the lymphocyte-enriched population for cell culture assays. Mice recta were also analysed by immune-histochemistry.

### 2.4.3 Cell count

Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). ADAM-MC automatic cell counter measures total cell numbers and cell viabilities by cutting edge detection technologies. Instead of trypan blue staining, which can lead to inaccurate data, ADAM-MC utilizes two sensitive fluorescence dye staining solutions, AccuStain Solution T (Propidium Iodide/lysis solution) and AccuStain Solution N (Propidium Iodide/PBS). AccuStain Solution T allows plasma membrane disruption and nucleus staining for measurement of total cell concentration. AccuStain Solution N allows staining of non-viable cells, thus leaving viable cells completely intact. A 532 nm optic laser is automatically focused onto the cell solution inserted into a disposable microchip and cell analysis is made by a CCD detection technology.

## 2.5 Flow cytometry analyses of CCR3 and CCR10 expression

CCR3 and CCR10 surface receptors were evaluated on splenocytes. Cells were resuspended in PBS (Organon Teknika Corp., Durham, NC) and stained for surface mAbs CD3e PE-Cy5 (Armenian Hamster IgG isotype, eBioscience, San Diego, USA), anti-mouse CD14 PE-Cy5 (rat IgG2a isotype, eBioscience, San Diego, USA), anti-mouse CD19 PE-Cy5 (rat IgG2a isotype, eBioscience, San Diego, USA), anti-mouse CCR3 FITC (rat IgG2a isotype, R&D Systems, Minneapolis, MN) and anti-mouse CCR10 PE (rat IgG2b isotype, R&D Systems, Minneapolis, MN). After 15 minutes incubation at room temperature in the dark, cells were washed in PBS (Organon Teknika Corp., Durham, NC) and fixed in 1% formaldehyde (Sigma).

All the cytometric analyses were performed using FC500 flow cytometer (Beckman-Coulter, Miami, FL) equipped with a double 15-mW argon ion laser operating at 456 and 488 interfaced with an Intercorp (Venice, Italy) computer. For each analysis 20,000 events were acquired and gated on CD3 or CD14 or CD19 expression and side scatter properties. Green fluorescence from FITC (FL1) was collected through a 525-nm band-pass filter, orangered fluorescence from R-PE (FL2) was collected through a 575-nm bandpass filter and red fluorescence from Cy5PE (FL4) was collected through a 670-nm band-pass filter. Data were collected using linear amplifiers for forward and side scatter and logarithmic amplifiers for FL1, FL2, FL4, and FL5 (for details, see Appendix).

## 2.6 Cell-mediated and humoral immunity

### 2.6.1 Cytokine assay

IFN- $\gamma$ , IL-4 and IL-5 production was evaluated in the supernatants from cultivated splenocytes as well as in PP/colonic T-cells after *ex vivo* re-stimulation with recombinant HIV-1<sub>III<sub>B</sub></sub> Env gp120 (NIBSC) (4  $\mu$ g/ml) using commercial ELISA kits (R&D Systems, Minneapolis, MN) and following the procedures suggested by the manufacturers. Cytokine concentration was calculated from a standard curve obtained with the corresponding recombinant mouse cytokine.

### 2.6.2 Total IgA concentrations

Total IgA in vaginal secretions were determined by an enzyme-linked immunosorbent assay (ELISA) method. A microtiter ELISA plate was coated with 100  $\mu$ l of a goat anti-mouse IgA antibody (Kamiya Biomedical Company, Seattle, WA) in PBS (Organon Teknika Corp., Durham, NC), kept overnight at 4°C and washed in PBS-0,05% Tween 20 (Sigma). IgA antibodies in mucosal washings were detected using a goat horseradish peroxidase-conjugated  $\alpha$ -chain specific anti-mouse IgA antibody (Sigma, St. Louise, MN).

### 2.6.3 Serological assays

HIV-specific IgG and IgA in sera and vaginal secretions were measured by an ELISA method based on a recombinant HIV-1 envelope protein. 96 well ELISA plates were coated overnight at 4°C with 100  $\mu$ l of 4  $\mu$ g/ml recombinant HIV-1<sub>III<sub>B</sub></sub> Env gp120 (Baculo) (NIBSC). After washing the plates three times with Tween-20 0.05 % buffer (in PBS), plates were incubated for 2 hours at 37°C, 5% CO<sub>2</sub> with PBS containing 3% of bovine serum albumin (BSA) (Sigma, St Louis, MO) to block non-specific protein binding sites. Serum dilutions 1/10 to 1/10000 and vaginal wash dilutions 1/2 to 1/1000 were incubated for 2 hours at 37°C, 5%

CO<sub>2</sub>. Plates were then washed three times and a goat HRP-conjugated  $\alpha$ -chain specific anti-mouse IgA antibody (Sigma, St. Louise, MN) or a goat HRP-conjugated anti-mouse IgG antibody (Jackson Immuno-Research, West Grove, PA) was added to the plates diluted 1:1000 or 1:30000 in PBS/BSA, respectively. Following 1 hour of incubation at 37°C, 5% CO<sub>2</sub>, the plates were washed and incubated with tetramethylbenzidine (TMB, R&D Systems, Minneapolis, MN) substrate solution for 30 min at RT. The colour reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub> 1.8 M. The optical density was measured at an absorbance of  $\lambda = 490$  nm and the concentrations of IgG or sIgA were calculated from the established standard curve.

### **2.7 HIV-1 neutralization assay**

HIV-1 neutralization assay was performed in a pooled donor PBMC-based assay. Briefly, pooled sera and vaginal secretions from pre-immunized and immunized mice were complement depleted by heat inactivation at 56°C for 60 minutes. Serial two-fold dilutions starting from 1/20 were incubated (in a humidified 5% CO<sub>2</sub> incubator at 37°C for 60 minutes) in duplicate in 96-well plates with HIV-1 IIIB and HIV-1 DU174 strains (NIBSC) of 40 and 20 TCID<sub>50</sub>, respectively. One hundred thousand PBMCs pooled from HIV-negative donors were stimulated with PHA for 48 h and subsequently added to each well and incubated overnight (18 h) at 37°C. Cells were extensively washed with RPMI 1640 (Organon Teknika Corp., Durham, NC), centrifuged at 400 g for 10 min and fresh medium (RPMI 1640, 200 mM L-glutamine, 20% fetal calf serum, 10U Interleukin-2, 1% penicillin and 2% streptomycin) was replaced on day 1 and day 3. After 7 days, supernatants were harvested and p24 antigen levels were determined by ELISA according to the manufacturer's instructions (Perkin-Elmer, Waltham, MA).

### **2.8 Immune-histochemistry analyses**

Tissues obtained from the colon of mice (2-cm specimens from the anus toward the left colon) were fixed in buffered-formalin for 24 hr at room temperature and then embedded in paraffin. Haematoxylin-eosin stained sections were used for histological evaluation. The evaluation of IgA<sup>+</sup> plasma cells was made on 3  $\mu$ m paraffin embedded slides; the sections were dewaxed in xylene, rehydrated in an ascending ethanol scale and pre-treated in a microwave oven (two cycles for 5 minutes each at 780 W, in EDTA buffer, pH 8). Endogen biotin and aspecific signals were blocked by using the appropriate reagents. For immune-histochemistry, a goat anti-mouse IgA (dilution 1:400, AbD Serotec) was used; slides were incubated for 2 hr at room temperature in a humid chamber; washed in PBS and then revealed by biotinylated anti-goat IgG (dilution 1:50, 1 hr incubation, R&D Systems, MN) followed by HRP-conjugated streptavidin (30 min incubation, R&D systems, MN). The chromogen was 3,3'-diaminobenzidine free base (DAB).

### 3. H7N1 VLPs-RECEIVING MICE

#### 3.1 H7N1 VLPs production

VLPs that mimic the overall structure of Influenza virus H7N1 particles without containing infectious genetic material were provided as a part of a wide collaboration with Dr. Francisco Veas of the Institute for Research and Development/UR178, University of Montpellier, France. From a passage of the H7N1 (A/Chicken/FPV/Rostock/1934) [179] in MDCK cells, viral RNA was isolated from total RNA using the High Pure RNA isolation kit (Roche Molecular Biochemicals, Mannheim, Germany). HA, NA and M2 coding sequences were then amplified from total viral RNA using Superscript Reverse transcriptase and specific primers. PCR products were inserted into the expression plasmid pHCMV. H7N1-VLPs were assembled on replication-defective core particles derived from murine leukaemia virus (MLV). VLP used in this work consisted in an empty core particles generated by the expression of MLV Gag proteins [180]. H7N1-VLPs harboring at their surface HA, NA and M2 proteins derived from the H7N1 virus were obtained after a transient expression in HEK 293T cells of 3 plasmids encoding for the surface HA (together with Gag), NA, M2 proteins. From cell supernatant, VLP were concentrated to 1/100 and purified by ultracentrifugation at 110,000 x g for 2 hours on a 20% sucrose cushion. VLP production was confirmed by standard electron microscopy analysis.

#### 3.3 Immunization procedure

Adult inbred female Balb/c mice, 6-8 weeks old, were purchased from the Charles River Laboratories (Calco, Italy). Mouse colonies were maintained on a 12-hours light-dark cycle in cages of 5 animals with water and food provided *ad libitum*.

Mice were randomized in six groups (5 mice/group) to receive VLPs (H7N1 VLPs 100 µl/mouse) in combination with pCCL28 (50 µg/mouse) or VLPs (H7N1 VLPs 100 µl/mouse) in combination with pCCL19 (50 µg/mouse) or VLPs alone (H7N1 VLPs 100 µl/mouse) or pCCL28 alone (50 µg/mouse) or pCCL19 alone (50 µg/mouse). Control mice were treated with endotoxin-free phosphate-buffered saline (PBS, Organon Teknika Corp., Durham, NC).

H7N1 VLPs were administrated intraperitoneally; the CCL28- and the CCL19-expressing plasmids were administrated intramuscularly in the quadriceps muscle.

Mice were immunized on day 0, boosted on day 14 and sacrificed on day 28. Serum and saliva samples were collected on week 0, 2, and 4, whereas splenocytes, BALs, and lungs were collected on week 4.

#### 3.4 Sample collection and processing

##### 3.4.1 Blood, saliva and BALs

Blood samples were collected by tail bleeding; serum was obtained by standard methods and stored at -80°C until use. See 2.4.1 paragraph for details about the method.

Saliva is the most commonly used fluid for measurements of humoral mucosal responses in mice. However, because it is not naturally produced in the quantity needed for experimentation, chemical stimulants are often used to increase its production. The most used stimuli are carbachol (carbamyl choline chloride) and pilocarpine-HCl, which are injected at concentrations of 50-100 µg and 2.5-5.0 mg/kg body weight, respectively, depending on the mouse strain. Because carbachol (acetylcholine agonist) rapidly initiates a great, but short-lived, increase in saliva flow, it facilitates an easy and quick collection of saliva. Even more importantly, it does not require the anesthetization of mice, a considerable advantage because anesthesia, at least in rats, has been reported to affect the salivary flow rate.

In this regard, mice were intraperitoneally injected with 10-30 µg/ml carbachol (Sigma) in PBS to induce mice drooling. Saliva samples were gently aspirated with a plastic Pasteur, avoiding any gingival damage, rapidly pipetted into tubes placed on ice and centrifuged for 5 minutes at 800 x g to remove potential bacteria and food particles. Supernatants were added with 100X-concentrated protease inhibitor cocktail (1%, v/v) (O-complete, Roche Applied Science, Germany) and subsequently frozen at -70°C.

BALs generally represent the mucosal samples used to test the immune responses elicited by infections or immunization of the respiratory tract.

Mice were euthanized by an overdose of anesthetic (i.p. injection with 150 mg xylazine/kg body weight) and placed on their back. After making an incision from the abdomen to the maxilla, the skin was removed and a midline incision over the anterior aspect of the trachea slightly superior to the thoracic inlet was made. Trachea was clamped off at the level of thoracic inlet and a blunt animal-feeding needle (Popper & Sons Inc., New Hyde Park, NY) attached to a tuberculin syringe was inserted into the tracheal lumen. 500-1000 µl of PBS (Organon Teknika Corp., Durham, NC) were repeatedly instilled and aspirate three times. BALs were placed in tubes set on ice and centrifuged for 10 minutes at 800 x g to remove potential bacteria. Supernatants were added with 100X-concentrated protease inhibitor cocktail (1%, v/v) (O-complete, Roche Applied Science, Germany) and frozen at -70°C.

### 3.4.2 Tissue harvesting

Splenocytes were collected from fresh specimens and used in cell culture assays. See 2.4.2 paragraph for details about the method.

The lungs were crushed into a single cell suspension using a Seward Stomacher 80 (Metrohm Inc. USA, Riverview, FL), put through a 40 µm cell strainer (BD Falcon 2350, BD Biosciences, Bedford, MA), washed with RPMI 1640 (Organon Teknika Corp., Durham, NC) for 10 minutes at 800 x g, pelleted and incubated for 5-10 minutes at room temperature in ACK lysing buffer, resulting in the lysis of RBCs. Cells were then washed in RPMI 1640 (Organon Teknika Corp., Durham, NC) for 10 minutes at 800 x g, resuspended in medium, counted and used in cell culture assays. Furthermore, whole-lung extracts prepared as homogenates were centrifuged for 15 minutes at 800 x g to collect supernatants for immunoglobulin assays. The lungs were also analysed by immune-histochemistry.

### 3.4.3 Cell count

Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). See 2.4.3 paragraph for details about the method.

## 3.5 Flow cytometry analyses of CCR3 and CCR10 expression

CCR3 and CCR10 surface receptors were evaluated on CD3<sup>+</sup>, CD14<sup>+</sup> and CD19<sup>+</sup> splenocytes by cytofluorimetric analyses. See 2.5 paragraph for details about the method.

## 3.6 Cell-mediated and humoral immunity

### 3.6.1 Cytokine assay

IFN-γ, IL-4 and IL-5 production was evaluated in splenic and lung T-cells after *ex vivo* re-stimulation with recombinant Influenza A/Chicken/FPV/Rostock/1934 (H7N1) HA (Sino Biological Inc., Beijing, China) (5 µg/ml) using commercial ELISA kits (R&D Systems, Minneapolis, MN) and following the procedures suggested by the manufacturers. Cytokine concentration was calculated from a standard curve obtained with the corresponding recombinant mouse cytokine.

### 3.6.2 Total IgA concentrations

Total IgA in lung homogenates, BALs and saliva samples were determined by an enzyme-linked immunosorbent assay (ELISA) method. A microtiter ELISA plate was coated with 100 µl of a goat anti-mouse IgA antibody (Kamiya Biomedical Company, Seattle, WA) in PBS (Organon Teknika Corp., Durham, NC), kept overnight at 4°C and washed in PBS-0,05% Tween 20 (Sigma). IgA antibodies in mucosal washings were detected using a goat horseradish peroxidase-conjugated α-chain specific anti-mouse IgA antibody (Sigma, St. Louise, MN).

### 3.6.3 Serological assays

Influenza HA-specific IgG and IgA in sera, lung homogenates, BALs and saliva samples were measured by an ELISA method based on a recombinant Influenza A hemagglutinin protein. 96 well ELISA plates were coated overnight at 4°C with 100 µl of 5 µg/ml Influenza virus (A/Chicken/FPV/Rostock/1934 H7N1) HA (Sino Biological Inc., Beijing, China). After washing the plates three times with Tween-20 0.05 % buffer (in PBS), plates were incubated for 2 hours at 37°C, 5% CO<sub>2</sub> with PBS containing 3% of bovine serum albumin (BSA) (Sigma, St Louis, MO) to block non-specific protein binding sites. Serum dilutions 1/10 to 1/10000, lung homogenates dilutions 1/10 to 1/1000, BAL dilutions 1/5 to 1/500 and saliva sample dilutions 1/2 to 1/200 were incubated for 2 hours at 37°C, 5% CO<sub>2</sub>. Plates were then washed three times and a goat HRP-conjugated α-chain specific anti-mouse IgA antibody (Sigma, St. Louise, MN) or a goat HRP-conjugated anti-mouse IgG antibody (Jackson Immuno-Research, West Grove, PA) was added to the plates diluted 1:1000 or 1:30000 in PBS/BSA, respectively. Following 1 hour of incubation at 37°C, 5% CO<sub>2</sub>, the plates were washed and incubated with tetramethylbenzidine (TMB, R&D Systems, Minneapolis, MN) substrate solution for 30 minutes at room temperature. The colour reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub> 1.8 M. The optical density was measured at an absorbance of λ=490 nm and the concentrations of IgG or sIgA were calculated from the established standard curve.

## 3.7 Hemagglutination (HA) and hemagglutination inhibition (HAI) test

HA and HAI tests were performed as described in the WHO manual on Animal Influenza Diagnosis and Surveillance using 0.5% turkey RBCs but employing H7N1 VLPs as the agglutinating agent in place of infectious virus, as described elsewhere [181].

A series of 2-fold dilutions of H7N1-VLPs in PBS (Organon Teknika Corp., Durham, NC) were prepared and incubated at 25°C for 2 hours with 50 µl of 0.5% turkey blood cells. The extent of hemagglutination was inspected visually, and the highest dilution capable of agglutinating turkey red blood cells was determined.

Mouse sera and BALs were heat-inactivated at 56°C for 1 hour and then treated with receptor destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) at 37°C overnight according to the manufacturer's instruction. After treatment, 25 µl of VLPs containing four HA units of Influenza virus A/Chicken/FPV/Rostock/1934 at 37°C for 1 hour, followed by incubation with 50 µl of 0.5% turkey RBCs (Lampire Biological Laboratories, Pipersville, PA) at 25°C for 30-60 minutes. Reference antiserum to A/Chicken/FPV/Rostock/1934 was used as a positive control.

Hemagglutination occurs when the RBCs are in suspension after the RBC control has settled completely as a compact button. The HAI titer was defined as the reciprocal of the highest serum or BAL dilution that inhibited hemagglutination. Spots indicating H7N1 VLP-neutralizing activity were documented by photography.

### **3.8 Immune-histochemistry analyses**

The lungs were fixed in buffered-formalin for 24 hours at room temperature and then embedded in paraffin. Haematoxylin-eosin stained sections were used for histological evaluation.

The evaluation of IgA<sup>+</sup> plasma cells was made on 3 µm paraffin embedded slides; the sections were dewaxed in xylene, rehydrated in an ascending ethanol scale and pre-treated in a microwave oven (two cycles for 5 minutes each at 780 W, in EDTA buffer, pH 8). Endogen biotin and aspecific signals were blocked by using the appropriate reagents. For immune-histochemistry, a goat anti-mouse IgA (dilution 1:400, AbD Serotec) was used; slides were incubated for 2 hours at room temperature in a humid chamber; washed in PBS and then revealed by biotinylated anti-goat IgG (dilution 1:50, 1 hr incubation, R&D Systems, MN) followed by HRP-conjugated streptavidin (30 min incubation, R&D systems, MN). The chromogen was 3,3'-diaminobenzidine free base (DAB).



## 4. HPV-16 VLPs-RECEIVING MICE

### 4.1 HPV-16 VLPs production

HPV-16 VLPs were provided as a part of collaboration with Dr. Lutz Gissmann, German Cancer Research Center of Heidelberg, Germany.

The HPV-16 L1 ORF was excised from plasmid HPV16-114/k-L1/L2-pSynxtVI [120], using *Bgl*II and subcloned into pUC19 (New England Biolabs, Hitchin, United Kingdom) using the single *Bam*HI site of this vector. *Spodoptera frugiperda* (Sf9) cells were grown in suspension or monolayer cultures at 27°C in TNMFH medium (Sigma-Aldrich), supplemented with 10% fetal calf serum and 2 mM glutamine. For HPV 16 L1-based recombinant baculovirus construction, 10 mg of transfer-plasmid were used to transfect Sf9 cells together with 2 mg of linearized Baculo-Gold DNA (Pharmlingen). Recombinant viruses were purified by standard methods as suggested by the manufacturer. To test for expression of HPV-16 capsid protein, 10<sup>6</sup> Sf9 cells were infected with baculovirus recombinants at a MOI of 5 to 10. After incubation, medium was removed and cells were washed with PBS. The cells were then lysed in SDS-sample buffer and analyzed by SDS-page and immune blotting assay.

*Trichoplusia ni* (TN) High Five cells were grown to a density of 1-2 x 10<sup>6</sup> cells/ml in Ex-Cell 405 serum-free medium (JRH, Biosciences). Approximately 2 x 10<sup>8</sup> cells were pelleted at 1000 x g for 15 min, resuspended in 20 ml of medium and infected with recombinant baculoviruses at a MOI of 2-5 for 1 hour at room temperature. After addition of 200 ml of medium, cells were plated on round dishes and incubated for 3-4 days. Cells were then harvested, pelleted and resuspended in 10 ml of extraction buffer. The following steps were performed at 4°C. After sonication for 45 seconds at 60 watts, the extract was pelleted at 10,000 rpm in a Sorvall SS34 rotor. The pellet was resuspended in 6 ml of extraction buffer, sonicated again for 30 seconds at 60 watts, and centrifuged again. Supernatants were layered onto a two-step gradient with 14 ml of 40% sucrose on top of 8 ml of CsCl solution (4.6 g CsCl per 8 ml extraction buffer) and centrifuged in a Sorvall AH629 swinging bucket rotor for 2 hours at 27,000 rpm (10°C). The interphase between CsCl and sucrose and the complete layer of CsCl were collected and filled into 13.4 ml Quickseal tubes. Extraction buffer was added to adjust the volume to 13.4 ml. Samples were centrifuged overnight at 50,000 rpm at 20°C in a Beckman 70 Ti rotor. Gradients were fractionated (1 ml per fraction) by puncturing tubes on top and bottom with a 21-gauge needle. Then 2.5 ml of each fraction were analyzed by a 10% SDS-polyacryl amide gel and Western blotting. VLP fraction were dialyzed for 2 hours against 10 mM HEPES (pH 7.5, 300 mM NaCl).

### 4.3 Immunization procedure

Adult inbred female Balb/c mice, 6-8 weeks old, were purchased from the Charles River Laboratories (Calco, Italy). Mouse colonies were maintained on a 12-h light-dark cycle in cages of 5 animals with water and food provided *ad libitum*.

Mice were randomized in six groups (5 mice/group) to receive VLPs (HPV-16 VLPs 50 µg/mouse) in combination with pCCL28 (50 µg/mouse) or VLPs (HPV-16 VLPs 50 µg/mouse) in combination with pCCL19 (50 µg/mouse) or VLPs alone (HPV-16 VLPs 50 µg/mouse) or pCCL28 alone (50 µg/mouse) or pCCL19 alone (50 µg/mouse). Control mice were treated with endotoxin-free phosphate-buffered saline (PBS, Organon Teknika Corp., Durham, NC). HPV-16 VLPs, the CCL28- and the CCL19-expressing plasmids were separately administrated intramuscularly in the quadriceps muscle.

Mice were immunized on day 0, boosted on day 14 and sacrificed on day 28. Serum and vaginal washes were collected on week 0, 2, and 4, whereas splenocytes and genital tract were collected on week 4.

### 4.4 Sample collection and processing

#### 4.4.1 Blood and vaginal secretions

Blood samples were collected by tail bleeding; serum was obtained by standard methods and stored at -80°C until use. Vaginal secretions were collected by standard methods and stored at -20°C until use. See 2.4.1 paragraph for details about the methods.

#### 4.4.2 Tissue harvesting

Splenocytes and cervical lymphocytes were collected from fresh specimens and used in cell culture assays. Lymphocyte-rich population from cervical mucosa was isolated following the procedure used for PP/colonic cells collection. See 2.4.2 paragraph for details about the method. Furthermore, the cervix was analyzed by immune-histochemistry.

#### 4.4.3 Cell count

Cell count was performed with the automated cell counter ADAM-MC (Digital Bio, NanoEnTek Inc, Corea). See 2.4.3 paragraph for details about the method.

#### 4.5 Flow cytometry analyses of CCR3 and CCR10 expression

CCR3 and CCR10 surface receptors were evaluated on CD3<sup>+</sup>, CD14<sup>+</sup> and CD19<sup>+</sup> splenocytes by cytofluorimetric analyses. See 2.5 paragraph for details about the method.

#### 4.6 Cell-mediated and humoral immunity

##### 4.6.1 Cytokine assay

IFN- $\gamma$ , IL-4 and IL-5 production was evaluated in splenic and cervical T-cells after *ex vivo* re-stimulation with recombinant HPV-16 L1 protein (Meridian Life Science Inc., Saco, ME) (4  $\mu$ g/ml) using commercial ELISA kits (R&D Systems, Minneapolis, MN) and following the procedures suggested by the manufacturers. Cytokine concentration was calculated from a standard curve obtained with the corresponding recombinant mouse cytokine.

##### 4.6.2 Total IgA concentrations

Total IgA in vaginal secretions were determined by an enzyme-linked immunosorbent assay (ELISA) method. A microtiter ELISA plate was coated with 100  $\mu$ l of a goat anti-mouse IgA antibody (Kamiya Biomedical Company, Seattle, WA) in PBS (Organon Teknika Corp., Durham, NC), kept overnight at 4 °C and washed in PBS-0,05% Tween 20 (Sigma). IgA antibodies in vaginal washings were detected using a goat horseradish peroxidase-conjugated  $\alpha$ -chain specific anti-mouse IgA antibody (Sigma, St. Louise, MN).

##### 4.6.3 Serological assays

Papillomavirus L1-specific IgG and IgA in sera and vaginal secretions were measured by an ELISA method based on a recombinant HPV L1 protein. 96 well ELISA plates were coated overnight at 4 °C with 100  $\mu$ l of 4  $\mu$ g/ml HPV-16 L1 protein (Meridian Life Science Inc., Saco, ME). After washing the plates three times with Tween-20 0.05 % buffer (in PBS), plates were incubated for 2 hours at 37 °C, 5% CO<sub>2</sub> with PBS containing 3% of bovine serum albumin (BSA) (Sigma, St Louis, MO) to block non-specific protein binding sites. Serum dilutions 1/10 to 1/10000 and vaginal secretion dilutions 1/2 to 1/1000 were incubated for 2 h at 37 °C, 5% CO<sub>2</sub>. Plates were then washed three times and a goat HRP-conjugated  $\alpha$ -chain specific anti-mouse IgA antibody (Sigma, St. Louise, MN) or a goat HRP-conjugated anti-mouse IgG antibody (Jackson Immuno-Research, West Grove, PA) was added to the plates diluted 1:1000 or 1:30000 in PBS/BSA, respectively. Following 1 hour of incubation at 37 °C, 5% CO<sub>2</sub>, the plates were washed and incubated with tetramethylbenzidine (TMB, R&D Systems, Minneapolis, MN) substrate solution for 30 min at room temperature. The colour reaction was stopped by adding H<sub>2</sub>SO<sub>4</sub> 1.8 M. The optical density was measured at an absorbance of  $\lambda$ =490 nm and the concentrations of IgG or sIgA were calculated from the established standard curve.

#### 4.7 Hemagglutination inhibition test (HAI)

To verify the specificity and neutralizing ability of the antibodies, HAI assays were done [182].

Mouse blood was collected by tail bleeding in a heparinised tube and the RBCs were separated by centrifugation at 800 x g for 5 minutes at 4 °C, washed twice with PBS and suspended in dilution buffer (PBS, Organon Teknika Corp., Durham, NC) at a concentration of 1% (vol/vol). They were stored at 4 °C and used for up to 3 days.

VLPs were incubated with 2-fold dilutions of experimental sera (1/20 to 1/640) and vaginal secretion samples (1/10 to 1/320) at room temperature for 2 hours, after which the samples were mixed with an equal volume of the 1% RBC suspension. Aliquots (100  $\mu$ l) of the mixtures were transferred to a round-bottomed, 96 well plate and incubated for 3 hours at 4 °C. The monoclonal antibody H16.V5, kindly provided by Prof. Neil Christensen, Penn State University, Pennsylvania, was used as a positive control [183]. Spots indicating HPV-16 VLP-neutralizing activity were documented by photography.

#### 4.8 Immune-histochemistry analyses

The genital tract was fixed in buffered-formalin for 24 hours at room temperature and then embedded in paraffin. Haematoxylin-eosin stained sections were used for histological evaluation.

The evaluation of IgA<sup>+</sup> plasma cells was made on 3  $\mu$ m paraffin embedded slides of the cervical mucosa; the sections were dewaxed in xylene, rehydrated in an ascendent ethanol scale and pre-treated in a microwave oven (two cycles for 5 minutes each at 780 W, in EDTA buffer, pH 8). Endogen biotin and aspecific signals were blocked by using the appropriate reagents. For immune-histochemistry, a goat anti-mouse IgA (dilution 1:400, AbD Serotec) was used; slides were incubated for 2 hours at room temperature in a humid chamber; washed in PBS and then revealed by biotinylated anti-goat IgG (dilution 1:50, 1 hr incubation, R&D Systems, MN) followed by HRP-conjugated streptavidin (30 min incubation, R&D systems, MN). The chromogen was 3,3'-diaminobenzidine free base (DAB).

## **5. STATISTICAL ANALYSES**

Comparisons between groups were analyzed to evaluate immunological differences. Kruskal & Wallis analysis of variance was performed for each variable; Bonferroni correction was applied to the results. Two-sided p-values were considered. Data analysis was performed using the SPSS statistical package (SPSS Inc. Chicago, Illinois, USA).

## ***Results and Discussion***

## 1. HIV-1<sub>IIIIB</sub> VLPs-RECEIVING MICE

### 1.1 CCR3- and CCR10- expressing CD19<sup>+</sup> cells

The tissue-specific migration of lymphocytes is tightly regulated by a complex network of chemokines [44]. CCL28 has been shown to direct homing ASC, and in particular of IgA<sup>+</sup> ASCs to the gastro-intestinal, the upper aero-digestive and the mammary glands. CCL28 binds to CCR3 and CCR10 receptors; CCR10 in particular is considered to be a unifying chemokine receptor playing a pivotal role in homing of plasma blasts to extra-intestinal effector sites [57].

Inbred female Balb/c mice were immunized with a prime-boost regimen using HIV-1<sub>IIIIB</sub> VLPs in the presence or in the absence of the CCL28-expressing plasmid. The murine CCL19 chemokine gene inserted into a pCpG-free expression vector was used as negative control (for further details about immunization procedure, see Materials and Methods, paragraph 2.3).

To analyze the expression of CCL28 chemokine receptors on circulating lymphocytes, flow cytometry assessment of CCR3 and CCR10 was performed in CD3<sup>+</sup>, CD14<sup>+</sup> and CD19<sup>+</sup> purified splenocytes.

Results indicated that the CCR3- and CCR10-expression levels in CD3<sup>+</sup> splenocytes as well as CD14<sup>+</sup> cells were similar in the six analyzed groups (data not shown). In contrast, CD19<sup>+</sup>/CCR3<sup>+</sup> and CD19<sup>+</sup>/CCR10<sup>+</sup> splenocytes were significantly augmented in HIV-VLP<sub>IIIIB</sub>-CCL28 treated mice as compared with either HIV-VLP<sub>IIIIB</sub>-CCL19 ( $p=0.014$  and  $0.043$ , respectively), HIV-VLP<sub>IIIIB</sub> alone ( $p=0.005$  and  $0.008$ , respectively), CCL28 alone ( $p=0.004$  and  $0.006$ , respectively), CCL19 alone ( $p=0.001$  and  $0.004$ , respectively) or saline controls ( $p=0.002$  and  $0.005$ , respectively) (Fig. 25).

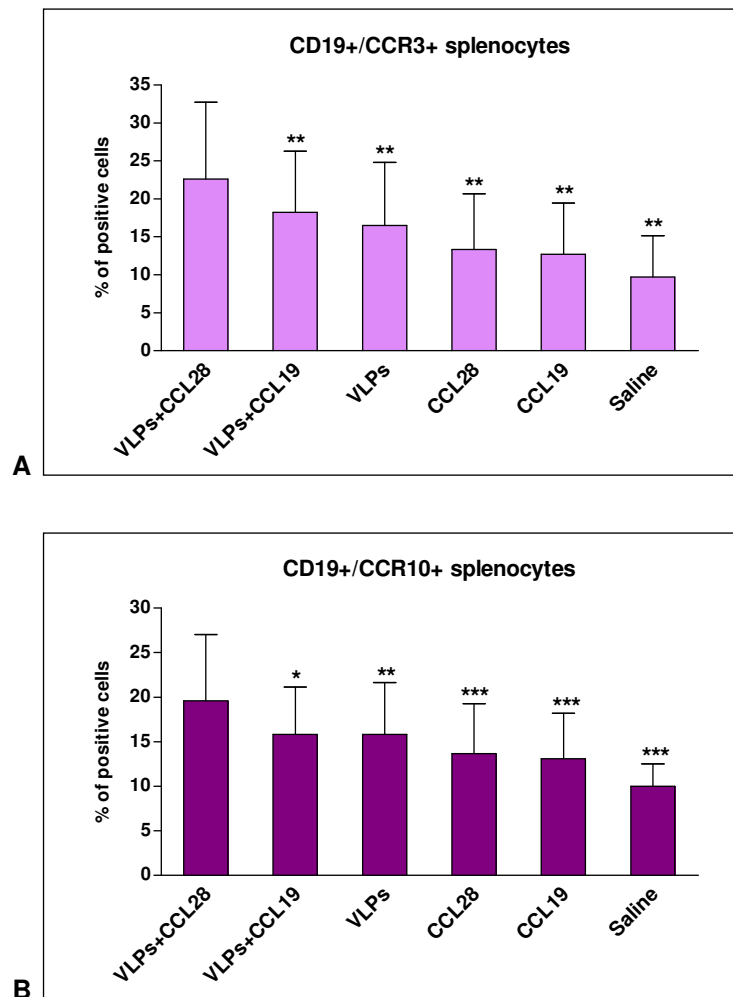


Figure 25: expression of CCR3 and CCR10 on CD19<sup>+</sup> splenocytes; **(A)** CCR3-expressing, CD19<sup>+</sup> splenocytes, **(B)** CCR10-expressing, CD19<sup>+</sup> splenocytes. Mean values, standard deviations and statistically significant differences are shown (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HIV-VLP<sub>IIIIB</sub>-CCL28 mice).

### 1.2 Surface density of CCR3 and CCR10 on CD19<sup>+</sup> cells

Evaluation of the mean fluorescence intensity (MFI), a relative measure of the surface density on a cellular level, showed that the CCR3 MFI on CD19<sup>+</sup> splenocytes was significantly augmented in HIV-VLP<sub>IIIIB</sub>-CCL28

treated mice as compared with either HIV-VLP<sub>III</sub>B-CCL19 ( $p=0.029$ ), HIV-VLP<sub>III</sub>B alone ( $p=0.015$ ), CCL28 alone ( $p=0.005$ ), CCL19 alone ( $p=0.009$ ) or saline controls ( $p<0.001$ ) (Fig. 26, panel A).

The CCR10 MFI on CD19<sup>+</sup> lymphocytes was similarly up-regulated in HIV-VLP<sub>III</sub>B-CCL28 treated mice as compared with either HIV-VLP<sub>III</sub>B-CCL19 ( $p=0.004$ ), HIV-VLP<sub>III</sub>B alone ( $p=0.002$ ), CCL28 alone ( $p=0.001$ ), CCL19 alone ( $p<0.001$ ) or saline controls ( $p<0.001$ ) (Fig. 26, panel B).

Results presented herein put in evidence that immunization of mice with Env-expressing VLPs in the presence of the CCL28-expressing plasmid results in the modulation of the whole CCL28-CCR3/CCR10 circuit correlating with a robust up-regulation of chemokine receptor expression on circulating CD19<sup>+</sup> cells.

As CCL28 is expressed by a variety of mucosal sites, the up-regulation of CCR3 and CCR10 observed on circulating CD19<sup>+</sup> lymphocytes could be dependent on the recirculation of lymphocytes between the mucosal and haematic compartments. Nevertheless, intramuscular delivery of the CCL28-expressing plasmid results in a massive recruitment of cognate chemokine receptor positive cells [184]; thus the up-regulation of CCR3 and CCR10 could be also secondary to CD19<sup>+</sup>/CCR3<sup>+</sup> or CD19<sup>+</sup>/CCR10<sup>+</sup> cell recruitment at the site of vaccine inoculation, favouring the migration of lymphocytes to mucosal effector sites. It remains to verify whether the expression and the surface density of CCR3 and CCR10 is up-regulated as well in CD19<sup>+</sup> lymphocytes isolated from mucosal secretions.

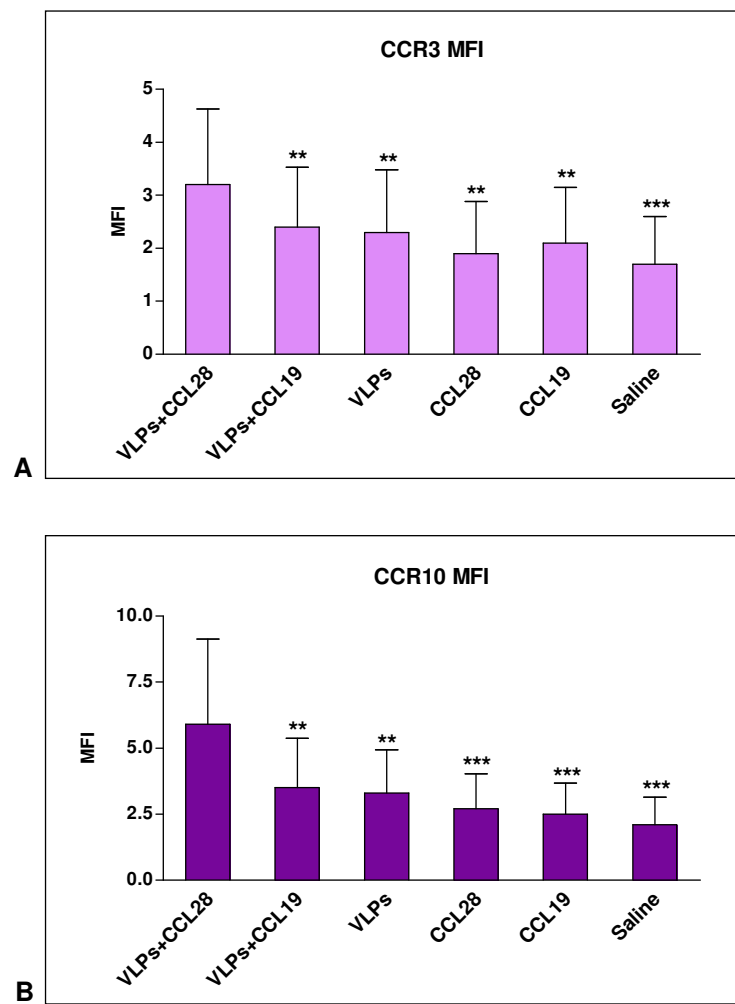


Figure 26: CCR3 and CCR10 mean fluorescence intensity (MFI) on CD19<sup>+</sup> splenocytes; (A) CCR3<sup>+</sup>/CD19<sup>+</sup> splenocytes, (B) CCR10<sup>+</sup>/CD19<sup>+</sup> splenocytes. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HIV-VLP<sub>III</sub>B-CCL28 mice).

### 1.3 Induction of mucosal total and HIV-specific IgA

IgA are mostly mucosal antibodies that are responsible for the first line of defense of adaptive immunity against pathogens [1].

HIV-specific mucosal IgA are observed in HIV-infected individuals, and, notably, have been described to be present also in HIV-exposed seronegative individuals (HESN). IgA isolated from cervico-vaginal secretions of these individuals are capable of inhibiting virus transcytosis through epithelial layers in vitro and have a potent neutralizing activity [12,66]. Because IgA in HESN seem to contribute to the prevention of infection it

is reasonable to infer that vaccine procedures capable of eliciting a strong IgA response would be beneficial by contributing to the containment of HIV infection.

To assess the CCL28-induced migration of Ig-ASCs at mucosal effector sites, both total and Env-specific antibody responses were quantified in vaginal secretions of both immunized and control mice.

Total IgA levels were significantly augmented, compared to baseline values, in vaginal secretions of HIV-VLP<sub>III</sub>B-CCL28 mice compared to all the other groups of mice (VLP<sub>III</sub>B-CCL28 vs VLP<sub>III</sub>B-CCL19  $p=0.04$ , VLP<sub>III</sub>B-CCL28 vs VLP<sub>III</sub>B alone  $p=0.008$ , VLP<sub>III</sub>B-CCL28 vs CCL28 alone  $p<0.001$ , VLP<sub>III</sub>B-CCL28 vs CCL19 alone  $p<0.001$ , VLP<sub>III</sub>B-CCL28 vs saline  $p<0.001$ ) (Fig. 27).

More interestingly, Env-specific IgA levels were significantly increased as well in vaginal washes of VLP-CCL28 mice as compared with either HIV-VLP<sub>III</sub>B-CCL19 or HIV-VLP<sub>III</sub>B alone animals (VLP<sub>III</sub>B-CCL28 vs VLP<sub>III</sub>B-CCL19  $p=0.045$ , VLP<sub>III</sub>B-CCL28 vs VLP<sub>III</sub>B alone  $p=0.005$ ) (Fig. 28). Pre-immunization as well as control mice vaginal secretions did not contain specific antibodies against the HIV-1 Env protein. The observed specific mucosal anti-HIV IgA responses were normalized on the total IgA content.

Results presented herein demonstrate that the objective of up-regulating potentially beneficial mucosal immune responses is achievable in the mouse model by the use of CCL28 as an adjuvant.

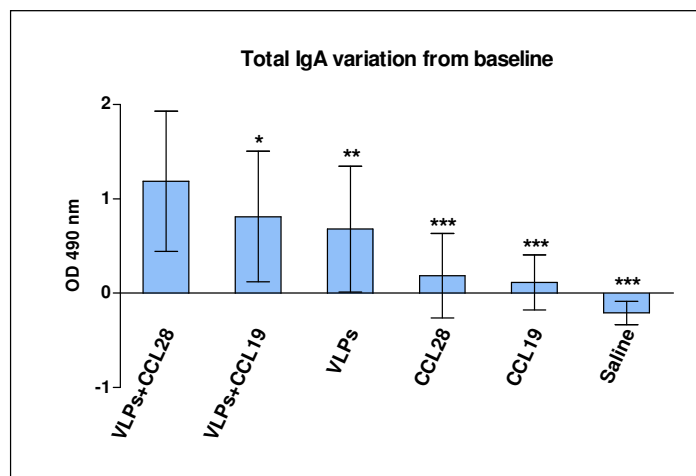


Figure 27: mucosal humoral immunity. Total IgA variation from baseline in vaginal washes is shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are represented (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HIV-VLP<sub>III</sub>B-CCL28 mice).

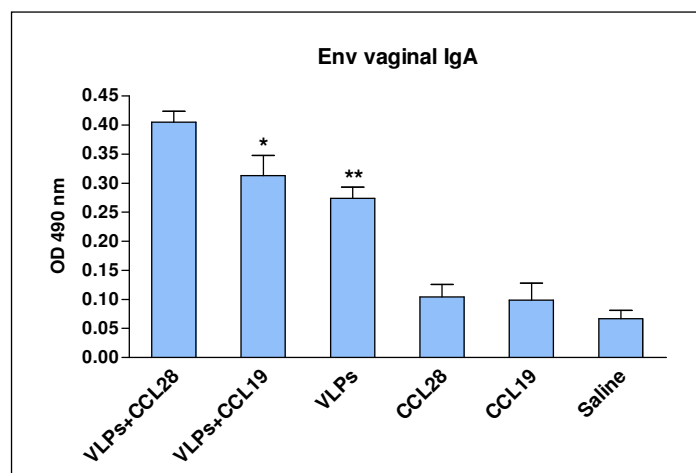


Figure 28: HIV-specific mucosal humoral immunity. Env-specific IgA levels in vaginal secretions are shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are represented (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HIV-VLP<sub>III</sub>B-CCL28 mice).

#### 1.4 Induction of HIV-specific systemic humoral response

Sera from immunized and control group mice were collected two weeks after final immunization to measure Env-specific antibodies. ELISA tests were performed on microwell plates coated with recombinant HIV-1<sub>III</sub>B Env gp120.

Results showed that serum anti-Env IgG and IgA levels were significantly increased in HIV-VLP<sub>III</sub>B-CCL28 mice as compared with either HIV-VLP<sub>III</sub>B-CCL19 ( $p=0.046$  and  $0.050$ , respectively) or HIV-VLP<sub>III</sub>B alone ( $p=0.040$  and  $0.042$ , respectively) (Fig. 29-30). Naïve and control mice did not show Env-specific antibodies. The observation that CCL28 exerts its adjuvant effects not only in the mucosal system but also systemically is important as it is likely that both lines of defense will be necessary to optimally constraint HIV infection [185].

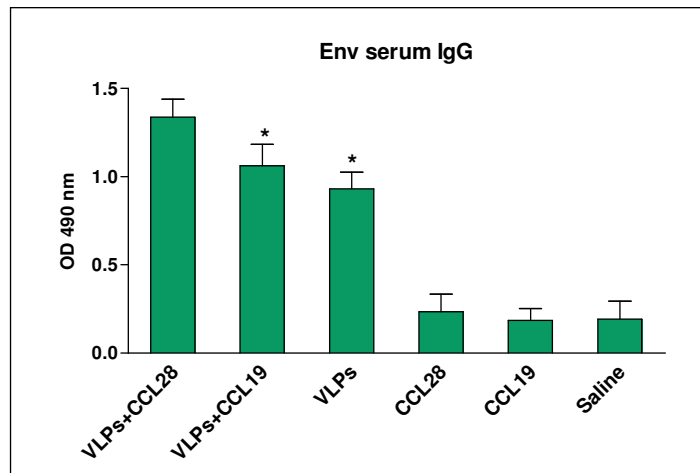


Figure 29: HIV-specific systemic humoral responses. Serum Env-specific IgG levels are shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HIV-VLP<sub>III</sub>B-CCL28 mice).

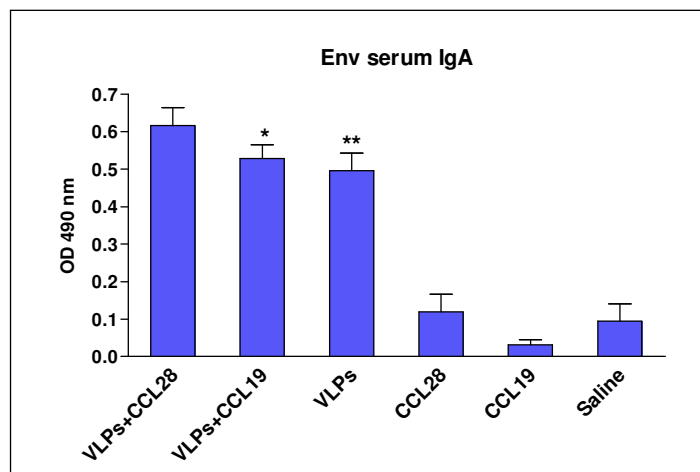


Figure 30: HIV-specific systemic humoral responses. Serum Env-specific IgA levels. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HIV-VLP<sub>III</sub>B-CCL28 mice).

### 1.5 Cytokine production by Env-stimulated splenic and colon cells

To investigate whether CCL28 administration would increase the magnitude of Env-specific cytokine production, splenic and colon T-cells were stimulated with recombinant HIV-1<sub>III</sub>B Env gp120 to quantify Env-specific production of Th1-type (IFN- $\gamma$ ) and Th2-type (IL-4 and IL-5) cytokines.

Results showed a significant increase of IFN- $\gamma$  production by both spleen and colon T-cells of HIV-VLP<sub>III</sub>B-CCL28 mice compared to HIV-VLP<sub>III</sub>B-CCL19 (spleen  $p=0.026$ ; colon  $p=0.033$ ) and VLP alone mice (spleen  $p<0.001$ ; colon  $p=0.002$ ) (Fig. 31).

In these conditions, similar results were observed when both the IL-4 and IL-5 production from HIV-VLP<sub>III</sub>B-CCL28 treated mice was compared with either HIV-VLP<sub>III</sub>B-CCL19 (spleen  $p=0.048$  and  $0.043$ , respectively; colon  $p=0.040$  and  $0.011$ , respectively) or HIV-VLP<sub>III</sub>B alone (spleen  $p=0.002$  and  $<0.001$ , respectively; colon  $p=0.01$  and  $<0.001$ , respectively) (Fig. 32). Naïve and control mice did not show Env-specific cellular responses.



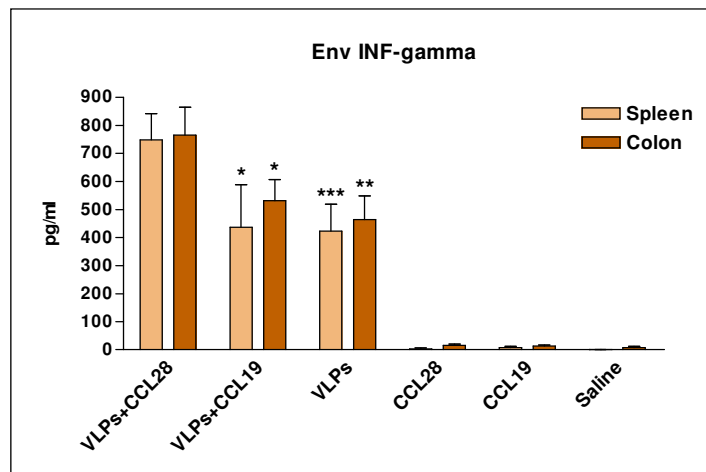


Figure 31: HIV-specific cell mediated responses. Env-specific IFN- $\gamma$  concentration (pg/ml) in the supernatants of splenic and colonic T-cells re-stimulated *ex vivo* with the recombinant HIV-1<sub>IIIB</sub> Env gp120. Splenocytes and cells from Peyer's patches/colon are represented by the orange bars and the brown bars, respectively. Mean values after medium is subtracted, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HIV-VLP<sub>IIIB</sub>-CCL28 mice).

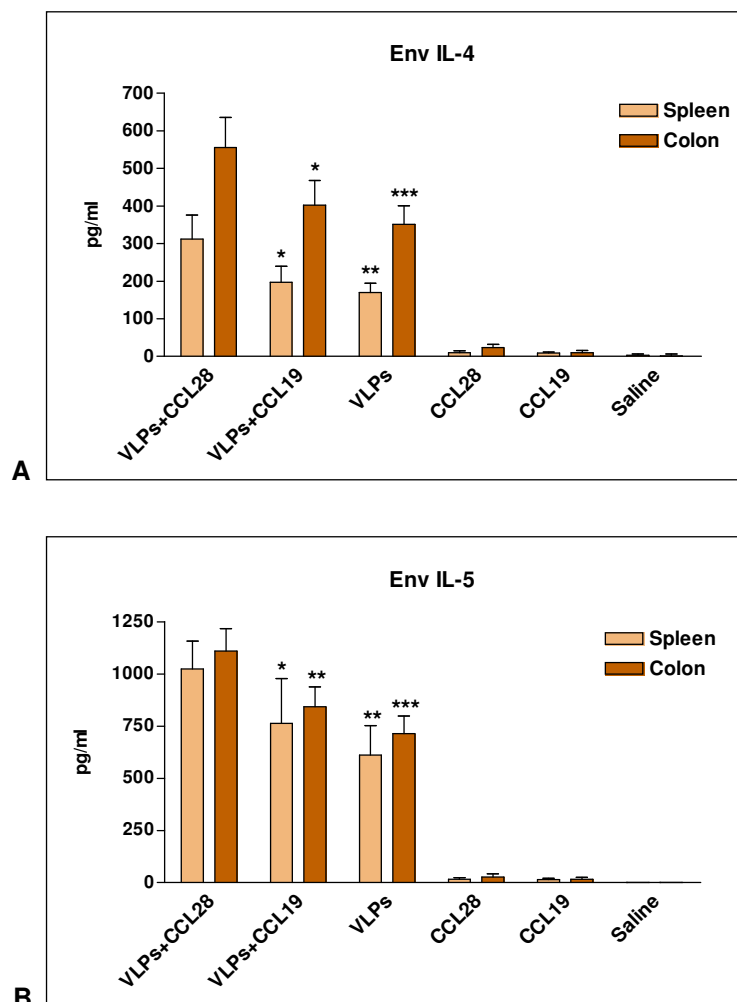


Figure 32: HIV-specific cell-mediated responses. Panel **A** and **B** represent, respectively, Env-specific IL-4 and IL-5 concentration (pg/ml) in the supernatants of splenic and colonic T-cells re-stimulated *ex vivo* with the recombinant HIV-1<sub>IIIB</sub> Env gp120. Splenocytes and cells from Peyer's patches/colon are represented by the orange bars and the brown bars, respectively. Mean values after medium is subtracted, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HIV-VLP<sub>IIIB</sub>-CCL28 mice).

In particular, the increase of Env-stimulated IFN- $\gamma$  production in spleens and colon would suggest the possible stimulation of both systemic and mucosal CTL-mediated responses in CCL28-receiving mice; preliminary results suggest that this is indeed the case. This is particularly relevant in the design of both protective and therapeutic vaccines, as HIV-specific CTLs have been described in several different ESN cohorts and many authors claim that these cells make a fundamental contribution to modulating resistance to HIV infection [185]. Notably, a correlation between IL-5 production in spleen and colon and both systemic and mucosal IgA levels was observed.

### 1.6 IgA<sup>+</sup> plasma cells in the gastro-intestinal mucosal lamina propria

Recent studies have shown that the GALT contains the vast majority of T-cells in the body, and that GALT is the preferential target for HIV replication during the acute phase of HIV infection. This phenomenon results in a massive and systematic destruction of CD4<sup>+</sup> T-cells strongly enhancing the pathogenesis of HIV infection. These observations have further strengthened the importance and urgency of developing mucosal vaccine for the prevention of HIV infection.

Since the gastro-intestinal mucosal lamina propria (MLP) is the principal site of HIV replication during primary infection, induction of adaptive mucosal immunity could obstacle the establishment of HIV infection. As the results herein demonstrated that the up-regulation of CCL28-CCR3/CCR10 circuit is correlated with increased concentrations of both systemic and mucosal HIV-specific IgA, we further verified these data with immune-histochemistry analyses of IgA-ASC distribution in the gastrointestinal (GI) MLP.

IgA<sup>+</sup> plasma cells were clearly identified by immunopositive staining in the cytoplasm of cells with plasma cell-like morphology. Results showed the presence of numerous IgA<sup>+</sup> cells clustered within the lamina propria of the colonic mucosa in HIV-VLP<sub>III</sub>-CCL28 treated mice (mean number and S.E. of IgA<sup>+</sup> plasma cells =  $98.2 \pm 6$ ); this increase was statistically significant compared to all the other groups of mice ( $p < 0.001$  in all cases). In HIV-VLP<sub>III</sub>-CCL19, VLP<sub>III</sub> alone ( $54.8 \pm 3$  plasma cells), CCL28 alone ( $48.2 \pm 2$  plasma cells), CCL19 alone or saline alone mice ( $42.6 \pm 6$  plasma cells), rare and isolated IgA<sup>+</sup> cells were identified by immune-histochemistry suggesting that the adjuvant role of CCL28 results in a massive chemokine-directed recruitment of IgA-ASCs at the mucosal level. Representative results are shown in Fig. 33.

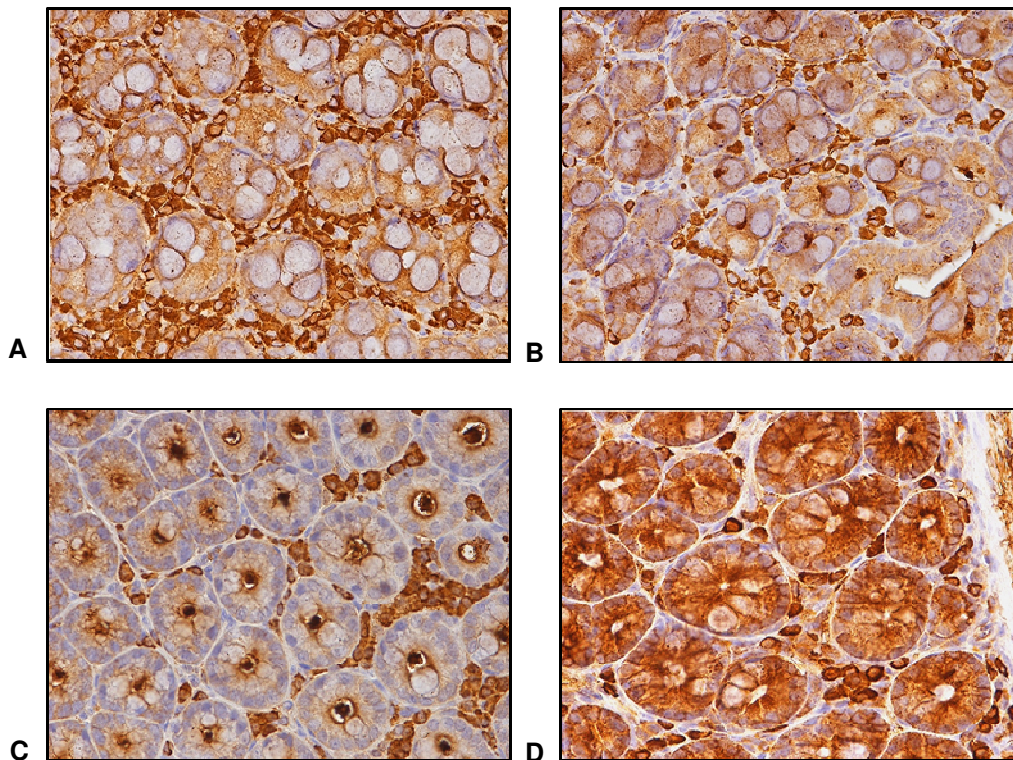


Figure 33: IgA<sup>+</sup> plasma cells. Balb/c mice immunized with HIV-VLP<sub>III</sub> in the presence of CCL28, HIV-VLP<sub>III</sub> in the presence of CCL19, HIV-VLP<sub>III</sub> alone and saline are shown in panels A, B, C and D, respectively. Representative results obtained in the lamina propria of colon mucosa (2-cm specimens from the anus toward the left colon) are presented.

Results presented here indicate that immunization in the presence of CCL28 significantly increases the quantity of mucosal ASCs in the rectum. Although in this study we did not directly measured rectal IgA, it is plausible that increased quantity of tissue IgA-producing ASCs would results in an augmented amount of

secreted antibodies, thus the situation of IgA in rectal washes would parallel what is observed in vaginal washes of CCL28-receiving mice. This could be extremely useful in the design of protective vaccines.

### 1.7 Neutralization activity of sera and mucosal secretions

To verify whether the adjuvant effect of CCL28 could result in an enhanced neutralizing activity of Env-specific antibodies, neutralization experiments against HIV-1<sub>III<sub>B</sub></sub> and HIV-1<sub>DU174</sub> were performed. HIV-1<sub>III<sub>B</sub></sub> is a subtype B CXCR4-tropic strain; HIV-1<sub>DU174</sub> is a subtype C CCR5-tropic strain.

A marginal neutralizing activity (<20%) was detected in both the pre-immune and immune sera of mice treated with either CCL28 alone, CCL19 alone or saline (data not shown). Immune sera from HIV-VLP<sub>III<sub>B</sub></sub>-CCL28 treated mice showed a neutralization activity titer of 200 (50% neutralization activity) against both HIV-1<sub>III<sub>B</sub></sub> and HIV-1<sub>DU174</sub> at a TCID<sub>50</sub> of 40 and 20, respectively. Conversely, immune sera from HIV-VLP<sub>III<sub>B</sub></sub>-CCL19 or VLP<sub>III<sub>B</sub></sub> alone mice showed a neutralization activity titer of 130 against both HIV-1<sub>III<sub>B</sub></sub> and HIV-1<sub>DU174</sub> at the same TCID<sub>50</sub> of 40 and 20, previously used (Fig. 34).

Importantly, immune vaginal secretions from VLP-CCL28-receiving mice showed a neutralization titer of 100 and 60 against, respectively, HIV-1<sub>III<sub>B</sub></sub> and HIV-1<sub>DU174</sub> (Fig. 35). Also in this case the neutralization activity against both clade B and C strains was lower in mice treated with HIV-VLP<sub>III<sub>B</sub></sub>-CCL19 or VLP alone, resulting in a neutralizing titer of 60 and 30, respectively (Fig. 35). Lacking of neutralization activity was observed when HIV-1<sub>III<sub>B</sub></sub> and HIV-1<sub>DU174</sub> were used at a double TCID<sub>50</sub>.

The observation that the neutralization ability of serum and vaginal washes of immunized mice in the presence of CCL28 was increased indicates that the immune modulation induced by this chemokine is associated with an augmented capacity to reduce HIV infectivity.

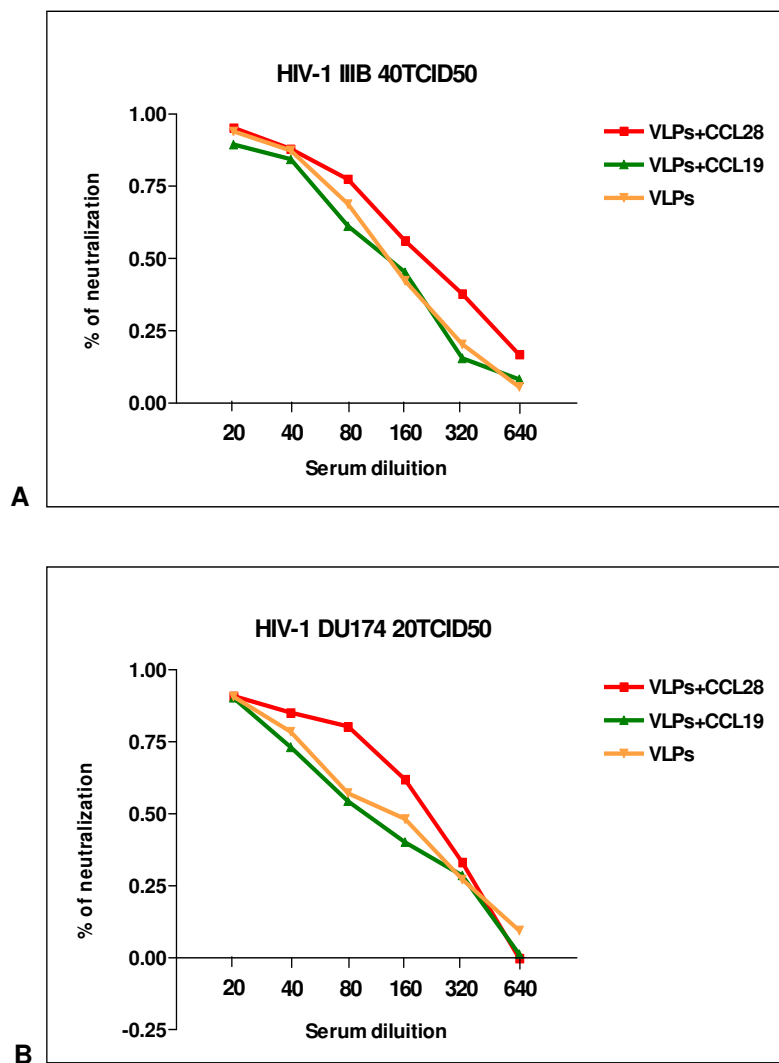


Figure 34: neutralization activity against both a HIV-1 lab strain from clade B and a primary isolate from clade C. Ex vivo neutralizing activity of immune sera against HIV-1<sub>III<sub>B</sub></sub> (A) and HIV-1<sub>DU174</sub> (B) is shown. The neutralization titer of serum antibodies is represented as percentages of the virus replication compared to control samples. The test has been scored positive with a 50% neutralizing activity. Mean titers are indicated.

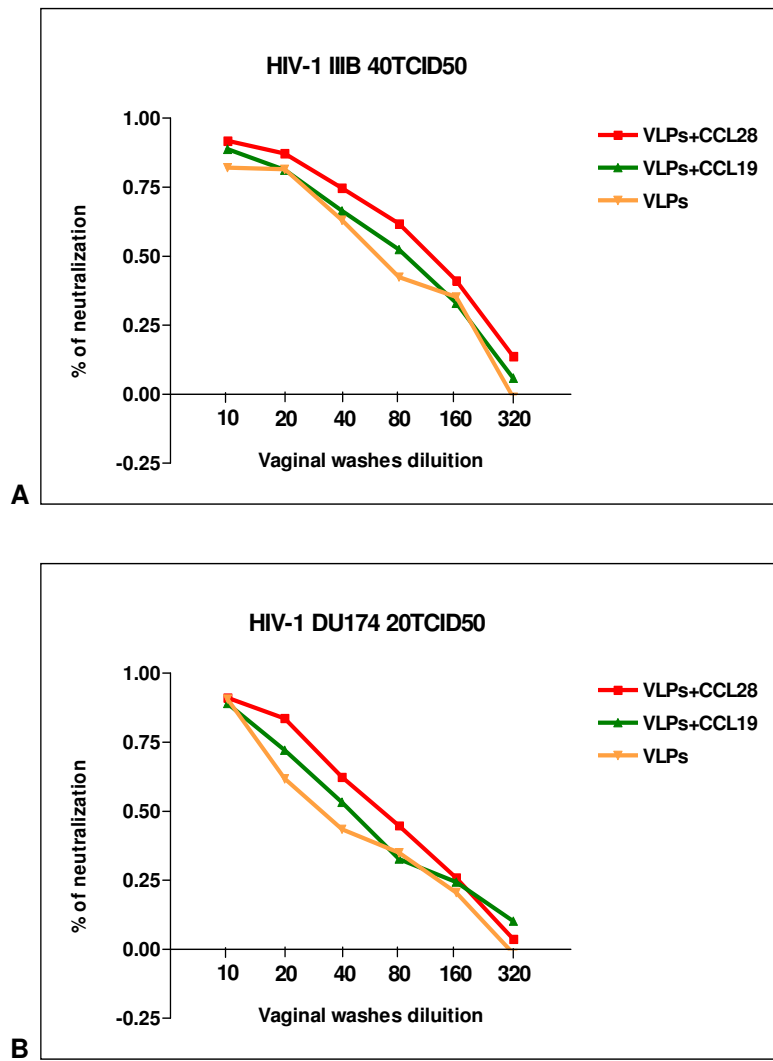


Figure 35: neutralization activity against both a HIV-1 lab strain from clade B and a primary isolate from clade C. Ex vivo neutralizing activity of immune vaginal secretions against HIV-1<sub>IIIIB</sub> (A) and HIV-1<sub>DU174</sub> (B) is shown. The neutralization titer of mucosal antibodies is represented as percentages of the virus replication compared to control samples. The test has been scored positive with a 50% neutralizing activity. Mean titers are indicated.

## 2. H7N1 VLPs-RECEIVING MICE

### 2.1 CCR3- and CCR10- expressing CD19<sup>+</sup> cells

Inbred female Balb/c mice were immunized with a prime-boost regimen using H7N1 VLPs in the presence or in the absence of CCL28-expressing plasmid. The murine CCL19 chemokine gene inserted into a pCpG-free expression vector was used as a negative control (for further details about immunization procedure, see Materials and Methods, paragraph 3.3).

To determine whether intramuscular delivery of the CCL28 expression plasmid would result in a modulation of cognate chemokine receptors on circulating lymphocytes, flow cytometry assessment of CCR3 and CCR10 was performed in CD3<sup>+</sup>, CD14<sup>+</sup> and CD19<sup>+</sup> purified splenocytes.

Results indicated that the CCR3- and CCR10-expression levels in CD3<sup>+</sup> splenocytes as well as CD14<sup>+</sup> cells were similar in the six analyzed groups (data not shown). In contrast, CD19<sup>+</sup>/CCR3<sup>+</sup> and CD19<sup>+</sup>/CCR10<sup>+</sup> splenocytes were significantly augmented in H7N1-VLP-CCL28 treated mice as compared with either H7N1-VLP-CCL19 ( $p=0.033$  and  $0.031$ , respectively), H7N1-VLP alone ( $p=0.005$  and  $0.006$ , respectively), CCL28 alone ( $p=0.003$  and  $<0.001$ , respectively), CCL19 alone ( $p=0.002$  and  $<0.001$ , respectively) or saline controls ( $p=0.003$  and  $<0.001$ ) (Fig. 36).

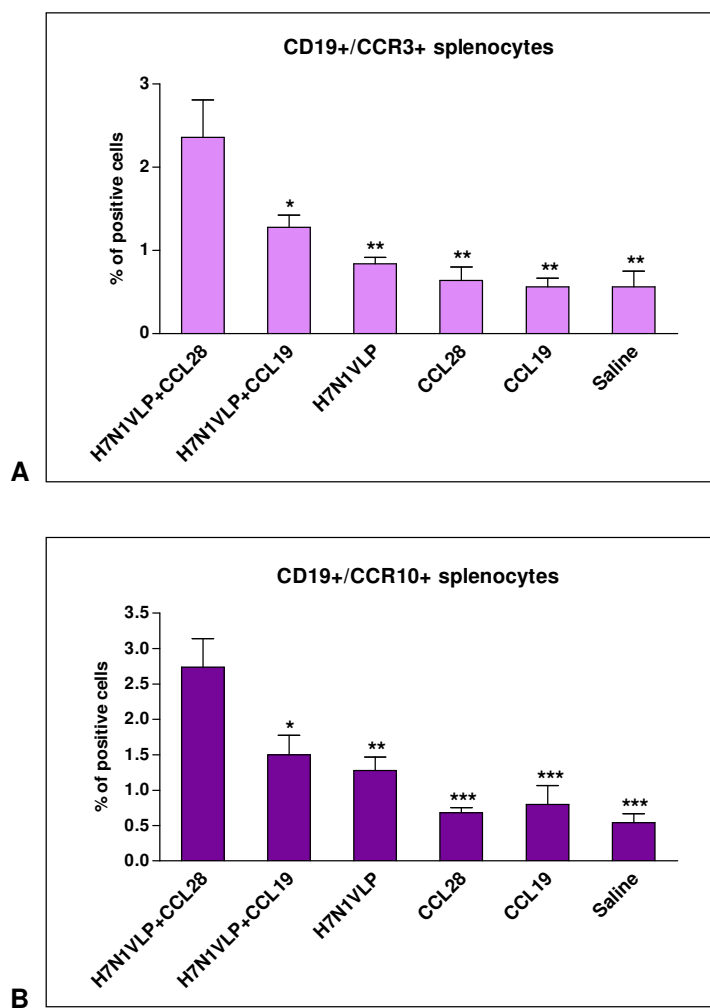


Figure 36: expression of CCR3 and CCR10 on CD19<sup>+</sup> splenocytes; (A) CCR3<sup>+</sup>/CD19<sup>+</sup> splenocytes. (B) CCR10<sup>+</sup>/CD19<sup>+</sup> splenocytes. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs H7N1-VLP-CCL28 mice).

### 2.2 Surface density of CCR3 and CCR10 on CD19<sup>+</sup> cells

Evaluation of the mean fluorescence intensity (MFI) showed that the CCR3 MFI on CD19<sup>+</sup> splenocytes was significantly augmented in H7N1-CCL28 treated mice as compared with either H7N1-CCL19 ( $p=0.041$ ), HIV-VLP<sub>III B</sub> alone ( $p=0.049$ ), CCL28 alone ( $p=0.008$ ), CCL19 ( $p=0.007$ ) or saline controls ( $p=0.007$ ) (Fig. 37, panel A).

The CCR10 MFI on CD19<sup>+</sup> lymphocytes was similarly up-regulated in H7N1-VLP-CCL28 treated mice as compared with either H7N1-VLP-CCL19 ( $p=0.044$ ), H7N1-VLP alone ( $p=0.004$ ), CCL28 alone ( $p<0.001$ ), CCL19 alone ( $p<0.001$ ) or saline controls ( $p<0.001$ ) (Fig. 37, panel B).

We concluded that immunization of mice with HA7-expressing VLPs in the presence of the CCL28-expressing plasmid results in an up-regulation of the whole CCL28-CCR3/CCR10 circuit. It remains to verify whether the expression and the surface density of CCR3 and CCR10 is up-regulated as well in CD19<sup>+</sup> lymphocytes isolated from mucosal secretions.

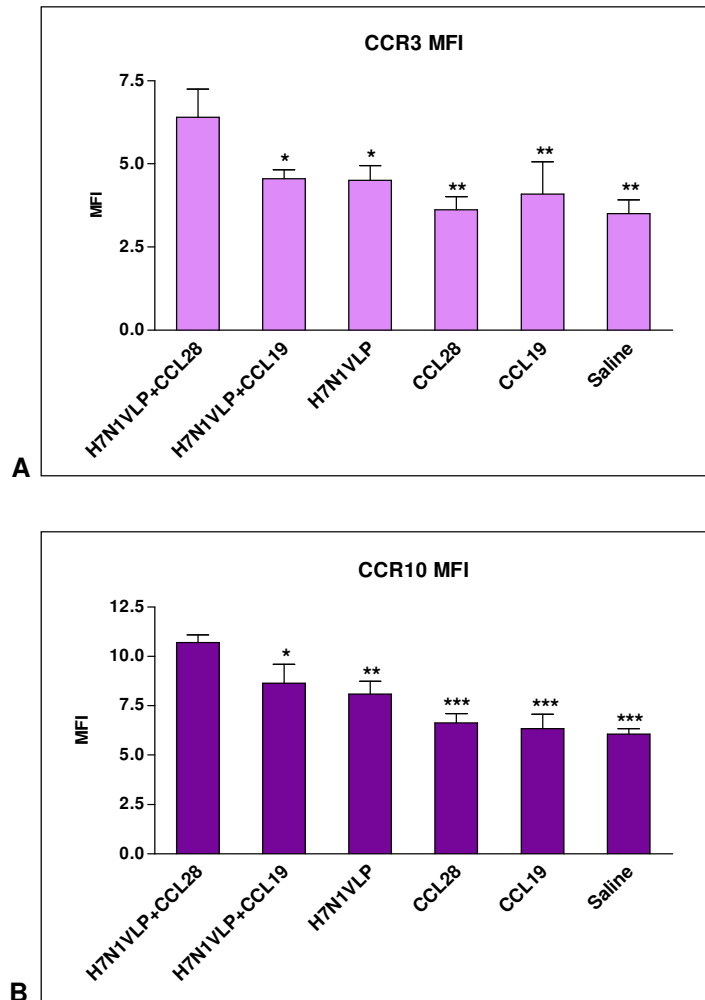


Figure 37: CCR3 and CCR10 mean fluorescence intensity (MFI) on CD19<sup>+</sup> splenocytes; (A) CCR3<sup>+</sup>/CD19<sup>+</sup> splenocytes, (B) CCR10<sup>+</sup>/CD19<sup>+</sup> splenocytes. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs H7N1-VLP-CCL28 mice).

### 2.3 Induction of mucosal total and HA7-specific IgA

Since the respiratory mucosal surfaces are the natural route of entry and the primary replication site of influenza virus, it is important to determine mucosal immune responses.

Mucosal samples from the oral cavity and the respiratory tract, including trachea and lungs, of both immunized and control mice were collected two weeks after each immunization and at week 4, respectively, to determine total and HA7-specific IgA antibodies. Saliva, BALs and lung homogenates are in fact the most commonly used fluids for measurements of humoral mucosal responses elicited by infections or immunization of the respiratory tract.

Total IgA levels were significantly augmented, compared to baseline values, in lung homogenates, BALs and saliva samples of H7N1-VLP-CCL28 mice compared to all the other groups of mice (lung homogenates:  $p<0.04$  in all cases; BALs  $p<0.04$  in all cases; saliva samples:  $p<0.05$  in all cases) (Fig. 38).

Importantly, HA7-specific IgA levels were significantly increased as well in lung homogenates and BALs of H7N1-VLP-CCL28 mice as compared with either H7N1-VLP-CCL19 ( $p=0.049$  and  $0.046$ , respectively) or H7N1-VLP alone ( $p<0.001$  and  $p=0.008$ , respectively) (Fig. 39). Also IgA levels in saliva samples of H7N1-VLP-CCL28 mice were augmented compared with either H7N1-VLP-CCL19 or H7N1-VLP alone ( $p=0.033$ )

(Fig. 39). Naïve and control mice did not show mucosal HA7-specific antibodies. The observed specific mucosal anti-HA7 IgA responses were normalized on the total IgA content. Results presented herein demonstrate that CCL28 efficiently modulates mucosal immune responses in mice immunized with H7N1-VLPs.

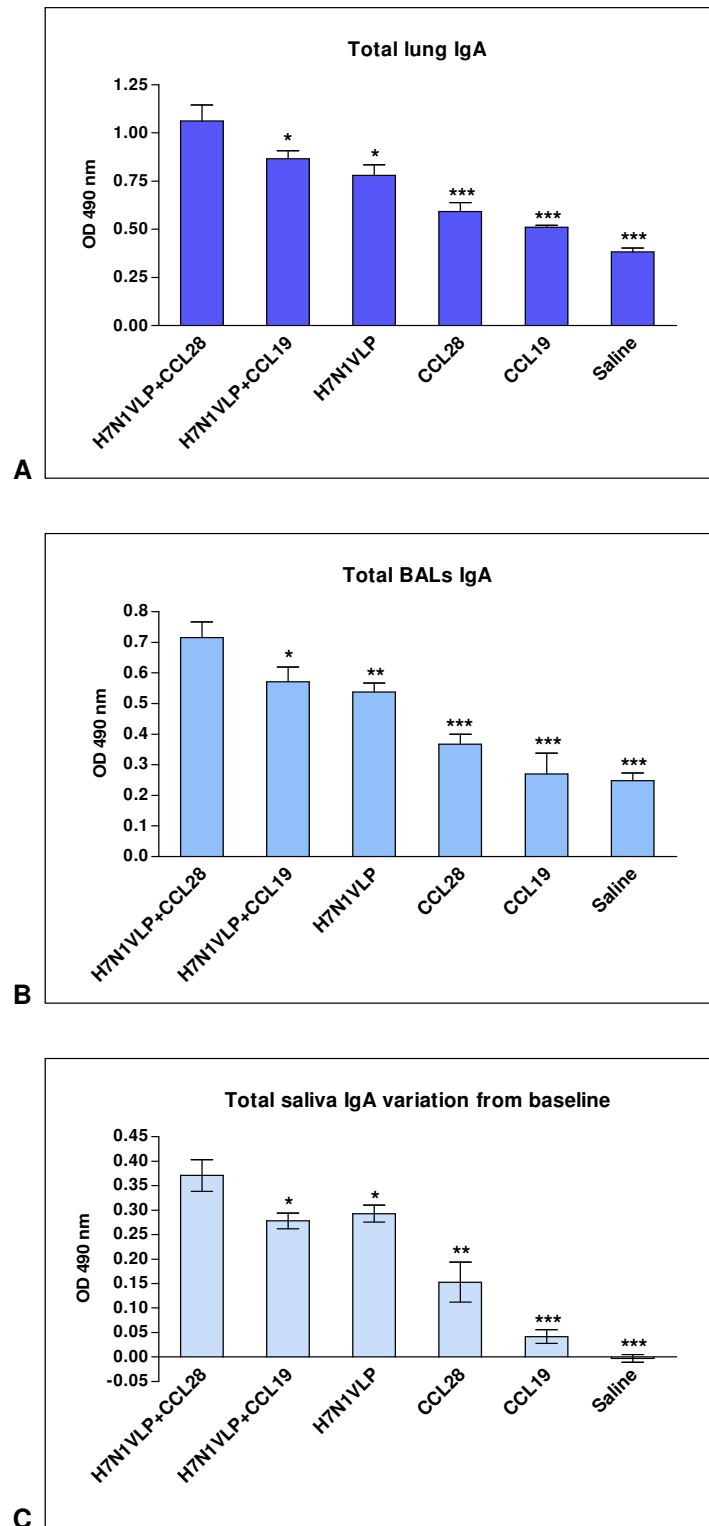


Figure 38: mucosal humoral immunity. Total IgA variation from baseline in lung homogenates (A), BALs (B) and saliva samples (C) is shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs H7N1-VLP-CCL28 mice).

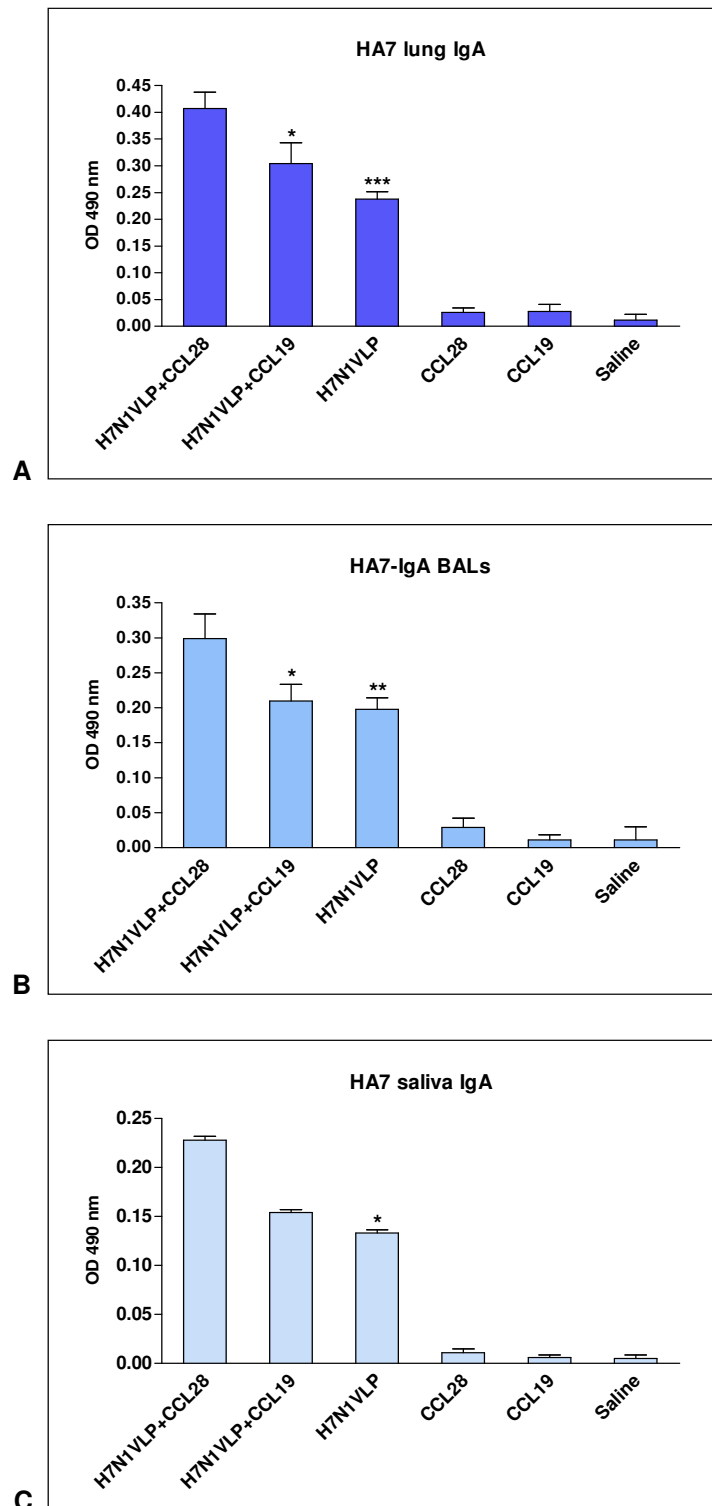


Figure 39: H7N1-specific mucosal humoral immunity. HA7-specific IgA levels in lung homogenates (**A**), BALs (**B**) and saliva samples (**C**) are shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs H7N1-VLP-CCL28 mice).

## 2.4 Induction of HA7-specific systemic humoral response

As protection of the respiratory tract has been recently correlated with the presence of both systemic IgG and mucosal SIgA antibodies [29], sera from immunized and control group mice were collected two weeks after final immunization to measure HA7-specific antibodies in ELISA. Serological tests were performed on microwell plates coated with recombinant HA from Influenza A virus H7N1.

Immunization with H7N1-VLPs in the presence of the CCL28-expressing plasmid resulted in the significant enhancement of serum anti-HA7 IgG and IgA levels as compared with either H7N1-VLP-CCL19 ( $p = 0.044$



and 0.041, respectively) or H7N1-VLP alone mice ( $p=0.03$  and  $0.008$ ) (Fig. 40-41). Naïve and control mice did not show HA7-specific antibodies.

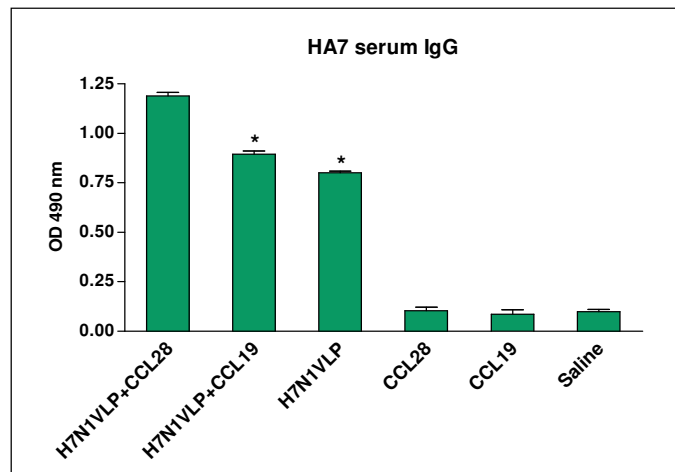


Figure 40: H7N1-specific systemic humoral responses. Serum HA7-specific IgG levels are shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs H7N1-VLP-CCL28 mice).

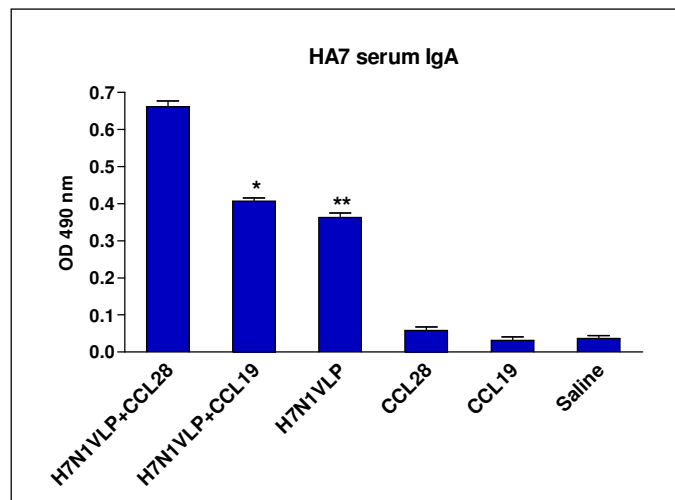


Figure 41: H7N1-specific systemic humoral responses. Serum HA7-specific IgA levels. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs H7N1-VLP-CCL28 mice).

It remains to investigate whether CCL28 immunization in combination with H7N1-VLPs maintains long-lasting protective immune responses, as during the differentiation and development of B-cell memory, a fraction of germinal center B-cells migrate to the bone marrow where they develop into long-lived antibody-secreting PCs.

## 2.5 Cytokine production by HA7-stimulated splenic and lung cells

The ability of the plasmid-encoded CCR3 and CCR10 chemokine ligand to modulate antigen-specific T-cell function in a relevant challenge model was examined next, as the rapid induction of cellular immune responses is important to control viral replication.

Splenic and lung T-cells were stimulated with recombinant HA from Influenza A virus H7N1 to quantify HA7-specific production of Th1-type (IFN- $\gamma$ ) and Th2-type (IL-4 and IL-5) cytokines. Recombinant Influenza HA7 was used because vaccine-induced HA-specific T-cell and antibody responses have been reported to impact Influenza A challenge in the lungs of mice.

Results showed a significant increase of IFN- $\gamma$  production by both spleen and lung T-cells of H7N1-VLP-CCL28 mice compared to H7N1-VLP-CCL19 (spleen  $p=0.049$ ; lungs  $p=0.044$ ) and H7N1-VLP alone mice (spleen  $p=0.46$ ; lungs  $p=0.018$ ) (Fig. 42). In these conditions, similar results were observed when both the IL-4 and IL-5 production from H7N1-VLP-CCL28 treated mice was compared with either H7N1-VLP-CCL19 (spleen IL-5  $p=0.041$ ; lungs IL-5  $p=0.037$ ) or H7N1-VLP alone (spleen  $p=0.043$  and  $0.032$ , respectively;

lungs  $p=0.039$  and  $<0.01$ , respectively) (Fig. 43). Naïve and control mice did not show HA7-specific cellular responses.

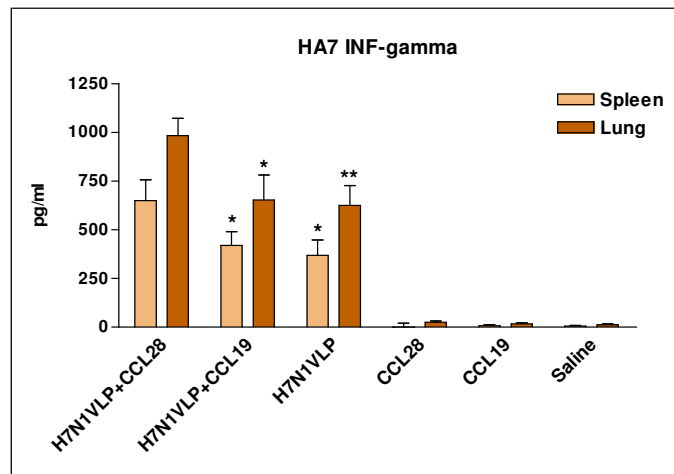
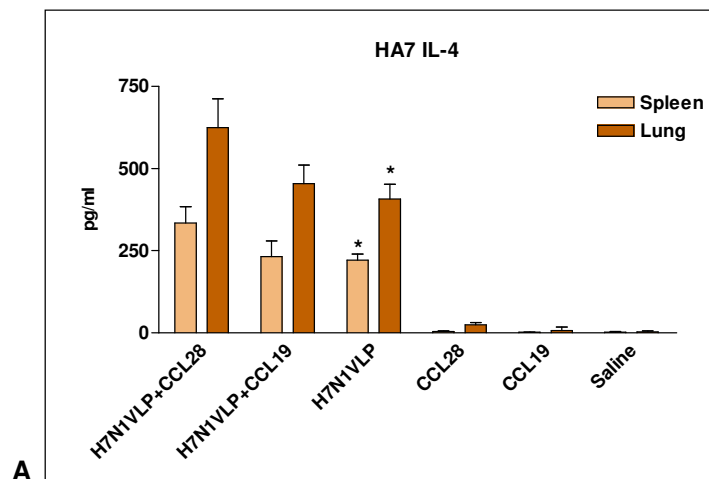
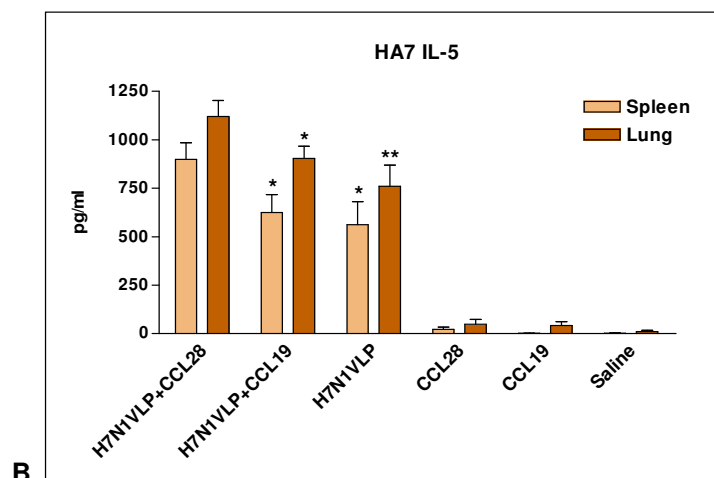


Figure 42: H7N1-specific cell mediated responses. HA7-specific IFN- $\gamma$  concentration (pg/ml) from splenic and lung T-cells re-stimulated ex vivo with the recombinant HA protein from Influenza A virus H7N1. Splenocytes and cells from the lungs are represented by the orange bars and the brown bars, respectively. Mean values after medium is subtracted, standard deviations and statistically significant differences are represented (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs H7N1-VLP-CCL28 mice).



A



B

Figure 43: H7N1-specific cell-mediated responses. Panel A and B represent, respectively, HA7-specific IL-4 and IL-5 concentration (pg/ml) from splenic and lung T-cells re-stimulated ex vivo with the recombinant HA protein from Influenza A H7N1. Splenocytes and cells from lungs are represented by the orange bars and the brown bars, respectively. Mean values after medium is subtracted, standard deviations and statistically significant differences are represented (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs H7N1-VLP-CCL28 mice).

We can conclude that CCL28 chemokine induces both Th1- and Th2-type cellular immune responses at the systemic as well as at the mucosal level. Furthermore, the enhanced HA7-specific IFN- $\gamma$  production in spleen and in lungs would suggest the possible stimulation of CTL-mediated responses in both systemic and mucosal compartments of CCL28-receiving mice; preliminary results suggest that this is indeed the case. In addition, it has been reported that induction of IFN- $\gamma$  secreting T-cells responses may correlate with reduced viral replication in lungs and host protective immunity [186]. Also, there seems to be a correlation between IL-5 production in spleen and lungs and both systemic and mucosal IgA levels.

## 2.6 IgA<sup>+</sup> plasma cells in lungs

Since the natural route of entry of influenza virus is represented by the respiratory mucosal surfaces, including the nose, the oral cavity, trachea and the lungs, induction of adaptive mucosal immunity could prevent the establishment of influenza virus infection.

As the results herein demonstrated that the up-regulation of CCL28-CCR3/CCR10 circuit is correlated with increased concentrations of both systemic and mucosal HA7-specific IgA, we further verified these data with immune-histochemistry analyses of IgA-ASC distribution in the lungs.

IgA<sup>+</sup> plasma cells were clearly identified by immunopositive staining in the cytoplasm of cells with plasma cell-like morphology. Results showed the presence of BALT structures and numerous IgA<sup>+</sup> cells in the lungs of H7N1-CCL28 treated mice (mean number and S.E. of IgA<sup>+</sup> plasma cells =  $78.5 \pm 6$ ); this increase was statistically significant compared to all the other groups of mice ( $p < 0.005$  in all cases). In H7N1-VLP-CCL19 ( $45.1 \pm 4$  plasma cells), H7N1-VLP alone ( $31.2 \pm 2$  plasma cells), CCL28 alone ( $28.4 \pm 4$  plasma cells), CCL19 alone ( $14.3 \pm 2$  plasma cells) or saline alone mice ( $2.1 \pm 4$  plasma cells), the BALT was incompletely formed or totally absent and rare IgA<sup>+</sup> cells were identified by immune-histochemistry, suggesting that the adjuvant role of CCL28 results in a massive chemokine-directed recruitment of IgA-ASCs at the mucosal level. Representative results are shown in Fig. 44.

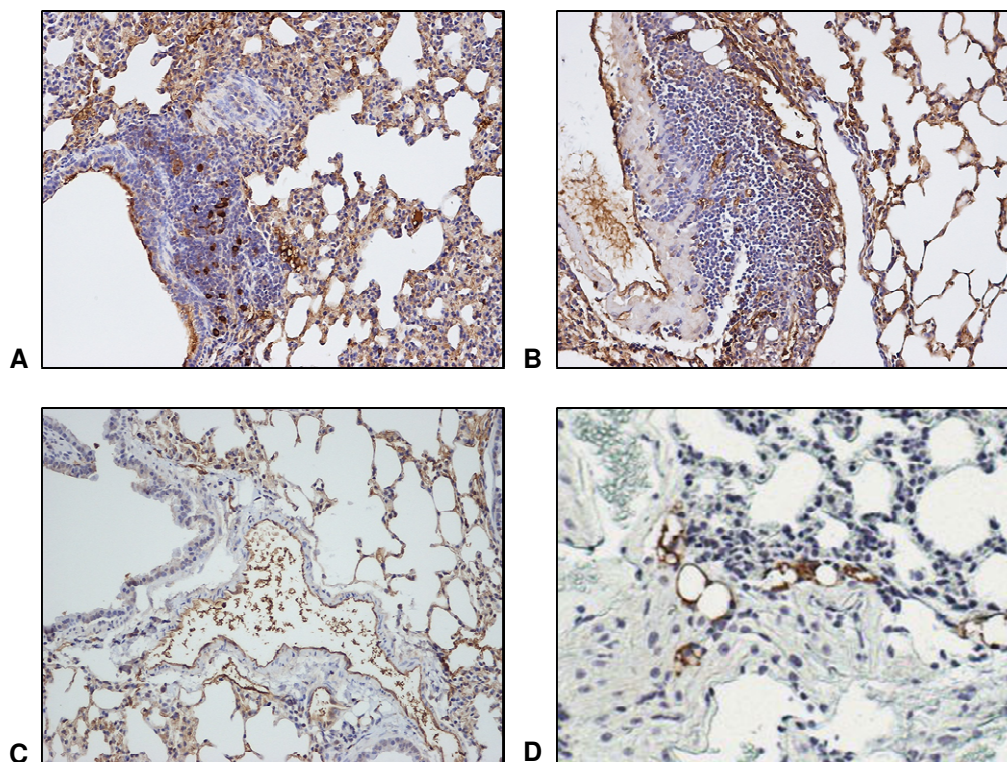


Figure 44: IgA<sup>+</sup> plasma cells. Balb/c mice immunized with H7N1-VLP in the presence of CCL28, H7N1-VLP in the presence of CCL19, HIV-VLP alone and saline are shown in panels A, B, C and D, respectively. Representative results obtained in the lungs are presented.

Results presented here indicate that immunization in the presence of CCL28 significantly increases the quantity of mucosal ASCs in the lungs. This could be extremely useful in the design of protective vaccines.

## 2.7 Neutralization activity of sera and mucosal secretions

Since neutralization activities against influenza virus are an indicator of induction of functional antibodies most likely conferring protective immunity, we performed HAI tests.

The basis of the HAI test is that influenza viruses will hemagglutinate erythrocytes through the interaction of sialic acid and sialic acid receptors on the HA protein. Since the influenza virion can attach to more than one erythrocyte at a time, this allows for cross-linking or clumping of erythrocytes by the virus. This hemagglutination can be inhibited by antibodies directed against the HA protein. Antibodies to the HA are subtype specific, so that antibodies against one subtype will not typically react with another subtype. Therefore, the HAI test has been used as the primary and classical method of identifying the HA subtype of an unknown virus.

Antibodies elicited by the vaccine in pooled sera and in pooled mucosal secretions collected 4 weeks after the first immunization were evaluated for the ability to prevent H7N1 VLPs-induced agglutination of turkey RBCs.

As shown in Fig. 45, panel B, H7N1-VLP-CCL28 mice had serum titer of hemagglutination inhibition antibodies against the Influenza A virus H7N1 of 1:480. In contrast, H7N1-VLP-CCL19 and H7N1-VLP alone mice had HAI titers of 1:160 and 1:120, respectively, and none of the other groups of mice had any detectable HAI antibodies. Interestingly, BALs from H7N1-VLP-CCL28 mice showed an HAI titer of 1:80 that were 2-3 fold higher than the HAI titers from either H7N1-CCL19-VLP or H7N1-VLP animals (1:40 and 1:30, respectively) (Fig. 45, panel C). Immune BALs from either CCL28 alone, CCL19 alone or saline mice showed no HAI antibodies. Naïve mice did not have any detectable HAI antibodies.

HAI antibody titers in the range of 1:40 are required to confer 50% protection against infection and are generally considered an immunological correlate of protection threshold beyond which it is unlikely that serious illness will occur [187]. Delivery of the CCL28-expressing plasmid in combination with H7N1-VLPs results in HAI antibodies that were well above the level to protect 90% of infected mice against Influenza virus A/Chicken/FPV/Rostock/1934.

It remains to verify whether mice vaccinated with the adjuvanted protocol are protected against a challenge with homologous or heterologous viral strains and whether VLP-CCL28-induced responses can lower viral load *in vivo*.

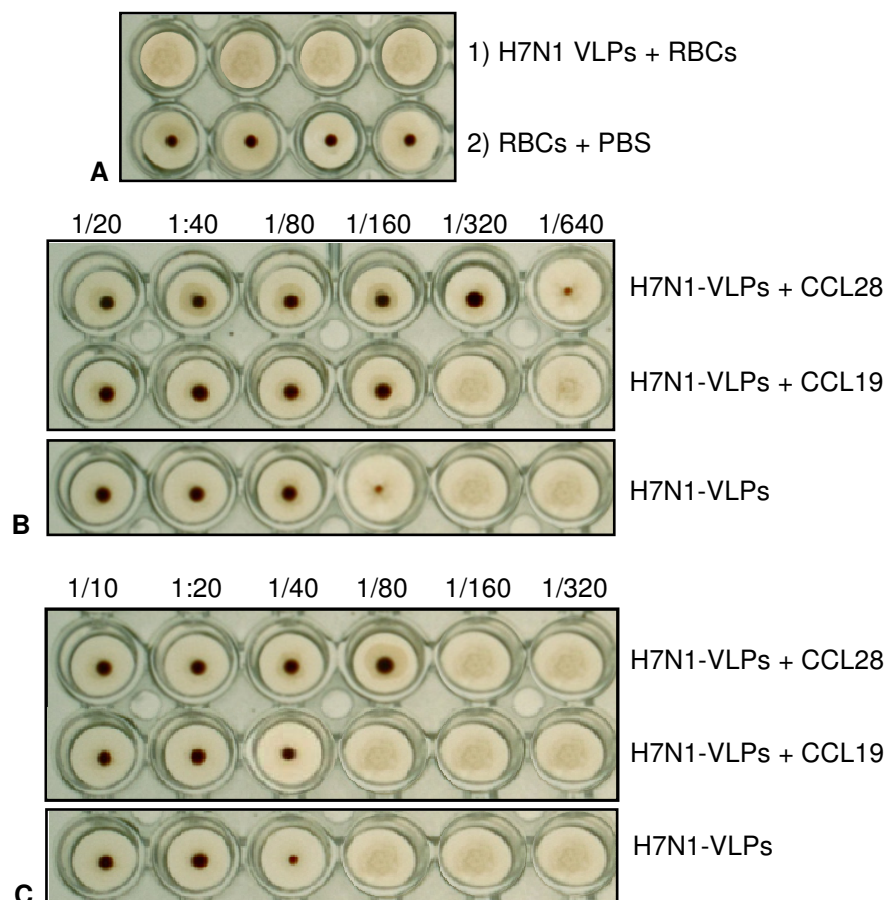


Figure 45: HAI test. Two negative controls are shown in panel **A**: 1) RBC control: RBC + PBS; 2) Agglutination control: H7N1-VLPs + RBC. The VLPs bind to RBC, forming a spot at the bottom of the culture well. When the VLPs were bound to the specific antibodies, the spot disappeared. Panels **B** and **C** show HAI titers of immune sera and BALs at different 2-fold dilutions, respectively.

### 3. HPV-16 VLPs-RECEIVING MICE

#### 3.1 CCR3- and CCR10- expressing CD19<sup>+</sup> cells

Inbred female Balb/c mice were immunized with a prime-boost regimen using HPV-16 VLPs in the presence or in the absence of the CCL28-expressing plasmid. The murine CCL19 chemokine gene inserted into a pCpG-free expression vector was used as a negative control (for further details about immunization procedure, see Materials and Methods, paragraph 4.3).

To analyze the expression of CCL28 chemokine receptors on circulating lymphocytes, flow cytometry assessment of CCR3 and CCR10 was performed in CD3<sup>+</sup>, CD14<sup>+</sup> and CD19<sup>+</sup> purified splenocytes.

CCR3- and CCR10-expression levels in CD3<sup>+</sup> splenocytes as well as CD14<sup>+</sup> cells were similar in the six analyzed groups (data not shown). In contrast, CD19<sup>+</sup>/CCR3<sup>+</sup> and CD19<sup>+</sup>/CCR10<sup>+</sup> splenocytes were significantly augmented in HPV16-VLP-CCL28 treated mice as compared with either HPV16-VLP-CCL19 ( $p=0.005$  and  $0.036$ , respectively), HPV16-VLP alone ( $p=0.009$  and  $0.018$ , respectively), CCL28 alone ( $p=0.004$  in both cases), CCL19 alone ( $p<0.001$  in both cases) or saline controls ( $p<0.001$  in both cases) (Fig. 46).

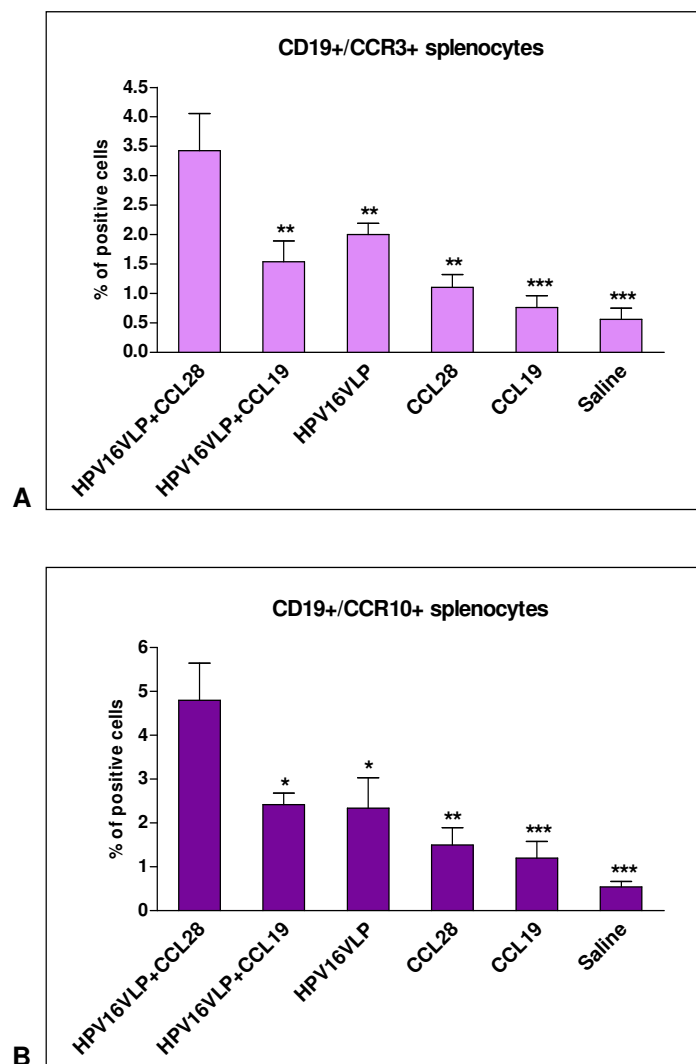


Figure 46: expression of CCR3 and CCR10 on CD19<sup>+</sup> splenocytes; (A) CCR3<sup>+</sup>/CD19<sup>+</sup> splenocytes, (B) CCR10<sup>+</sup>/CD19<sup>+</sup> splenocytes. Mean values, standard deviations and statistically significant differences are indicated represented (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HPV16-VLP-CCL28 mice).

#### 3.2 Surface density of CCR3 and CCR10 on CD19<sup>+</sup> cells

CCR3 mean fluorescence intensity (MFI) on CD19<sup>+</sup> splenocytes was significantly increased in HPV16-VLP-CCL28 treated mice as compared with either HPV16-VLP-CCL19 ( $p=0.026$ ), HPV16-VLP alone ( $p=0.023$ ), CCL28 alone ( $p=0.020$ ), CCL19 alone or saline controls ( $p=0.001$ ) (Fig. 47, panel A).

Similarly, CCR10 MFI on CD19<sup>+</sup> lymphocytes was significantly augmented in HPV16-VLP-CCL28 treated mice as compared with either HPV16-VLP-CCL19 ( $p=0.046$ ), HPV16-VLP alone ( $p=0.035$ ), CCL28 alone ( $p<0.001$ ), CCL19 alone ( $p<0.001$ ) or saline controls ( $p<0.001$ ) (Fig. 47, panel B).

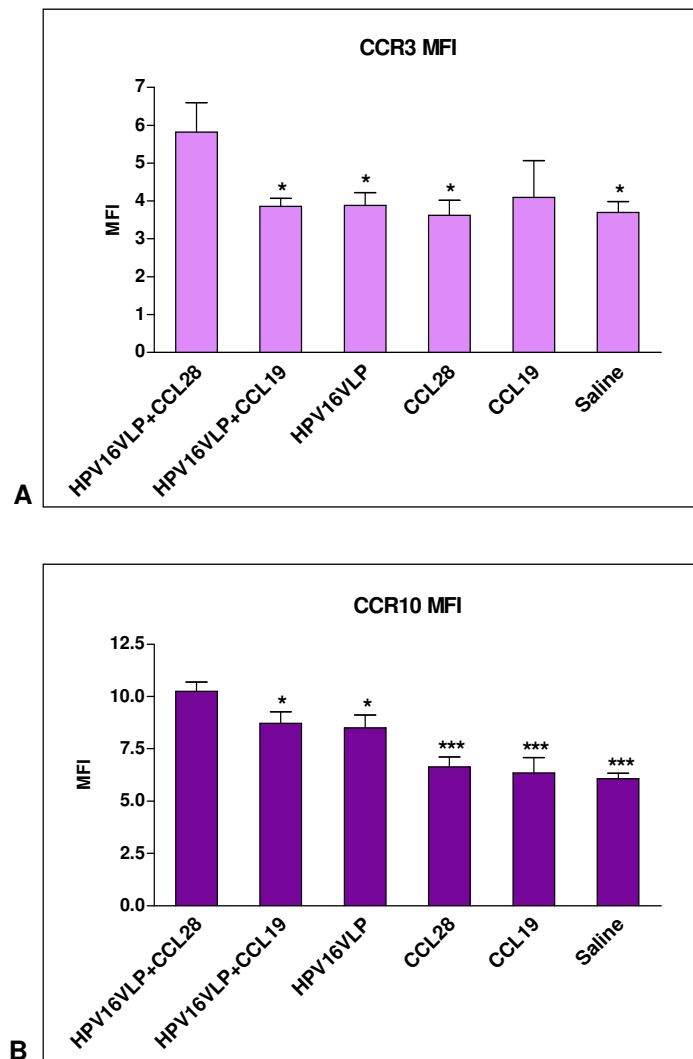


Figure 47: CCR3 and CCR10 mean fluorescence intensity (MFI) on CD19<sup>+</sup> splenocytes; (A) CCR3<sup>+</sup>/CD19<sup>+</sup> splenocytes, (B) CCR10<sup>+</sup>/CD19<sup>+</sup> splenocytes. Mean values, standard deviations and statistically significant differences are indicated (\*  $p<0.05$ , \*\*  $p<0.01$  and \*\*\*  $p<0.001$  vs HPV16-VLP-CCL28 mice).

Results presented herein demonstrated that immunization of mice with L1-expressing VLPs in the presence of the CCL28-expressing plasmid results in the modulation of the whole CCL28-CCR3/CCR10 circuit correlating with a relevant up-regulation of chemokine receptor expression on circulating CD19<sup>+</sup> cells. Nevertheless, the expression and the surface density of CCR3 and CCR10 in CD19<sup>+</sup> lymphocytes isolated from mucosal secretions need to be further investigated.

### 3.3 Induction of mucosal total and L1-specific IgA and IgG antibodies

Studies of HPV-16-specific antibody responses at the genital mucosa have confirmed that type-specific IgA antibodies are detectable at the cervix and are associated with the presence of viral DNA. Cervical IgA from women with active HPV-16 infections have been shown to have virus neutralizing capabilities but do not seem to correlate with viral clearance in healthy women or patients with CIN [188]. Nevertheless, a protective role of IgA antibodies in HPV-16 exposure or early infection cannot be excluded.

Thus, to assess the CCL28-induced enhancement of mucosal humoral responses, total and L1-specific antibody responses were quantified in vaginal secretions of both immunized and control mice.

Total IgA levels were significantly augmented, compared to baseline values, in vaginal secretions of HPV16-VLP-CCL28 mice compared to all the other groups of mice ( $p<0.04$  in all cases) (Fig. 48).

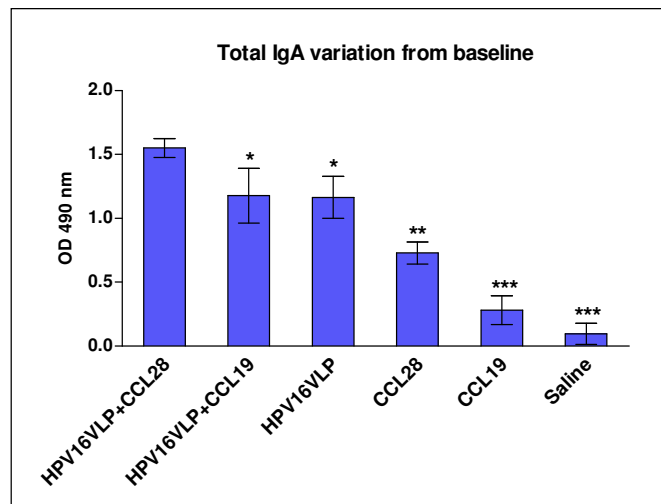


Figure 48: mucosal humoral immunity. Total IgA variation from baseline in vaginal secretions is shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HPV16-VLP-CCL28 mice).

Importantly, L1-specific IgA levels were detectable and significantly increased as well in vaginal secretions of HPV16-VLP-CCL28 mice as compared with either HPV16-VLP-CCL19 ( $p = 0.040$ ) or HPV16-VLP alone mice ( $p = 0.002$ ) (Fig. 49). Vaginal secretions from pre-immunized as well as control mice did not have specific antibodies against the HPV-16 L1 protein. The observed specific mucosal anti-L1 IgA responses were normalized on the total IgA content. Also L1-specific IgG levels were significantly augmented in vaginal secretions of HPV16-VLP-CCL28-receiving mice compared with the all other groups, most likely reflecting serum IgG levels (data not shown).

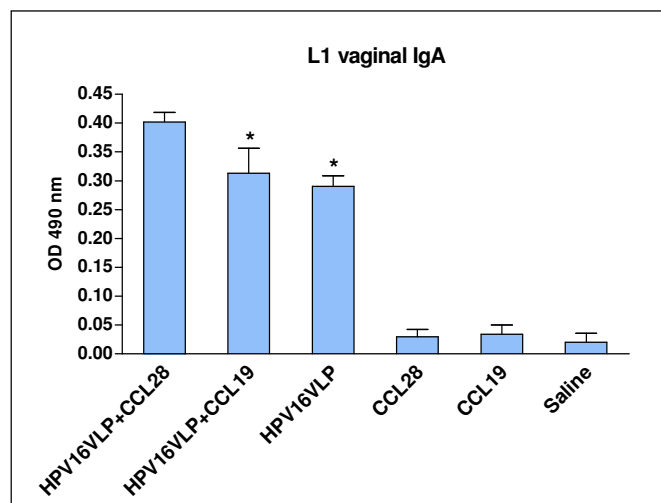


Figure 49: HPV-16-specific mucosal humoral immunity. L1-specific IgA levels in vaginal secretions are shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HPV16-VLP-CCL28 mice).

Results presented herein demonstrate that immunization of mice with L1-expressing VLPs in the presence of CCL28 up-regulate potentially beneficial mucosal immune responses against HPV-16 infection.

### 3.4 Induction of L1-specific systemic humoral response

Sera from immunized and control group mice were collected two weeks after final immunization to measure L1-specific antibodies. ELISA tests were performed on microwell plates coated with recombinant L1 protein from HPV-16.

Serum anti-L1 IgG and IgA levels were significantly increased in HPV16-VLP-CCL28 mice as compared with either HPV16-VLP-CCL19 ( $p = 0.042$  and  $0.05$ , respectively) or HPV16-VLP alone animals ( $p = 0.002$  and  $0.004$ , respectively) (Fig. 50-51). Pre-immunized and control mice did not have serum L1-specific antibodies.

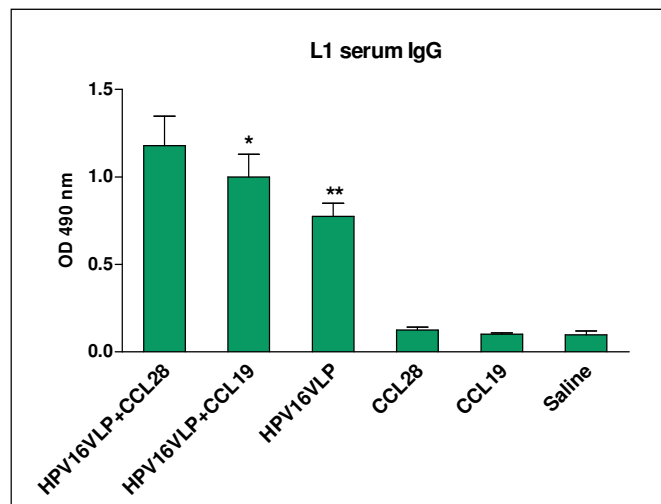


Figure 50: HPV-16-specific systemic humoral responses. Serum L1-specific IgG levels are shown. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are indicated (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HPV16-VLP-CCL28 mice).

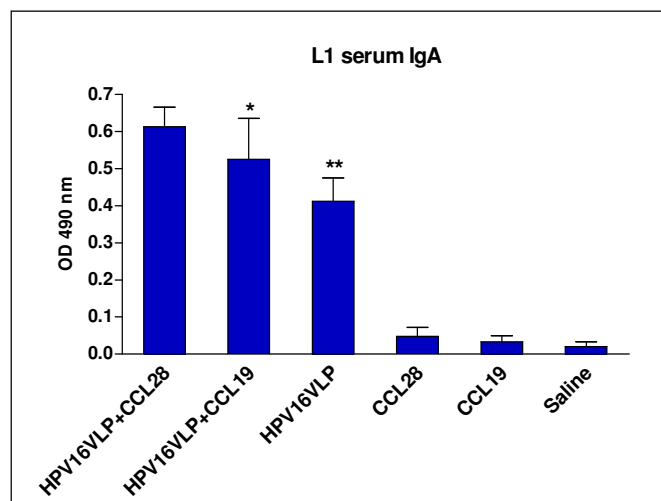


Figure 51: HPV-16-specific systemic humoral responses. Serum L1-specific IgA levels. Absorbance was read at 490 nm. Mean values, standard deviations and statistically significant differences are indicated (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HPV16-VLP-CCL28 mice).

The high levels of systemic antibodies detected in sera of immunized mice is in contrast to the situation after natural infection that leads only 50-70% of infected individuals to detectable antibodies of low titers. This difference may be explained by the much higher dose of antigen that is administered during vaccination. More importantly, it is based upon the exposure of the viral particles to the immune system in the non-natural compartment as happens after intramuscular immunization.

The observation that CCL28 exerts its adjuvant effects not only in the mucosal system but also systemically is important as it is likely that both lines of defense will be necessary to optimally constraint HPV-16 infection [189]. Furthermore, circulating antibodies induced in the vaccination, particularly IgG, reach the mucosal surface most likely by diffusion ("transudation") into the cervical mucosa, thus preventing infection [190].

### 3.5 Cytokine production by L1-stimulated splenic and cervical cells

To investigate whether CCL28 administration would affect L1-specific cytokine production, splenic and cervical T-cells were stimulated with recombinant HPV-16 L1 protein to quantify L1-specific production of Th1-type (IFN- $\gamma$ ) and Th2-type (IL-4 and IL-5) cytokines.

Results showed a significant increase of IFN- $\gamma$  production by both spleen and cervical cells of HPV16-VLP-CCL28 mice compared to HPV16-VLP-CCL19 (spleen  $p = 0.040$ ; cervix  $p = 0.028$ ) and VLP alone mice (spleen  $p = 0.026$ ; cervix  $p = 0.01$ ) (Fig. 52). The IL-4 and IL-5 production was also augmented in HPV16-VLP-CCL28 treated mice as compared with either HPV16-VLP-CCL19 (spleen IL-5  $p = 0.048$ ; cervix  $p = 0.039$  and  $0.045$ , respectively) or HPV16-VLP alone (spleen  $p = 0.041$  and  $0.008$ , respectively; cervix  $p = 0.032$  and  $0.005$ , respectively) (Fig. 53). Naïve and control mice did not show L1-specific cell-mediated responses.



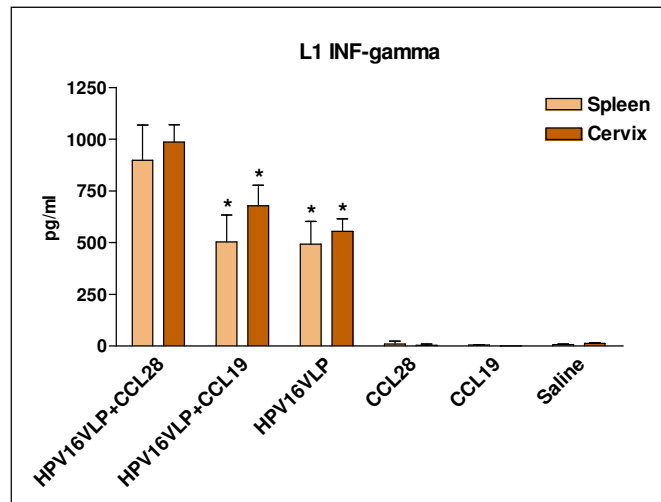


Figure 52: HPV-16-specific cell mediated responses. L1-specific IFN- $\gamma$  concentration (pg/ml) in the supernatants of splenic and cervical T-cells re-stimulated *ex vivo* with the recombinant L1 protein from HPV-16. Splenocytes and cells from the uterine cervix are represented by the orange bars and the brown bars, respectively. Mean values after medium is subtracted, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HPV16-VLP-CCL28 mice).

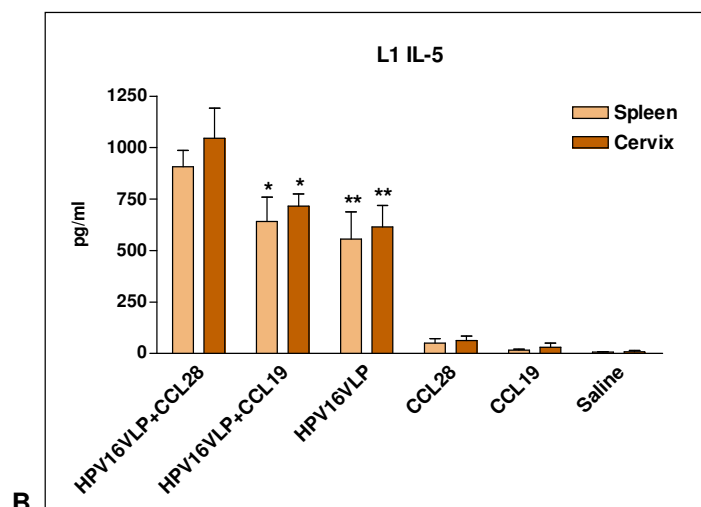
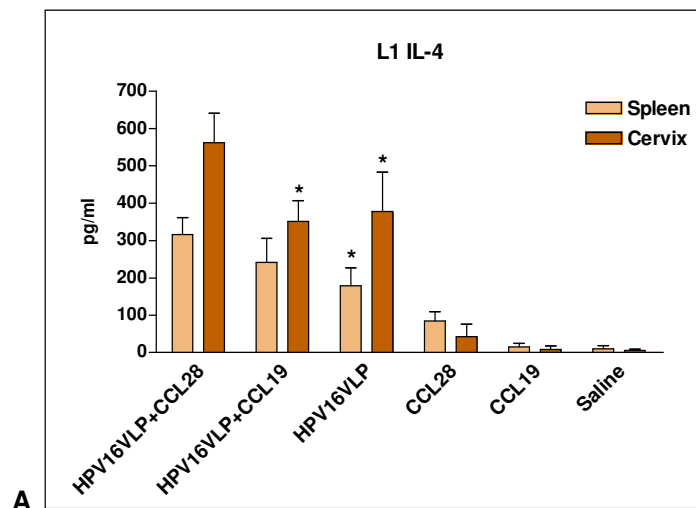


Figure 53: HPV-16-specific cell-mediated responses. Panels **A** and **B** represent, respectively, L1-specific IL-4 and IL-5 concentration (pg/ml) from splenic and cervical T-cells re-stimulated *ex vivo* with the recombinant L1 protein from HPV-16. Splenocytes and cells from the uterine cervix are represented by the bars and the bars, respectively. Mean values after medium is subtracted, standard deviations and statistically significant differences are represented (\*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$  vs HPV16-VLP-CCL28 mice).

Preliminary results suggest that the increase of L1-stimulated IFN- $\gamma$  production in the spleen and in the uterine cervix also depends on the stimulation of CTL-mediated responses in CCL28-receiving mice. HPV-16 L1-specific Th1-type T-cell immunity is induced in human subjects by either natural exposure to transient HPV-16 infections, the persistence of this virus in low- or high-grade cervical lesions or by L1-VLP vaccinations. Studies on the rate of genital wart clearance have shown that persisting warts are characterized by the absence of immune infiltration, whereas wart regression is associated with both CD4+ and CD8+ T-cell infiltration into the wart stroma and epithelium [191]. The presence of L1-specific Th1 T-cell responses may therefore assist in protective immunity, although the detection of these T-cells, unlike that of Th1 T-cell immunity against E2 and/or E6, is not indicative of successful control of this virus [192]. Importantly, the increased production of IL-5 correlates with the higher levels of IgA detected in the peripheral blood and in vaginal secretions of HPV16-VLP-CCL28 treated mice.

### 3.6 IgA<sup>+</sup> plasma cells in the cervical mucosa

HPV type 16 is the most prevalent HPV type associated with cervical intraepithelial neoplasia and cervical cancer worldwide, with >50% of women with cervical disease being infected with HPV-16. Since the cervical tract is the site of HPV-16 infection and replication, induction of adaptive mucosal immunity could therefore serve a major role in protection against HPV.

As the results herein demonstrated that the up-regulation of CCL28-CCR3/CCR10 circuit is correlated with increased concentrations of both systemic and mucosal L1-specific IgA, we further verified these data with immune-histochemistry analyses of IgA-ASC distribution in the cervical mucosa.

IgA<sup>+</sup> plasma cells were clearly identified by immunopositive staining in the cytoplasm of cells with plasma cell-like morphology. The number of IgA<sup>+</sup> cells in the stroma of the uterine cervix of HPV16-VLP-CCL28 treated mice (mean number and S.E. of IgA<sup>+</sup> plasma cells =  $25.6 \pm 3$ ) was higher than that of all the other groups ( $p < 0.05$  in all cases). In HPV16-VLP-CCL19 ( $18.4 \pm 3$  plasma cells), HPV16-VLP alone ( $2.8 \pm 2$  plasma cells), CCL28 alone ( $16 \pm 1$  plasma cells), CCL19 alone ( $8.8 \pm 3$  plasma cells) or saline alone mice ( $10 \pm 2$  plasma cells), rare or none IgA<sup>+</sup> cells were identified by immune-histochemistry suggesting that the adjuvant role of CCL28 results in an enhanced chemokine-directed recruitment of IgA-ASCs at the mucosal level. Representative results are shown in Fig. 54.

Results presented herein indicate that immunization in the presence of CCL28 increases the quantity of mucosal ASCs in the genital tract.

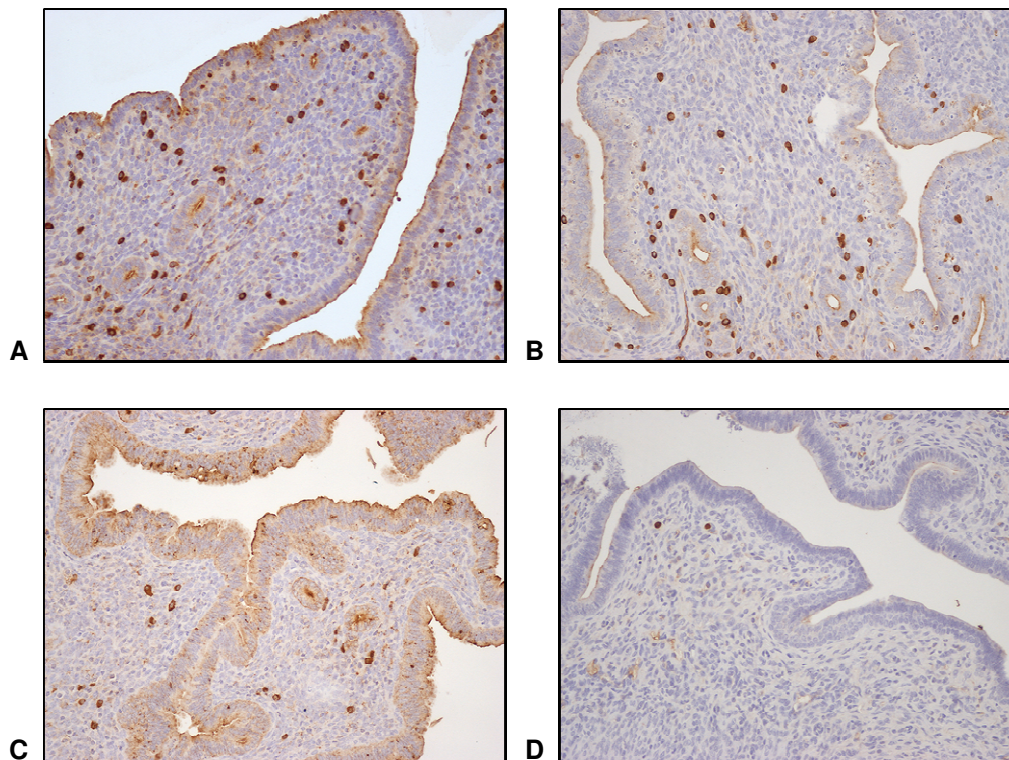


Figure 54: IgA<sup>+</sup> plasma cells. Balb/c mice immunized with HPV-16-VLP in the presence of CCL28, HPV-16-VLP in the presence of CCL19, HIV-VLP alone and saline are shown in panels A, B, C and D, respectively. Representative results obtained in the uterine cervix are presented.

### 3.7 Neutralization activity of sera and mucosal secretions

Protection against virally infections is typically conferred by neutralizing antibodies. There are several arguments that this is also true for papillomaviruses.

Antibodies against mucosal HPVs protect in some cases against reinfection by the same type, the efficacy towards protection probably depends upon the titer [193]. The original definition of HPV types made on the basis of the nucleotide sequence of their DNA (genotypes) seems to correlate with the antigenic properties of the respective virus particles (serotypes). Sera from naturally infected individuals of from vaccinated women show no or only a limited response to other HPV types, suggesting that during evolution different HPV types have developed as a consequence of the selection pressure by the humoral immune system. Furthermore, direct proof for a protective role of antibodies stems from experiments with animal HPVs in their natural host. The lesions induced by these viruses that closely resemble the HPV-related diseases were shown to be preventable by previous immunization with virus particles [194].

Thus, in order to assess neutralizing activity of the detected L1-specific antibodies of immunized mice, pooled sera and pooled vaginal secretions were tested in a hemagglutination inhibition assay as previously described [182].

Sera from HPV16-VLP-CCL28 mice showed a neutralization activity at a dilution of 1:320, that was 4-5 fold higher than the HAI titers from either HPV16-VLP-CCL19 or HPV16-VLP alone mice (1:80 and 1:60, respectively) (Fig. 55, panel B). Importantly, vaginal secretions showed a neutralizing activity at a dilution of 1:40, that was 2-fold higher than the HAI titers from either HPV16-VLP-CCL19 or HPV16-VLP alone mice (1:20) (Fig. 55, panel C). Pre-immune samples as well as immune sera and vaginal washes from either CCL28 alone, CCL19 alone or saline mice showed no HAI antibodies.

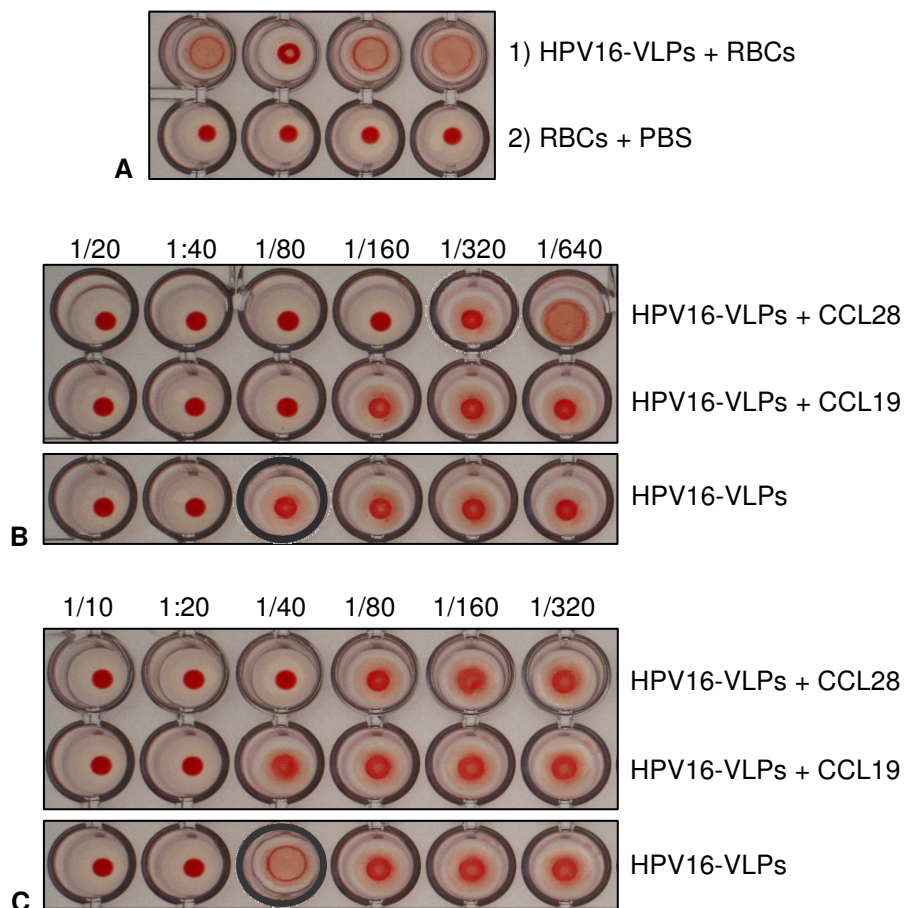


Figure 55: HAI test. Two negative controls are shown in panel **A**: 1) RBC control: RBC + PBS; 2) Agglutination control: HPV16-VLPs + RBC. The VLPs bind to RBC, forming a circle at the bottom of the culture well. When the VLPs were bound to the specific antibodies, the circles disappeared. Panels **B** and **C** show HAI titers of immune sera and vaginal secretions at different 2-fold dilutions, respectively.

Neutralizing activity of vaginal secretions from HPV16-VLP-CCL28 mice might be referred not only to IgG [193] but also to IgA antibodies specific for HPV-16 L1 protein, suggesting that CCL28 delivery as an expression plasmid in combination with HPV-16 VLPs could prevent HPV-16 infection of epithelial cells and induction of HPV-related diseases. These data provide the basis for further investigations on the induction of mucosal immune responses against sexually transmitted diseases.

## ***Conclusions***

Mucosal surfaces provide portals of entry for pathogens. A large number of mucosal infections continue to represent a challenge for the development of both prophylactic and therapeutic vaccines. Pathogens in this category include respiratory viruses, such as Influenza virus, and sexually transmitted disease (STD) pathogens, particularly HIV and HPV.

Influenza A is one of the major threats to human and animal health due to its high transmissibility and the potentially severe morbidity and mortality among susceptible hosts. Early vaccination is one of the most effective means to mitigate a future influenza pandemic. Nowadays, the two types of licensed influenza vaccines provide incomplete protection, due in part to the limited mucosal immunity and cytotoxic T-cell response, and the short duration of the protective immunity elicited by the vaccines.

The vast majority of newly acquired HIV infections are sexually transmitted. In this modality of infection the virus initially targets the vaginal or rectal mucosa, thus preventative vaccines or microbicides should be designed to protect such mucosae. With the exception of moderately promising results of the RV144 trial (the so called "Thai trial"), no candidate vaccine for HIV infection has been successful. Preventive approaches based on the prevention of HIV infection through the use of topical microbicides have also been unsuccessful, even if possible, breakthrough results were recently shown (Vienna AIDS conference). Notably, the necessity to protect mucosal tissues notwithstanding, as most of vaccine strategies are designed to play a therapeutic role via the elicitation of systemic immune responses.

Cervical cancer is the second leading cause of cancer mortality in women worldwide. The causal role of a subset of HPVs in cervical carcinogenesis has now been clearly established, since high-risk HPV DNA has been found in virtually all cervical cancer samples, HPV-16 being the most prevalent. A prophylactic vaccine that targets these HPV types might therefore reduce the incidence of this cancer and precursor lesions. The leading candidate is a subunit HPV VLP vaccine that has proven to be very efficient in preventing both persistent genital HPV infection and associated lesions. However, although VLP vaccination induces readily detectable IgG at the cervix, the level of antibody decreases several fold during ovulation, which might impair the long term protective effects of the vaccine. Indeed, induction of locally produced specific SIgA in mucosal secretions, in addition to serum-derived Igs, could represent a potential approach to overcome these difficulties.

In the design of effective prophylactic strategies against the viral infections mentioned above, VLPs represent an antigen delivery system currently under investigation as potential vaccine for different human viruses. VLPs are non-replicating particles that are similar in conformation to intact virus and are able to stimulate both arms of the immune system by entering both MHC class I and II presentation pathways.

Furthermore, one strategy that could improve immunogenicity of mucosal vaccines is the use of chemokine adjuvants. Genetic adjuvants encoding cytokines or chemokines represent a means of modifying the magnitude, duration and nature of the immune response generated *in vivo*. Few studies have measured the effects of genetic adjuvants encoding chemokines on induction of systemic and local immunity, and specifically of the CCR3 and CCR10 receptor ligand, CCL28 (also known as MEC, Mucosae-associated Epithelial Chemokine). Expression of CCR10 and its ligand within mucosal and epithelial tissue at the gastrointestinal and lung bronchial sites has been reported to have an important role in the trafficking of specific immune cells to these sites of pathogen exposure. In particular, CCR10, in response to signals delivered by CCL28, contributes to the broader localization of IgA-ASCs to the small intestine, colon and bronchi. It is well established that activation or differentiation of T-cells can alter the expression of specific chemokine receptors and thereby increase their sensitivity or responsiveness to cognate chemokine ligands. In addition, chemokines and their receptors can be up-regulated during inflammatory stimulation, leading to preferential recruitment of effector lymphocytes to specific tissue microenvironments.

We analyzed therefore a systemically administered vaccination strategy characterized by intramuscular vaccination with CCL28 plasmid adjuvant in three mouse models receiving HIV-1<sub>IIIIB</sub> VLPs, H7N1 VLPs and HPV-16 VLPs, respectively.

Results presented herein demonstrate that immunization of mice with either HIV-1<sub>IIIIB</sub> Env-, Influenza A HA7- or HPV-16 L1-expressing VLPs in the presence of CCL28 results in the modulation of the whole CCL28-CCR3/CCR10 circuit correlating with a robust up-regulation of the immunization-induced antigen-specific immune response.

Thus, cytokine secretion by spleen and colon or lung or uterine cervix, depending on the primary site of viral replication, as well as total and antigen-specific IgA and IgA<sup>+</sup> plasma cells were significantly augmented in the presence of CCL28. Notably, antigen-specific IgA and IgG levels were significantly increased in serum as well, suggesting that, systemic as well as mucosal immune responses are efficiently modulated by CCL28. Finally, the observation that the neutralization ability of serum and vaginal secretions of immunized mice in the presence of CCL28 was significantly increased indicates that the immune modulation induced by this chemokine is associated with an augmented capacity to reduce HIV or Influenza A virus H7N1 or HPV-16 infectivity.

The observation that CCL28 exerts its adjuvant effects not only in the mucosal system but also systemically is important as it is likely that both lines of defense will be necessary to optimally constraint viral infection. Thus, both the level of antigen-specific IgA and the neutralization ability of immune sera were significantly up-regulated in mice receiving CCL28. In addition, antigen-specific cytokine responses were increased in

spleen and mucosal tissues of these mice, indicating that this vaccine protocol might be able to elicit both types of immune responses. In particular, the increase of antigen-specific IFN- $\gamma$  production would suggest the possible stimulation of CTL-mediated responses both in the peripheral blood and in MALT structures of CCL28-receiving mice; preliminary results suggest that this is indeed the case.

Recent studies have shown that the GALT contains the vast majority of T-cells in the body and represents the preferential target for HIV replication during the acute phase of HIV infection. This phenomenon results in a massive and systematic destruction of CD4<sup>+</sup> T-cells strongly enhancing the pathogenesis of HIV infection. The respiratory tract, including trachea and lungs, is the natural route of entry of Influenza A viruses and is at high risk of developing severe complications especially from Influenza A viruses, which may include hemorrhagic bronchitis, pneumonia and death in people with chronic pulmonary or cardiac disease, or diabetes mellitus. Finally, the cervical tract, which represents the preferential replication site of HPV-16, is susceptible to the development of cervical intraepithelial neoplasia and cervical cancer worldwide, with >50% of women with cervical disease being infected with HPV-16. These observations have further strengthened the importance and urgency of developing mucosal vaccine for the prevention of HIV or Influenza A virus or HPV-16 infection.

Results presented here indicate that immunization on the presence of CCL28 significantly increases the quantity of mucosal ASCs in the rectum of HIV-1<sub>III<sub>B</sub></sub>-VLP-receiving mice, in the lungs of H7N1-VLP-receiving mice and in the uterine cervix of HPV16-VLP-receiving mice. This could be extremely useful in the design of protective vaccines.

As IgA are mostly mucosal antibodies that are responsible for the first line of defense of adaptive immunity against pathogens, it is reasonable to infer that vaccine procedures capable of eliciting a strong IgA response would be beneficial by contributing to the containment of viral infection.

Our results show that the objective of up-regulating potentially beneficial mucosal immune responses is achievable in the mouse model by the use of CCL28 as an adjuvant. The increased neutralization ability toward at least two different HIV-1 clades detected in immune sera and in vaginal secretions of HIV-1<sub>III<sub>B</sub></sub>-VLP-receiving mice indicates that the reduction of HIV infectivity *in vivo* could be achieved by such adjuvant. This is also desirable for Influenza A virus and HPV infection, as vaccination in the presence of CCL28 results in an enhanced neutralizing activity of sera and mucosal secretions against the homologous H7N1 clade A strain and HPV type 16, respectively.

The cell-mediated and humoral responses were not detected with the suboptimal vaccine doses used to critically evaluate adjuvant effect on B-cell responses. However, *in vivo* effects on B-cell proliferation and isotype switching consistent with the high dose *in vitro* results might well occur at levels of T-cell activity too low to be measured by the insensitive *in vitro* assays.

In conclusion, results presented herein indicate that CCL28 used as an adjuvant has a robust immunomodulatory effect on potentially beneficial mucosal and systemic immune responses. Therefore, the development of effective mucosal chemokine DNA vaccine adjuvants that can be delivered parenterally has implications for prophylactic immunization strategies that require both peripheral and mucosal physiologically relevant antigen-specific immunity.

Nevertheless, the efficacy evaluation of CCL28 adjuvanticity requires further experiments to define immune correlates of mucosal responses to vaccination with VLPs in combination with chemokine adjuvants.

***Appendix***

## PRINCIPLES OF CYTOMETRY

The flow cytometry allows automatic analysis of monodisperse cell suspensions, by measuring the physical and/or biochemical in a laminar flow that is hit by a beam of monochromatic light. By cytometry you can capture and store multiple parameters for each measured cell, such as volume, granularity and fluorescence, using different windows electronic analysis on specific populations, discriminated according to different parameters brands.

The principle underlying the cytometry is the possibility to analyze each individual event of the test sample, thanks to a fluidic system that generates a single line of cells. While proceeding, the individual cells through a measuring point where interacting with the beam of excitation. The fluorescent emission signal generated (due to the phenomena of diffraction, refraction and reflection), is collected by a system of lenses, dichroic mirrors and optical filters and sent to the sensors (photomultiplier) that measures the amplitude of signal. The signals from each sensor are digitized, combined with each other and sent to a computer that provides the presentation of data and their statistical definition.

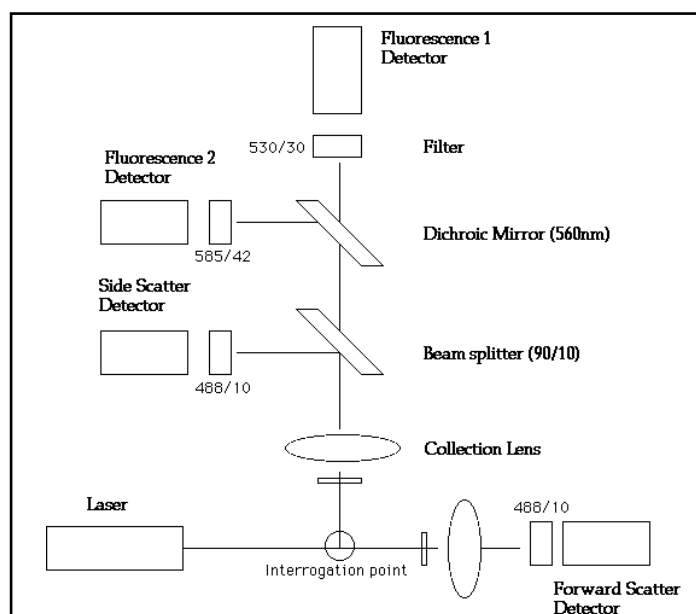


Figure 1: schematic representation of a cytometer: laser, dichroic filters, band pass filter and photomultiplier are in evidence.

The cytometer consists of four main components (Figure 1), detailed explaining in the following paragraphs:

- fluidics system, that allows to obtain a monodisperse flux of cells;
- optics and detectors, that allow the excitation of the fluorochrome and the detection of fluorescent signal and of the physical and/or biochemical characteristic of cells;
- signal processing, that allows the conversion of light in analogical signals, that can be received by a computer;
- data analysis, that allows to display data acquired in different graphic representations.

Another special component of the flow cytometer (not described here) permits to separate cells according to subtype or epitope expression for further biological studies. This process is called cell sorting or FACS™ analysis. After the sample is hydrodynamically focused by fluidic system, each particle is probed with a beam of light: the scatter and fluorescence signal is compared to the sort criteria set on the instrument. If the particle matches the selection criteria, the fluid stream is charged as it exits the nozzle of the fluidics system. In the following paragraphs, explanations of the single part of the flow cytometer are presented.

### Fluidic System

When a sample in solution is injected into a flow cytometer, the particles are randomly distributed in three-dimensional space. The sample must therefore be ordered into a stream of single particles that can be interrogated by the machine's detection system. This process is managed by the fluidics system.

Essentially, the fluidics system consists of a central channel/core through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This alters the velocity of the central fluid whose flow front becomes parabolic with greatest velocity at its centre and zero velocity at the wall (Figure 2). The effect



creates a single line of particles and is called hydrodynamic focusing. Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid.

Without hydrodynamic focusing the nozzle of the instrument (typically 70  $\mu\text{M}$ ) would become blocked, and it would not be possible to analyze one cell at a time.

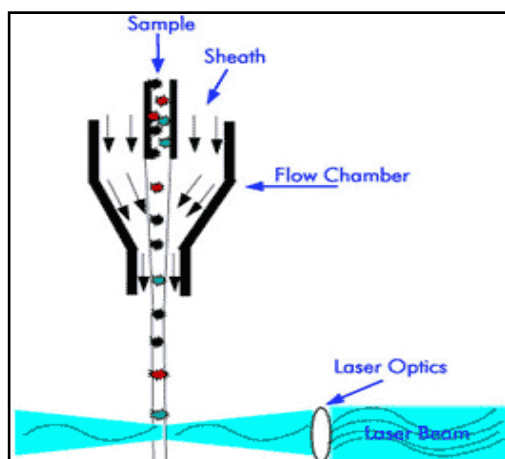


Figure 2: hydrodynamic focusing produces a single stream of particles

### Optics and detection

After hydrodynamic focusing, each particle passes through one or more beams of light. Light scattering or fluorescence emission (if the particle is labelled with a fluorochrome) provides information about the particle's properties. The laser and the arc lamp are the most commonly used light sources in modern flow cytometry (in particular, argon is the most used because its wavelength of 488 nm, that can excite the fluorochromes most common currently in use).

However, this produces unstable incoherent light of a mixture of wavelengths, which needs subsequent optical filtering. Light that is scattered in the forward direction, typically up to 20° offset from the laser beam's axis, is collected by a lens known as the forward scatter channel (FSC). The FSC intensity roughly equates to the particle's size and can also be used to distinguish between cellular debris and living cells.

Light measured approximately at a 90° angle to the excitation line is called side scatter. The side scatter channel (SSC) provides information about the granular content within a particle. Both FSC and SSC are unique for every particle, and a combination of the two may be used to differentiate different cell populations in an heterogeneous sample.

Fluorescence measurements taken at different wavelengths can provide quantitative and qualitative data about fluorochrome-labelled cell surface receptors or intracellular molecules such as DNA and cytokines. Flow cytometers use separate fluorescence (FL-) channels to detect light emitted. The number of detectors will vary according to the machine and its manufacturer. Detectors are either silicon photodiodes or photomultiplier tubes (PMTs). Silicon photodiodes are usually used to measure forward scatter when the signal is strong. PMTs are more sensitive instruments and are ideal for scatter and fluorescence readings.

The specificity of detection is controlled by optical filters, which block certain wavelengths while transmitting (passing) others. There are three major filter types. 'Long pass' filters allow through light above a cut-off wavelength, 'short pass' permit light below a cut-off wavelength and 'band pass' transmit light within a specified narrow range of wavelengths (termed a band width). All these filters block light by absorption (Figure 3).

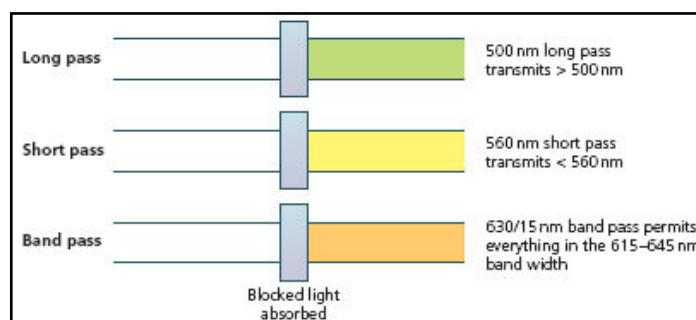


Figure 3: different type of optical filters

When a filter is placed at a 45 degrees angle to the oncoming light it becomes a dichroic filter/mirror. As the name suggests, this type of filter performs two functions, first, to pass specified wavelengths in the forward

direction and, second, to deflect blocked light at a 90 degrees angle. To detect multiple signals simultaneously, the precise choice and order of optical filters will be an important consideration (Figure 4)

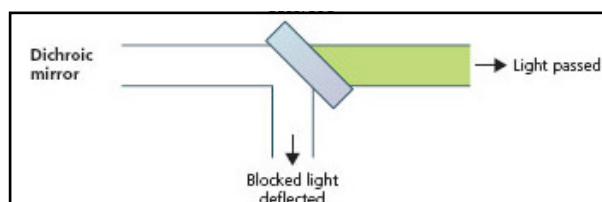


Figure 4: schematic representation of how work a dichroic mirror

### Signal processing

When light hits a photodetector a small current (a few microamperes) is generated. Its associated voltage has an amplitude proportional to the total number of light photons received by the detector. This voltage is then amplified by a series of linear or logarithmic amplifiers, and by analog to digital convertors (ADCs), into electrical signals large enough (5–10 volts) to be plotted graphically.

Log amplification is normally used for fluorescence studies because it expands weak signals and compresses strong signals, resulting in a distribution that is easy to display on a histogram. Linear scaling is preferable where there is not such a broad range of signals e.g. in DNA analysis.

The measurement from each detector is referred to as a 'parameter' e.g. forward scatter, side scatter or fluorescence. The data acquired in each parameter are known as the 'events' and refer to the number of cells displaying the physical feature or marker of interest.

### Data analysis

The data analysis software permits to analyse the data acquired also in presence of different colour (multicolour) of fluorescence and to represent the data themselves in different graphic formats. See now in details both of this important function of the software system.

One consideration to be aware of when performing multicolor fluorescence studies is the possibility of spectral overlap (Figure 5).

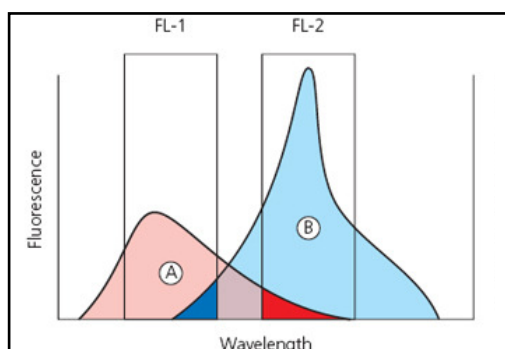


Figure 5: spectral overlap and fluorescent compensation (dark blue shade represents the proportion of B that overlaps into the FL1 channel. Red shade represents the proportion of A that interfere in channel FL2; compensation is able to eliminate these interferences)

When two or more fluorochromes are used during a single experiment there is a chance that their emission profiles will coincide, making measurement of the true fluorescence emitted by each difficult. Instead, a process called fluorescence compensation is applied during data analysis, which calculates how much interference (as a %) a fluorochrome will have in a channel that was not assigned specifically to measure it (Figure 5; in this examples, the spill-over fluorescent of A in FL2 is 17%, whereas of B in FL1 is about 5%). So, this mean that when the two fluorochromes are used for a dual colour experiment, the true reading for fluorochrome A is (total fluorescence measured in FL1) minus (5% of fluorochrome B's total fluorescence). Similarly, for B, the true signal is (total fluorescence measured in FL2) minus (17% of fluorochrome A's total fluorescence). Fortunately, modern flow cytometry analytical software applies fluorescence compensation mathematics automatically, which simplifies matters considerably.

An important principle of flow cytometry data analysis is to selectively visualize the cells of interest while eliminating results from unwanted particles e.g. dead cells and debris. This procedure is called gating. Cells have traditionally been gated according to physical characteristics. For instance, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Also, dead cells have lower forward scatter and higher side scatter than living cells. Lysed whole blood cell analysis is the most

common application of gating, and Figure 6 depicts typical graphs for SSC versus FSC when using large cell numbers. The different physical properties of granulocytes, monocytes and lymphocytes allow them to be distinguished from each other and from cellular contaminants.

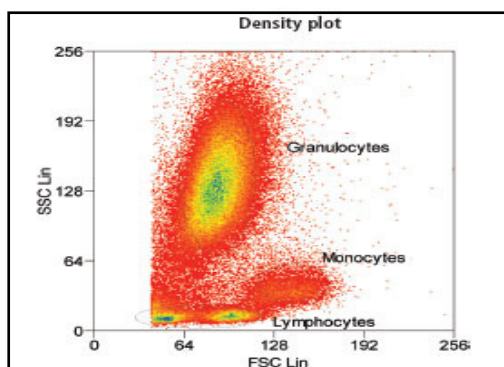


Figure 6: analysis of lysed whole blood using FSC/SSC

On the density plot, each dot or point represents an individual cell that has passed through the instrument. Yellow/green hotspots indicate large numbers of events resulting from discrete populations of cells. The colours give the graph a three-dimensional feel.

To represent data deriving from fluorochrome labelling, two different ways are used: the single-parameters histograms and two-parameters histograms.

The first type display a single measurement parameter (relative fluorescence or light scatter intensity) on the x-axis and the number of events (cell count) on the y-axis: the histogram is a very basic representation but is useful for evaluating the total number of cells in a sample that possess the physical properties selected for or which express the marker of interest. Cells with the desired characteristics are known as the positive dataset. Ideally, flow cytometry will produce a single distinct peak that can be interpreted as the positive dataset. However, in many situations, flow analysis is performed on a mixed population of cells resulting in several peaks on the histogram. In order to identify the positive dataset, flow cytometry acquisition should be repeated in the presence of an appropriate negative control.

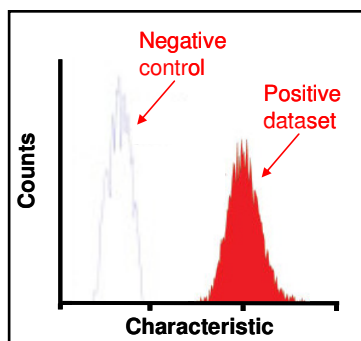


Figure 7: schematic representation of a single parameter histogram

The two-parameter histograms are graphs that display two measurement parameters, one on the x-axis and one on the y-axis (Figure 8), and the cell count as a density (dot) plot or contour map. The parameters could be SSC, FSC or fluorescence. This graph permits to evaluate the correlation between two data set acquired.

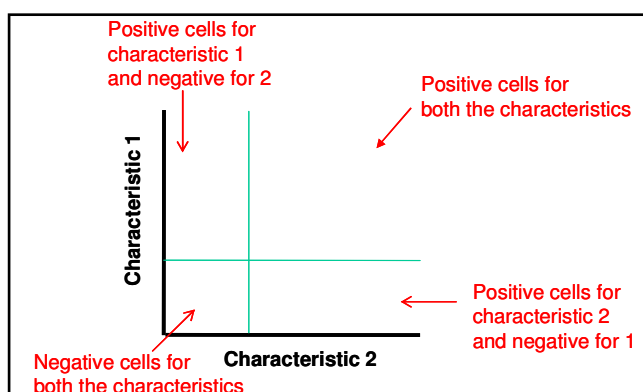


Figure 8: schematic representation of two-parameters graph

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