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PhD thesis

Adjuvant mucosal immunization of mice against influenza virus

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CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF PAPERS

This PhD thesis is based on the following papers:

ZANVIT P, HAVLÍČKOVÁ M, TÁČNER J, JIRKOVSKÁ M, PETRÁSKOVÁ P, NOVOTNÁ O, ČECHOVÁ D, JULÁK J, ŠTERZL I, PROKEŠOVÁ L: Immune response after adjuvant mucosal immunization of mice with inactivated influenza virus. Immunol Lett. 2005 Mar 15; 97(2):251-9.

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Submitted to Folia Microbiologica

ZANVIT P, TICHOPÁD A, HAVLÍČKOVÁ M, TÁČNER J, NOVOTNÁ O, JIRKOVSKÁ M, KOLOŠTOVÁ K, ČECHOVÁ D, JULÁK J, ŠTERZL I AND PROKEŠOVÁ L: Adjuvant effect of *Bacillus firmus* on the expression of cytokines and toll-like receptors in mice nasopharynx-associated lymphoid tissue (NALT) after intranasal immunization with inactivated influenza virus type A.
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ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cells
BAL	Bronchoalveolar lavage
BALT	Bronchus-Associated Lymphoid Tissue
BCR	B cell receptor
BD2	Beta defensin 2
BF	*Bacillus firmus*
BIR	Baculoviral inhibitor-of-apoptosis-protein repeat-containing domain
CALT	Conjunctiva-Associated Lymphoid Tissue
CARD	Caspase-recruitment domain
CDC	Centers for disease control
CFR	Complement-fixation reaction
CLN	Cervical lymph nodes
CpG	2´deoxyribo(cytidine-phosphate-guanosine)
CRAMP	Cathelicidin-related antimicrobial peptide
CRS	Cryptdin-related sequence peptides
CT	Cholera toxin
CTL	Cytotoxic T-lymphocyte
CTLA	Cytotoxic T lymphocyte antigen
DALT	Salivary duct-Associated Lymphoid Tissue
DBF	Delipidated *Bacillus firmus*
DCs	Dendritic cells
dsRNA	Double-stranded RNA
EC	Epithelial cell
FAE	Follicle-associated epithelium
G-	Gram negative bacteria
G+	Gram positive bacteria
GALT	Gut-Associated Lymphoid Tissue
GITR	Glucocorticoid-induced tumor necrosis factor receptor
HA	Haemagglutinin
HBD-1	Human beta-defensin 1

HIT	Hemagglutination inhibition test
HIV	Human immunodeficiency virus
HNP	Human neutrophil peptide
HSV	Herpes simplex virus
iBALT	Inducible bronchus-Associated Lymphoid Tissue
IELs	Intraepithelial lymphocytes
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IRF	Interferon regulatory factor
ISCOM	Immunostimulating complex
iTreg	Induced Treg
KIRs	Killer inhibitory receptors
LAIV	Live attenuated influenza vaccine
LALT	Larynx-Associated Lymphoid Tissue
LDALT	Lacrimal duct-Associated Lymphoid Tissue
LRRs	Leucine-rich repeats
M	Matrix protein
MAC	Membrane attack complex
MAdCAM-1	Mucosal addressin cellular adhesion molecule-1
MALT	Mucosa-Associated Lymphoid Tissue
MAMPs	Microbe-associated molecular patterns
MAPKKK	Mitogen-activated protein kinase kinase kinase
MBL	Mouse mannan-binding lectin
MCP	Monocyte chemotactic protein
mDCs	Myeloid dendritic cells
MHC	Major histocompatibility complex
MLNs	Mesenteric lymph nodes
NA	Neuraminidase
NALT	Nasopharynx-Associated Lymphoid Tissue
NK	Natural killer
NKRs	Natural killers receptors
NKT	Natural killer T-cells
NLRs	NOD-like receptors

NOD	Nucleotide-binding oligomerization domain
NP	Nucleoprotein
nTreg	Natural Treg
ODN	Oligodeoxyribonucleotides
OVA	Ovalbumin
PAMPs	Pathogen-associated molecular patterns
PCA	Principal component analysis
PCs	Plasma cells
pDCs	Plasmacytoid dendritic cells
pIgA	Polymeric IgA
pIgR	Polymeric immunoglobulin receptor
PLA2	Phospholipase A2
Poly I:C	Polyionosinic-polycytidylic acid
PPs	Peyer´s patches
PRRs	Pattern recognition receptors
qPCR	Quantitative polymerase-chain reaction
RNP	Ribonucleoproteins
RQ	Relative quantification
SC	Secretory component
SI	Stimulation index
SIgA	Secretory immunoglobulin A
SPs	Surfactant proteins
ss	Single stranded
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T-helper
TIR	Toll/interleukin receptor
TLRs	Toll-like receptors
Tregs	Regulatory T cells
VCAM	Vascular cell adhesion molecule
WHO	World Health Organisation

INTRODUCTION

1. Mucosal immune system

The mucosal immune system protects the body mucosal surfaces against invasion of microbial pathogens and in mammalian species covers a surprisingly large surface area. The mucosa of the adult human has a surface area of about 400 m^2 (airway epithelium covers approximately 80 - 120 m^2) compared with only 1.5 m^2 of skin. These mucosal surfaces are generally covered by a physically vulnerable monolayered epithelium, which is persistently exposed to airborne and ingested molecules and particles, including numerous harmful substances and pathogenic microorganisms. In fact, the majority of pathogens use the mucosa as their portal of entry. In addition to providing a physical barrier to the environment, the epithelium is equipped with several other innate defense mechanisms to prevent attachment, colonization, and possible damage by foreign agents. Many of these mechanisms cooperate with mucosal adaptive immune system represented by secretory immunoglobulins (mainly SIgA) and mucosal lymphocytes. [1]. The main constituents of mucosal immune system are connected with the gastrointestinal, respiratory and genitourinary tracts. While there are major similarities between these tissues, there are also considerable differences among them. The similarities generally pertain to the antigenic load (gastrointestinal and respiratory tracts) and the one layer of epithelial cells separating the lumen from the interior lamina propria. The differences are mainly in the expression of cellular homing receptors, antigen sampling, and unique cell types.

1.1. Compartmentalization of mucosal immune system

Mucosa-associated lymphoid tissue (MALT) is a general term comprising a collection of lymphoid tissues located at multiple sites throughout the body. Most well-known representatives are gut-associated lymphoid tissue (GALT), nasopharynx-associated lymphoid

tissue (NALT), and bronchus-associated lymphoid tissue (BALT); however, conjunctiva-associated lymphoid tissue (CALT), lacrimal duct-associated lymphoid tissue (LDALT), larynx-associated lymphoid tissue (LALT) and salivary duct-associated lymphoid tissue (DALT) have also been described. These tissues share, to varying degrees, a common characteristic structure [2].

The mucosal immune system can be divided into two functionally distinct parts. The inductive sites consisting of organized mucosa-associated lymphoid tissue (MALT) – where antigens sampled from mucosal surfaces stimulate naïve T-lymphocytes and B-lymphocytes. The effector sites are formed by dispersed lymphoid tissue in lamina propria and in stroma of exocrine glands. Here, B-cells terminally differentiate into plasma cells (PCs) that efficiently produce polymeric IgA (pIgA) which is exported on mucosal surfaces by polymeric immunoglobulin receptor (pIgR) mediated transcytosis. The inductive sites for mucosal immunity are organized lymphoid structures with B-cell follicules containing germinal centers, intervening T-cell areas, and a variety of antigen-presenting cells (APC) [1]. Such sites of the gut include Peyer's patches (PPs), the appendix, and isolated lymphoid follicles, whereas the unpaired nasopharyngeal tonsil (often called adenoids) and the paired palatine tonsils constitute inductive sites in the airways. All these lymphoid structures lack afferent lymphatics and sample exogenous antigens directly from mucosal surfaces through a characteristic follicle-associated epithelium (FAE), which contains (M) cells [3]. These specialized thin cells have been shown to be especially effective in the uptake of microorganisms and other particulate antigens.

After primary stimulation of mucosal inductive sites, B-cells exit through local regional LNs by draining lymphatics to ductus thoracicus into the blood circulation and "home" to mucosal effector sites for further maturation. B-Cells activated in one inductive site might seed distant effector sites, and the term "common mucosal immune system" refers

to the possibility of providing secretory antibodies of given specificity at all mucosal surfaces of the body [1]. However, the homing of B-cells activated in one mucosal microenvironment is not uniform to all mucosal surfaces, and lymphocytes preferentially migrate back to the region where they were originally stimulated [1;4;5]. This compartmentalization within the integrated mucosal immune system is supported by regionalized secretory immunity obtained after local immunization [6]. Thus, nasal antigen challenge will preferentially induce an immune response in the upper airways and saliva but, surprisingly, also in the female genital tract. Feeding of antigens (oral route) preferentially induces an immune response in the intestine but also in salivary and lactating mammary glands. Rectal antigen uptake preferentially induces an immune response in the large intestine and to some extent in the female genital tract. **Figure 1** summarize interactions between inductive and effector sites of mucosal immune system.

1.2. Homing of lymphocytes

The homing of lymphocytes depends on cell surface expression of homing receptors, adhesion molecules and chemokine receptors that bind complementary molecules on vascular endothelial cells [7]. Homing to the gut LP depends on strong lymphocyte surface expression of α4β7, CCR9 or CCR10, whereas a combined expression of α4β1, L-selectin, and CCR7 appears to direct B-cells to the upper respiratory and uterine cervical mucosae [4]. B-lymphocytes involved in the systemic antibody responses express mainly L-selectin and little α4β7 [8] and are presumably guided to the bone marrow by expression of CXCR3 and CXCR4 [9]. The counter-receptor for the α4β7 integrin is the unmodified mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) expressed on endothelial cells of the intestinal LP microenvironment [10;11]. By contrast, the α4β1 integrin binds vascular cell adhesion molecule (VCAM)-1 [12;13]. Homing to the respiratory tract appears to involve α4β1–VCAM-1 interactions, the same interactions that are involved in recruiting systemic

lymphocytes to sites of inflammation [14] but CCR10 is upregulated on the cells destined to home to the respiratory tract and is absent on systemic lymphocytes [15]. Similarly, CCL28, the chemokine ligand for CCR10, is expressed preferentially by mucosal epithelial cells [16]. Thus, CCR10 – CCL28 interactions appear to direct trafficking of lymphocytes generally to mucosal tissues, and α4β1–VCAM-1 interactions ensure homing to the respiratory tract. In the gut, expression of different chemokines in the small and large intestines explains the selective recruitment of lymphocytes activated by the oral and rectal route [1;17]. The chemokine CCL25 (TECK) is selectively produced by the crypt epithelium in the small intestine and attracts lymphocytes expressing CCR9 [18;19]. During an immune response to orally fed antigens, dendritic cells (DCs) in PPs and mesenteric lymph nodes (MLNs) imprint lymphocytes with a high expression of CCR9 and α4β7 integrin, combined with a downregulation of L-selectin [20;21], thus directing these cells to the small intestine. In the large intestine, CCL28 (MEC) expression appears to be important for attracting IgA+ plasmablasts that express high levels of CCR10 as well as α4β7 [7;22].

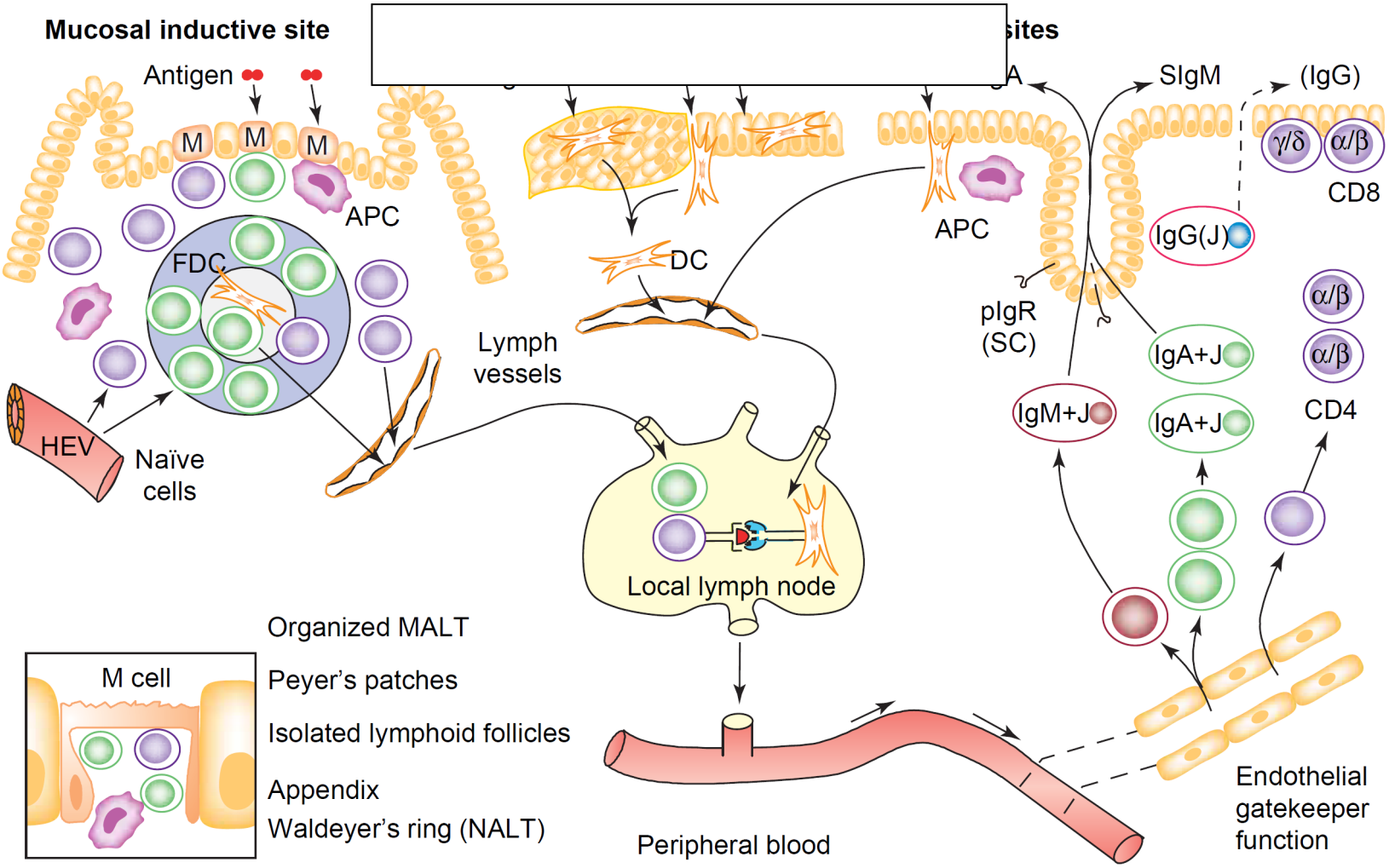


Figure 1. Scheme of inductive and effector sites of mucosal immune system [23].

2. Innate immunity in mucosal defense

All living organisms face the challenge of defending themselves against microorganisms in the environment. Although the adaptive immune system has been subject to considerable study, the contribution of the innate immune system to defense against microbial pathogens has been long time less appreciated. Nowadays, innate immunity is in centre of interest not only for the importance and efficiency of its primary defensive functions but also for its decisive impact on adaptive immunity. The components of the innate immune system are either cellular, consisting of various types of cells, or humoral, consisting of various secreted soluble peptides, proteins, enzymes and other mediators. The interactions of these components in the host with the invading microbes often, but not always, lead to a constellation of responses called inflammation. The purpose of inflammation is to amplify the body's defenses by increasing the number of leukocytes and blood supply to the site of infection, bringing antimicrobial proteins and white cells to defend the host and eliminate the pathogen or foreign antigen encountered. **Table 1** shows the components of cellular and humoral innate immunity and their functions.

Component	Function
Cellular	
Skin and Mucous membrane (epithelial cells)	Mechanical and chemical defenses
Phagocytic cells (neutrophils, macrophages)	Ingest and kill pathogens
Proinflammatory cells (macrophages, mast cells, eosinophils)	Innate defense and inflammation
Natural killer (NK) cells	Kill infected cells and tumor cells
Antigen-presenting cells (dendritic cells, macrophages, and B cells)	Recognize, process and present antigen to lymphocytes and initiate adaptive cellular immune response
Humoral	
Complement system	Enhances phagocytosis (opsonization), induces inflammation, and destroys some pathogens.
Cytokines	Secreted by many cells and influence innate and adaptive defenses
Chemokines	Secreted by many cells and attract as well as activate leukocytes
Antimicrobial peptides and proteins	Kill many microorganisms
Acute phase proteins	Enhance cellular and humoral defense
Enzymes	Kill and digest microorganisms

Table 1. Components of cellular and humoral innate immunity.

2.1. Mucosal barriers

Before microorganisms can enter the body, they must pass through the barriers of the skin, mucous membrane of the respiratory, alimentary, or urogenital tracts or the conjunctivae of the eyes. Each barrier has its own protective mechanisms, which can be broadly classified as mechanical, chemical, molecular, and microbial (**Table 2**). Mechanical barriers are highly effective, especially the skin, which is covered with a multilayer keratinized epithelium. Skin and mucous membranes are rich in chemical and molecular antimicrobial factors. Some of these factors are constitutively expressed, e.g., low pH in the stomach, fatty acids on the skin, and lysozyme (bacteriolytic enzyme) in all body fluids and granules of phagocytic cells. Initially, prevention of luminal antigen penetration is through a thick layer of mucus. Mucin 2 is a dominant intestinal mucus-formation molecule that is abundantly produced by goblet cells located at the intestinal villous epithelium. Mucus not only provides a physical and biological protective barrier, but also ensures maintenance of an appropriate concentration of antibodies at the mucosal surface by preventing Ag-specific SIgA antibodies from being physically carried away. Additionally, paracellular transport of luminal antigen is prevented by the juncture between adjacent ECs that is mediated by physical structures associated with the epithelium including the tight junctions and the subjacent desmosomes and adherence junctions [24]. The tight junctions are composed of a number of interacting cellular proteins, which include claudin, occuludin, ZO-1, ZO-2, and cingulin, among others. Under normal circumstances, the tight junctions exclude usually antigens greater than 500 to 900 Daltons. In addition to these physical barrier functions of epithelial cells, the epithelium of the MALTs of the lung, gut and genitourinary tracts, have been clearly shown to play an active role in both innate and adaptive types of mucosal immunity.

The innate immune system prevents colonization of the skin and mucous membranes by potential pathogens, but allows colonization by the nonpathogenic bacteria and fungi of the

normal flora. The flora (microbiota) is unique for each area of the body and is beneficial to the host because it stimulates the development of immune system, produces some metabolites utilized by the host, protects the skin and mucous membranes from colonization by pathogenic microorganisms and bacterial enzymes degrade some food constituents non-splittable by host enzymes. When a microorganism crosses the protective epithelia of mucous membranes, it encounters the next line of defense mechanisms: phagocytic cells, proinflammatory cells, natural killer (NK) cells, and antigen-presenting cells as well as humoral defenses.

Component	Function(s)
Mechanical	
Keratinized epithelium	Protection against microorganisms
Desquamation of stratified epithelium	Removes pathogens attached to its surface
Epithelial cells joined by tight junctions	Prevents entry of microbes
Mucus	Removes particles from the respiratory tract
Coughing and sneezing	Expel particles
Peristalsis	
Chemical and molecular	
Low pH in the stomach	Inactivates many bacteria, fungi, parasites and viruses
Antimicrobial peptides (Defensins, Cathelicidins)	Kill many microorganisms
Fatty acids (from sebaceous glands of the skin)	Inhibits bacterial growth
Enzymes	
Lysozyme	Hydrolyses bacterial cell wall peptidoglycan
Amidase (in the skin and serum)	
Ribonucleases (on skin)	Kill Bacteria and fungi
Phospholipases	Kill Bacteria
Enzymes of gastrointestinal tract	Kill and digest microorganisms

Table 2. Defense mechanisms (mechanical, chemical and molecular) of the skin or mucous membranes.

2.2. Antimicrobial peptides and proteins

Members of the antimicrobial peptide family are widely distributed in nature, existing in organisms from plants and insects to non-mammalian vertebrates and mammals. The epithelium secretes a variety of antimicrobial peptides (defensins, cathelicidins, cryptdin-related sequence peptides (CRS), antibacterial enzymes (lysozyme, secretory phospholipase-A2 [PLA2] or peroxidase), lactoferrin and others. In the intestine, these molecules are produced mainly by epithelial cells, Paneth cells, and PMNs [25;26]. Paneth cells reside at the

base of the crypts of the small intestine, but not in stomach or colon. They produce α-defensins constitutively [27;28]. In contrast, β-defensins are produced by epithelial cells of the whole intestine after microbial stimulation [25;26]. Defensins are small peptides (3 to 4 kDa) and in humans are subdivided into two families, α- and β-defensins based on their structure. Six α-defensins and four β-defensins have been well characterized in humans, but recent analysis of the human genome revealed 34 defensin genes, which highlights the significance of this family of antimicrobial peptides. Defensins, like most other antimicrobial peptides, are highly cationic, enabling them to bind to the negatively charged cell walls of bacteria and fungi and to kill them by pore formation and permeabilization of their cell membranes [29]. α-defensins are also secreted by tracheal epithelial cells and are homologous to peptides that function as mediators of nonoxidative microbial cell killing in human neutrophils (termed human neutrophil peptides; HNPs). β-defensins and in particular human β-defensin-1 (HBD-1) are expressed in the epithelial cells of the oral mucosa, trachea, bronchi, as well as mammary and salivary glands [30;31]. HNP-1, -2, -3 shares more than 60% homology and 40% identity with murine crypt cell defensins (cryptins) [32;33]. Defensins also inhibit viral infection (e.g., human immunodeficiency virus [HIV], herpes simplex virus [HSV], and influenza virus) by interupting their invasion at an early step, such as receptor binding [25;26]. In addition to the antimicrobial properties, defensins have chemotactic activities for monocytes, T cells, and B cells, implying that defensins may bridge between mucosal innate and acquired immunity via the augmentation of T and B cell interactions.

The cathelicidin is also a cationic small peptide containing a cathelin-like domain produced by epithelial cells, PMNs, and keratinocytes [25;26]. The expression of cathelicidin by epithelial cells is regulated by butyrate and other short-chain fatty acids produced by fermenting bacteria. Cathelicidin peptides have been isolated from many different species of

mammals. In humans, only cathelicidin LL-37 has been well characterized. In mice, mouse cathelicidin-related antimicrobial peptide (CRAMP) was described. The expression of LL-37 and CRAMP in skin keratinocytes varies with infection and/or injury [34]. Cathelicidins were originally found in neutrophils but have since been found in many other cells including epithelial cells and macrophages activated by bacteria, viruses or fungi. There is also some indication that cathelicidins and defensins can act at the interface of innate and adaptive immunity [35], modulating DC function [36] and antigen-specific immune responses. LL-37 induces differentiation of primary monocyte-derived DCs, increases endocytic capacity, modifies phagocytic receptor expression and function, upregulates co-stimulatory molecules expression (CD86) and enhances pro - Th1 cytokine secretion (IL-12) by LPS stimulated DCs. Likewise, mouse β-defensin-2 (BD2) stimulates DC maturation and upregulates their expression of co-stimulatory molecules (CD40, CD80 and CD86), major histocompatibility complex class II and chemokine receptor CCR7 [37]. These peptides might be effective adjuvants for the development of adaptive immunity [38]. The CRS peptide is produced by Paneth cells and shows antimicrobial activity through its cationic feature [25;26].

Antimicrobial enzymes are other molecules showing antimicrobial activities. PLA2 is a small enzyme produced by Paneth cells and PMNs, which degrades bacterial phospholipids and subsequently disrupts bacterial integrity [25;26]. Lysozyme is present in large amounts in all body fluids and in granules of phagocytic cells. It hydrolyses peptidoglycan, the main structural component of bacterial cell walls. Lysozyme acts mainly on Gram-positive (G+) bacteria, although many bacterial species have evolved resistant modifications of their cell wall chemistry. Gram-negative (G-) bacteria, however, are resistant because their peptidoglycan substrate is shielded by an outer membrane. In this case, lysozyme can work synergistically with other antimicrobial peptides and proteins which can damage the outer membrane of bacteria and allow lysozyme to access its substrate. Surfactant proteins A-D

(SPs) are highly hydrophobic proteins in the lung produced by alveolar type II cells. Several lines of evidence revealed that SPs are actively involved in lung innate immunity following bacterial penetration into the lower airways [39]. SPs bind to LPS and the interaction between SPs and CD14 may explain their ability to affect some LPS responses.

2.3. Cellular components of innate immunity

2.3.1. Neutrophils

Innate cellular response represents an extremely important branch of antimicrobial defense. Infection induces the influx of neutrophils, eosinophils, and monocytes/macrophages into afflicted tissues. Neutrophils are actively motile phagocytic cells produced in the bone marrow. They differentiate from stem cells over 2 weeks and enter then the blood stream. In case of need, they are attracted from blood to sites of infection. Neutrophils recognize and engulf microorganisms and produce a number of antimicrobial substances (enzymes, antimicrobial proteins and peptides) and reactive oxygen species (e.g., superoxide, hydrogen peroxide, hypochlorous acid, etc.). They contain two kinds of granules: peroxidase-positive (azurophil, or primary) and peroxidase-negative (specific or secondary). Their antimicrobial factors can kill and degrade microorganisms as well as damage host tissue after the death of short-living neutrophils.

2.3.2. Eosinophils

Differentiation of eosinophils occurs within the bone marrow in response to eosinopoietic cytokines, particularly IL-5. The mechanisms underlying mucosal tissue basophil/mast cell or eosinophil differentiation and/or accumulation are unclear. Eosinophil precursors (CD34/IL-5Rα+ cells) and IL-5 mRNA+ cells have been identified in the lungs of asthmatics, indicating that a population of eosinophils or basophil/mast cells may differentiate in situ [40]. The eosinophils are similar to neutrophils in many respects. However, they are

not efficient in phagocytosis, but can readily release the content of their granules to the outside. Their targets are usually parasites. Indeed, the increase of these cells in the circulation, eosinophilia, is the hallmark of diseases caused by metazoic parasites such as schistosomiasis or trichinosis. It has been shown that the cytoplasmic granules of the eosinophils carry large amounts of an enzyme known as eosinophil peroxidase, as well as specific cytotoxic cationic proteins. These compounds have the power to kill certain parasites. Thus, eosinophils have an anti-infectious property similarly as neutrophils but specifically target multi-cellular pathogens, which are too large to be phagocytized by neutrophils.

2.3.3. Macrophages

Macrophages have the most central and essential functions in the innate immune system, and have multiple roles in host defense. Mature, resident macrophages differentiate from circulating monocytes and occupy peripheral tissues and organs where they are most likely to encounter pathogens during the early stages of infection. Upon encounter with infectious agents, macrophages can employ a broad array of antimicrobial effector mechanisms, including phagocytosis of the pathogen and the induction of microbicidal effector systems, such as reactive oxygen and nitrogen intermediates and antimicrobial proteins and peptides. Tissue or resident macrophages exist throughout the body and have different names and functions, depending on the tissue. Thus, they are called Kupffer cells in the liver, alveolar macrophages in the lungs, osteoclasts in the bone, and microglia in the brain. Tissue macrophages contribute greatly to the inflammatory response by releasing main inflammatory cytokines: IL-1, which enhances the adherence of neutrophils to endothelia, TNF-α, which activates newly arrived neutrophils and monocytes, IL-6 and many chemokines that attract other leukocytes to bridge the innate with the adaptive immune system.

2.3.4. Natural killer cells

NK cells are large granular lymphocytes that have cytotoxic activity. In this aspect they resemble cytotoxic T cells. However, NK cells are neither T cells nor B cells because they do not have the markers characteristic of T or B-lymphocytes, mainly specific clonotypic receptors for antigen. They recognize their targets by inhibitory (KIRs, in mice Ly-49, CD94-NKG2) and stimulatory (NKR-P1, CD16) receptors without MHC restriction. NK cells can be divided into two phenotypically distinct functional subsets based on their cell surface expression of CD56 ($CD56^{bright}$ and $CD56^{dim}$). As mouse NK cells do not express CD56, comparable mouse NK cell subsets have proven difficult to identify [41;42]. Recently, mouse NK cells can be subdivided by the expression of CD27. The $CD27^{hi}$ and $CD27^{lo}$ mouse NK cell subsets show some similarities to but also some distinct differences from the human CD56 NK cell subsets in terms of their function and phenotype [43]. NK cells have several types of natural killer receptors (NKRs), which are lectins that bind to various glycoproteins present on many host cells. However, this would potentially induce the killing of many healthy cells. Therefore, NK cells also have killer inhibitory receptors (KIRs) recognizing own MHC class I antigens and hampering thus killing of own healthy cells. However, virus-infected cells or some tumor cells often have decreased surface expression of MHC class I molecules and prevailing signalization via NKRs causes the death of target cell. NK cells are stimulated by macrophage-derived IL-15 and IL-12. IL-15 is a growth factor for NK cells, and IL-12 induces IFN-γ production by NK cells and enhances their cytolytic activity. IFN-γ in turn activates macrophages and inhibits growth of viruses in host cells. NK cells also have Fc-γRIIIa receptors (CD16), which bind the Fc portion of IgG antibodies when complexed with antigen. This allows NK cells to exert antibody-dependent cell-mediated cytotoxicity (ADCC), which is an example of cooperation of innate and adaptive immunity.

2.3.5. Dendritic cells

Dendritic cells (DCs) provide a crucial connection between the innate and the adaptive immune system. Two main subsets of DCs have been identified (myeloid DCs (mDCs) [44] and plasmacytoid DCs (pDCs) [45], which differ in morphology, surface marker expression and function. mDCs have a typical dendroid morphology. They are CD11b+ and CD11c+. pDCs have a plasma cell-like morphology. They are $CD11c^{low}$ and $CD11b^{-}$. Human pDCs express CD123, whereas mouse pDCs express Ly6c. Both mDCs and pDCs are bone marrow derived. They show distinct migration patterns; immature mDCs migrate to peripheral tissues, whereas immature pDCs, which are $CD62L^{high}$, migrate to lymphatic tissues, where they enter the T-cell zones. Immature mDCs constantly monitor their environment by phagocytosing particles including viruses, but are unsuited to present antigen to naive T cells until they differentiate into mature DCs. Under the influence of cytokines such as TNF-α or type I IFNs and by activation of pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs). Interestingly, mDCs express many of the TLRs that recognize bacterial products such as TLR2, TLR4, and TLR5. They also express TLR3. In contrast, pDCs express TLRs that recognize viral genomes such as TLR7, TLR8 (expressed only by human pDC, not by mouse pDCs), and TLR9, indicating a pivotal role of pDCs in antiviral defense [45]. After antigen recognition and internalization, mDCs initially increase phagocytosis and start secreting chemokines that attract an additional inflammatory infiltrate, which includes cells of the innate and adaptive immune system and additional immature mDCs and pDCs. Activated mDCs then stop to take up antigen, upregulate CCR7 expression, which drives their migration to draining lymph nodes. Activated DCs furthermore upregulate their antigen-processing machinery as well as expression of MHC class I molecules, and translocate MHC class II determinants from intracellular vesicles to the cell surface. At this stage, MHC molecules are loaded with antigen-derived peptides. In addition, mature DCs start

to express or upregulate positive costimulatory molecules of the B7 family such as CD80 and CD86 as well as CD40, and secrete cytokines such as IL-12 and type I IFNs. When mature mDCs reach the lymph nodes, they migrate to the T-cell zones and initiate activation of naïve T cells. In contrast, upon activation, pDCs produce type I IFN at levels that are 10 to 100 times higher on a per-cell basis than those produced by mature mDCs being thus important players in anti-viral defense. In mDCs, stimulation by pathogen-associated molecular patterns (PAMPs), cytokines, or CD40 ligation activates IRF3, which leads initially to the production of low amounts of type I IFN. Upon binding to its receptor, type I IFN then increases its own production through a positive feedback mechanism, which requires upregulation of IRF7. pDCs constitutively express IRF7, which renders these cells independent of positive feedback, and they can therefore produce large amounts of type I IFN immediately upon activation [46]. pDCs can present antigen to naïve T cells as well, they upregulate MHC molecules and costimulatory molecules upon differentiation into mature pDCs. Nevertheless, levels of these crucial cell surface molecules are lower than those on mature mDCs. Furthermore, pDCs do not phagocytose antigen and are thus poorly suited to present antigen [47]. It is unclear whether pDCs indeed present antigen *in vivo* to cells of the adaptive immune system or if their primary role is to produce type I IFNs and to assist maturation of mDCs.

2.4. Pattern recognition receptors

2.4.1. Toll-like receptors

Innate immunity is often regarded as relatively non-specific. However, recent studies have shown that the innate immune system has a much greater specificity than previously thought, and can indeed respond differently to various antigens. The first step in innate immunity is the recognition of microorganisms by PPR – pattern recognition receptors that recognize specific molecular patterns present in the microbes but not in host tissues (PAMPs, or better MAMPs – pathogen or microbe associated molecular pattern) [48;49]. A family of

toll-like receptors (TLRs) conserved across species plays a central role in this discrimination, and currently 11 functional TLRs in human and 13 functional TLRs in mice have been identified. **Table 3** shows the list of 11 toll-like receptors and their ligands. TLRs 1, 2, 4, 5, 6 and 10 are localized on the cell surface and the TLRs 3, 7, 8 and 9 are localized in the endosomal compartments [50;51]. Upon ligand binding, all TLRs, except TLR3, signal through the MyD88-dependent pathway, whereas TLR3 activates the MyD88 independent pathway, TLR4 can use both mentioned pathways [52]. A series of molecular genetic studies have revealed the respective ligands for the TLRs which have been reviewed by a number of authors [50;51;53-55]. LPS from G- bacteria induces responses mainly via TLR4 ligation, whereas TLR2 in association with TLR1 or TLR6 recognizes peptidoglycan, lipopeptide and lipoprotein of G+ bacteria and mycoplasma lipopeptide [50]. TLR5 mediates the responses to bacterial flagellin, while TLR9 has been shown to recognize bacterial and viral oligodeoxynucletides containing unmethylated CpG dinucleotide motifs [56]. TLR3 responds to dsRNA, a byproduct of viral replication, and its synthetic homologue polyionosinic-polycytidylic acid (Poly I:C). TLR7 and TLR8 recognize synthetic antivirotic imidazoquinolines (R-848) and viral ssRNA [50]. TLR11, which recognizes uropathogenic *Escherichia coli* in mice, is found in the human genome, yet it may not be expressed due to the presence of stop codons found within the TLR11 open reading frame [57]. However, its recognition of *Toxoplasma gondii* profilin was described by some authors [58].

TLRs contain a number of leucine-rich repeats (LRRs) in its ectodomain, and a Toll/interleukin-1 receptor (TIR) domain in the cytoplasmic region. The TLR ectodomain is responsible for ligand binding, and the TIR domain recruits cytoplasmic adapter proteins to carry signals into the cytoplasm. Most TLRs activate the MyD88-dependent pathway, resulting in inflammatory response. Mainly because of their presence in dendritic cells, TLRs play an important role in the control of adaptive immune responses [59]. Virus infection is

sensed by the innate immune system. Following recognition, PRRs initiate signaling pathways that induce the production of a variety of cytokines including inflammatory cytokines such as IL-6 and TNF-α, anti-viral cytokines, chemokines, and IL-12. Type I IFN induces antiviral state of cells, activate natural killer (NK) cell cytotoxicity, and influence adaptive immunity by enhancing DC maturation. Type I IFN also upregulates transcription of many IFN-inducible genes that influence protein synthesis, growth arrest, and apoptosis to establish an antiviral state [60]. Within the TLR family, TLR3, TLR7, TLR8 and TLR9 represent a subfamily that recognizes viral nucleic acids in endosomes. Viral nucleic acids can be recognized also in cytoplasm by RIG-I-like RNA helicases (RLHs) such as RIG-I and Mda5.

2.4.2. Toll-like receptors responsible for sensing of viral nucleic acids

Double-stranded RNA (dsRNA) is synthesized during the course of replication of many viruses and serves as a potent activator of innate immune cells that induces production of type I IFNs. A synthetic analog of viral dsRNA, poly I:C, has been used extensively to mimic immune response during viral infection. Induction of type I IFNs and pro-inflammatory cytokines in response to poly I:C or genomic RNA purified from a dsRNA viruses, such as reovirus, was abrogated in macrophages derived from TLR3 deficient mice [61]. TLR3 deficient mice are consistently resistant to I:C-induced shock, indicating that TLR3 recognizes poly I:C and possibly senses viral dsRNA. TLR3 is also implicated in recognizing dsRNA derived from ssRNA viruses such as the respiratory syncytial, encephalomyocarditis, and West Nile viruses [62;63]. TLR3 is expressed on a CD4- CD8+ subset of DC which has high phagocytic activity. The apoptotic bodies of virus-infected or dsRNA-loaded cells are taken up by CD8+ DC, and dsRNA is recognized by their TLR3. This process triggers cross-presentation, a pathway important for the development of the CD8 cytotoxic T-cell response against viruses which do not infect DC.

Receptor	Ligand	Origin of ligand
TLR1	Triacyl lipopeptides Soluble factors	Bacteria and mycobacteria Neisseria meningitidis
TLR2	Lipoprotein/lipopeptides Peptidoglycan Lipoteichoic acid Lipoarabinomannan Phenol-soluble modulin Glycoinositolphospholipids Glycolipids Porins Atypical lipopolysaccharide Atypical lipopolysaccharide Zymosan Heat-shock protein 70*	Various pathogens Gram-positive bacteria Gram-positive bacteria Mycobacteria Staphylococcus epidermidis Trypanosoma cruzi Treponema maltophilum Neisseria Leptospira interrogans Porphyromonas gingivalis Fungi Host
TLR3	Double-stranded RNA (dsRNA)	Viruses
TLR4	Lipopolysaccharide Taxol Fusion protein Envelope protein Heat-shock protein 60* Heat-shock protein 70* Type III repeat extra domain A of fibronectin* Oligosaccharides of hyaluronic acid* Polysaccharide fragments of heparan sulphate* Fibrinogen*	Gram-negative bacteria Plants Respiratory syncytial virus Mouse mammary-tumour virus Chlamydia pneumoniae Host Host Host Host Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides Lipoteichoic acid Zymosan	Mycoplasma Gram-positive bacteria Fungi
TLR7	Imidazoquinoline Loxoribine Bropirimine Single-stranded RNA	Synthetic compounds Synthetic compounds Synthetic compounds Viruses
TLR8	Imidazoquinoline Single-stranded RNA	Synthetic compounds Viruses
TLR9	CpG containing DNA	Bacteria and viruses
TLR10	N.D.	N.D.
TLR11	N.D. Profilins	Uropathogenic bacteria Toxoplasma gondii

Table 3. Toll-like receptors and their ligands.

* It is possible that these ligand preparations, particularly those of endogenous origin, were contaminated with lipopolysaccharide and/or other potent microbial components, so more-precise analysis is required to conclude that TLRs recognize these endogenous ligands. N.D. - not determined [64].

Thus, the TLR3 dependent pathway is important for cross-presentation by CD8+ DC [62]. TLR7 was initially identified as a receptor that recognizes imidazoquinoline derivatives with antiviral activity, such as imiquimod and resiquimod (R-848), and guanine analogues

such as loxoribine [65]. Subsequently, guanosine or uridine-rich ssRNA derived from the human immunodeficiency virus (HIV) and the influenza virus was identified as a natural ligands for TLR7 [66;67]. TLR8 is phylogenetically similar to TLR7. Human TLR8 preferentially mediates the recognition of HIV-derived ssRNA and R-848, although mice deficient for TLR8 respond normally to these molecules, suggesting that mouse TLR8 may not be functional [66-68]. TLR9 recognizes unmethylated 2′−deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs that are frequently present in viruses and bacteria [69]. TLR7 and TLR9 are highly expressed in plasmacytoid DCs (pDCs) also known as IFN-producing cells - a subset of DCs having plasmacytoid morphology and primarily secreting vast amounts of type I IFN in response to viral infection [70;71]. In TLR7 deficient mice, IFN-α production by pDCs was decreased after infection with influenza virus [66;72]. Plasmacytoid DC rely on TLR7 and TLR9 to detect viral infection, but viral detection by pDCs does not seem to require viral replication within cells. Unlike other TLRs, TLR3, TLR7, TLR8 and TLR9 are not expressed at the plasma membrane and are exclusively localized in endosomes, suggesting that these intracellular TLRs recognize nucleic acids following the internalization and lysis of viruses [48]. Endosomal localization is also important to prevent contact with self DNA, because TLR9 can respond to self DNA if it is relocalized to plasma membranes [73]. It is notably evident that the TLR system is required for pDC induction of the antiviral response. Collectively, these observations indicate that the TLR system plays a pivotal role in the detection of viruses by pDCs.

2.4.3. TLR7 and TLR9 signaling pathway

TLRs contain extracellular LRRs that mediate ligand recognition, a transmembrane domain, and a cytosolic TIR domain required for downstream signaling pathways [74]. Upon recognition of nucleic acids, TLR7 and TLR9 recruit a TIR-containing MyD88 adaptor molecule that is universally utilized by all TLRs with the exception of TLR-3. The association

of TLRs and MyD88 results in the recruitment of members of the IRAK family, including IRAK1, IRAK2, IRAK4, and IRAK-M. In particular, IRAK4 and IRAK1 are sequentially phosphorylated and involved in activation of the MyD88-dependent signaling pathway, while IRAK-M negatively regulates the MyD88-dependent pathway. The function of IRAK2 remains unknown. Once phosphorylated, IRAK4 and IRAK1 dissociate from MyD88 and interact with TRAF6, an E3 ligase that forms a complex with ubiquitin-conjugating enzymes (Ubc13 and Uev1A) and promotes the synthesis of lysine 63-linked polyubiquitin chains. TAK1, a member of mitogen-activated protein kinase kinase kinase (MAPKKK), is activated by TRAF6-dependent ubiquitination, and in combination with TAB1, TAB2 and TAB3, activates two downstream pathways involving the IKK complex and the MAPK family. The IKK komplex composed of the catalytic subunits IKKα, IKKβ, and a regulatory subunit known as NEMO/IKKγ, induce the phosphorylation and subsequent degradation of the IκB proteins that allow the NF-κB transcription factor to translocate into the nucleus. The MAPK family (JNK, p38, ERK) phosphorylates and activates the NF-κB and AP-1 transcription factors. NF-κB and AP-1 play central roles in the induction of genes encoding inflammatory cytokines [74]. TLR7 and TLR9 mediated type I IFN induction in pDCs is dependent on MyD88. IRF7, structurally the most similar to IRF3, is present in the cytoplasm and translocates to the nucleus after phosphorylation by one or more virus-activated kinases. IRF7 potently activates the promoters of IFN-α and IFN-β genes. The expression of the IRF7 gene is weak under unstimulated conditions, but rapidly upregulates in response to TLR7 and TLR9 ligation or viral infection in most cell types, suggesting a positive feedback regulation of type I IFN induction. In pDCs, IRF7 is constitutively expressed and binds to MyD88 [75;76]. Plasmacytoid dendritic cells lacking IRF7 consistently fail to produce IFN-α in response to CpG DNA, whereas IRF3 is dispensable in these pathways [77]. IRF7 also forms a complex with IRAK1, IRAK4, IKKα, and TRAF6 in addition to MyD88 [75;76;78;79].

While mice deficient in MyD88, IRAK4, or TRAF6 exhibit defects in both IRF7 and NF-κB activation associated with impaired induction of type I IFN and inflammatory cytokines in response to CpG DNA, pDCs derived from IRAK1 or IKKα-deficient mice specifically show loss of IRF7 activation and type I IFN induction. Moreover, IRAK1 and IKKα (but not IRAK4) are capable of phosphorylating IRF7 [78;79]. Together, IRAK1 and IKKα are most likely the kinases that catalyze the phosphorylation of IRF7 in pDCs. However, the functional relationship of IRAK1 and IKKα remains unclear. It is possible that they function as a heterodimer to potentiate IRF7 activation, or they may phosphorylate different residues of IRF7, both of which are required for the activation. Several additional components of the MyD88–IRF7 complex have recently been identified. TRAF3 binds MyD88 and IRAK1, and is critical for type I IFN induction in TLR7 and TLR9 signaling [80;81]. TRAF3 is also necessary for the induction of the IL-10 anti-inflammatory cytokine, but not for the induction of pro-inflammatory cytokines, in response to TLR7 and TLR9 ligands [80]. IRF8 is also implicated in TLR9-mediated responses in pDCs. pDCs derived from IRF8 deficient mice show a loss of TLR9-mediated induction of type I IFN and inflammatory cytokines caused by impaired NF-κB DNA binding activity, suggesting the possibility that IRF8 facilitates NF-κB DNA-binding [82]. In pDC and mDC, different signaling pathways are activated after ligation of TLR9: while mDCs derived from IRF1-deficient mice display impaired induction of IFN-β, inducible nitric oxide synthase, and IL-12 p35 in response to a TLR9 ligand, pDCs derived from IRF1-deficient mice show normal induction of IFN-β and IFN-α [83]. IRF1 also interacts with MyD88 and is released into nuclei in response to ligand stimulation. Cytokine induction in response to TLR ligands is enhanced by pretreatment of cells with IFN-γ. Consistent with the findings that IFN-γ stimulation induces IRF1 expression, IFN-γ-mediated enhancement is impaired in IRF1-deficient mice. Thus, IFN-γ-induced IRF1 is recruited to MyD88 and translocated into nuclei in response to TLR stimulation to induce a set of genes

including IFN-β in mDCs. IRF5 is involved in TLR signaling as well. IRF5-deficient mDCs and macrophages exhibit impaired inflammatory cytokine production in response to multiple TLR ligands, but exhibit normal secretion of type I IFN by pDCs [84]. IRF5 binds MyD88 and TRAF6 and translocates to nuclei after phosphorylation. In the nucleus, IRF5 binds ISRE motifs found in the promoter regions of genes encoding inflammatory cytokines to cause their expression, presumably via collaborative activation with NF-κB. IRF5-mediated responses are negatively regulated by IRF4, which competes with IRF5 for interaction with MyD88 [85]. Studies of synthetic CpG oligodeoxyribonuleotides (ODN) led to their classification into three groups, based on their biological effects. D/A type ODN induces a secretion of type I IFN by pDCs but has a low ability to induce B cell activation and IL-12 production. In contrast, K/B type ODN stimulates B cell activation and IL-12 production, but poorly induces type I IFN; C type ODN has the ability to induce both type I IFN induction and B cell activation. A/D type CpG ODN colocalizes with TLR9, MyD88, and IRF7 in endosomes in pDCs and are rapidly transferred to lysosomes and degraded. However, when A/D type CpG ODN relocalizes to the endosomes in mDCs using a cationic lipid, these cells can produce IFN-α through activation of the MyD88-IRF7 pathway [46]. B/K type CpG ODN also induces secretion of IFN-α if they are manipulated to remain in the endosomes of mDCs for longer periods. These findings suggest that retention of the CpG DNA–TLR9 complex in endosomes may cause the induction of robust IFN-α production.

Host cells express multiple PRRs for the detection of viruses. These PRRs are expressed in different cellular compartments and recognize different types of nucleic acids. TLR7 and TLR9 are expressed by pDCs and act as sensors for viral ssRNA and DNA that trigger the production of large amounts of IFN-α. They use MyD88 as an adapter to induce type I IFN via IRAK1/IKKα-dependent phosphorylation of IRF7 **(Figure 2)**. Is very important to understand how PPRs detect nucleic acid and induce antiviral innate immune

responses. Such knowledge could improve therapeutic strategies for the treatment of infectious diseases and also autoimmune diseases associated with viral infection.

2.4.4. RIG-I-like RNA helicases

Because TLRs are localized in endosomes, they are unable to sense viruses that have entered the cytosol and initiated replication. Numerous studies implicated TLR-independent mechanisms in the detection of viral infection. For example, induction of IFN-β followed by transfection with poly I:C or infection with RNA viruses is normally observed in the absence of TLR3 or TRIF, suggesting that host cells have a mechanism to recognize actively replicating viruses in the cytoplasm [86;87]. RIG-I, a member of the RNA helicase family, was identified as a molecule that senses dsRNA and induces type I IFN responses [88]. RIG-I contains a DExD/H box RNA helicase and two caspase recruiting domain (CARD)-like domains. The helicase domain interacts with dsRNA, whereas the CARD-like domains are required for activating downstream signaling pathways. Furthermore, Mda5 and LGP2 were subsequently identified as members of the RIG-I like RNA helicases family [89;90]. Mda5 contains two CARD-like domains and a helicase domain. LGP2 lacks the CARD-like domains and is thought to negatively regulate RIG-I and Mda5. Studies of RIG-I- and Mda5-deficient mice revealed that RIG-I is essential for the recognition of a series of ssRNA viruses including flaviviruses, paramyxoviruses, orthomyxoviruses, and rhabdoviruses. Mda5 is required for the recognition of a different set of RNA viruses that includes picornaviruses [91;92]. Furthermore, Mda5 and RIG-I detect poly I:C and long dsRNA, respectively, indicating that these RNA helicases detect different RNA viruses [92]. RIG-I-mediated detection of RNA has recently been shown to depend on the 5’ triphosphate end of RNA generated by viral polymerases [93]. However, the production of type I IFN is observed even in pDCs derived from RIG-I or Mda5-deficient mice, although mDCs, macrophages, and

fibroblasts derived from these mice showed lower type I IFN induction after infection with corresponding RNA viruses [92].

2.4.5. NOD-like receptors

NOD-like receptors (NLRs) are a large family of about 20 intracellular proteins with a common protein-domain organization but diverse functions [94-96]. All NLRs contain a nucleotide-binding oligomerization domain (NOD) followed by a leucine-rich-repeat domain at the carboxy terminus. At the amino terminus, NLRs have one of three domains and are thereby categorized into three subfamilies: a caspase-recruitment domain (CARD), present in proteins in the NOD subfamily; a pyrin domain, in the NALP subfamily; or a BIR domain (baculoviral inhibitor-of-apoptosis-protein repeat-containing domain), in the NAIP subfamily [94-96]. The N-terminal domains engage distinct signaling pathways, which define the functional properties of the family members. The proteins of the NOD subfamily - NOD1 and NOD2 - are both involved in sensing bacterial peptidoglycans, although they recognize structurally distinct peptidoglycan fragments [94]. The sensing of peptidoglycan by NOD1 or NOD2 triggers the production of pro-inflammatory cytokines and chemokines and the recruitment of neutrophils to the site of infection [95]. In addition, these NOD proteins contribute to the initiation of the adaptive immune response [97;98]. NOD2 is also crucial for the production of defensins by Paneth cells. The NALP subfamily of NLRs has 14 members, and at least some of these are involved in the induction of the inflammatory response mediated by the IL-1 family of cytokines, which includes IL-1β, IL-18 and IL-33 [96]. These cytokines are synthesized as inactive precursors that need to be cleaved by the pro-inflammatory caspases: that is, caspase 1, caspase 4 and caspase 5 in humans, and caspase 1, caspase 11 and caspase 12 in mice. These caspases are activated in a multi-molecular complex called the inflammasome [99].

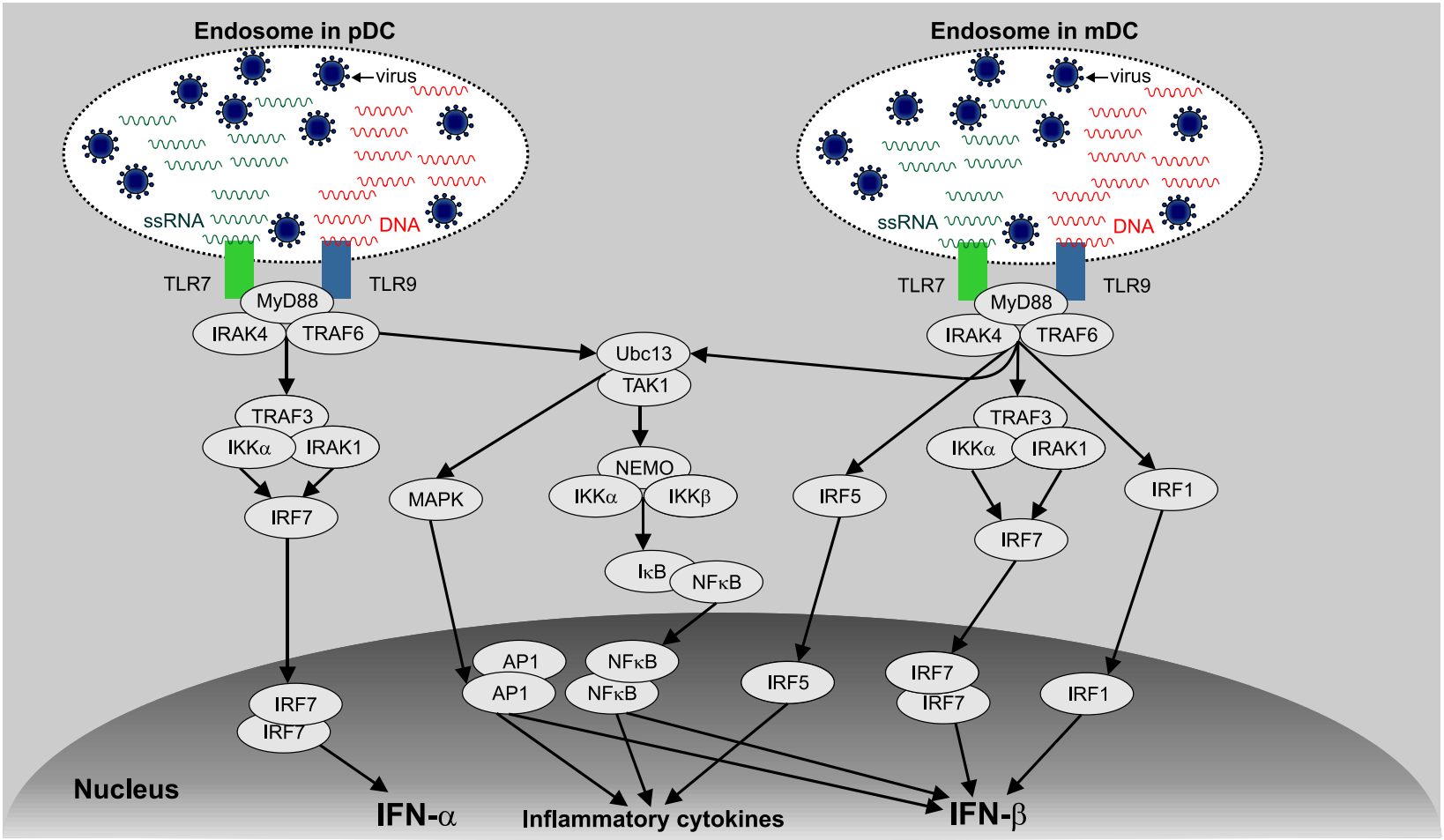


Figure 2. TLR7 and TLR9 signaling. TLR7 and TLR9 recruit MyD88, IRAK4, and TRAF6. TRAF6 then ubiquitin-dependently activates TAK1. The TAK1 complex activates the IKK complex consisting of IKKα, IKKβ, and NEMO/IKKγ to catalyze phosphorylation of IκB. IκBs are destroyed by the proteasome pathway, allowing NF-κB to translocate to the nucleus. TAK1 simultaneously activates the MAPK pathway, resulting in phosphorylation and activation of AP-1. NF-κB and AP-1 control inflammatory responses by inducing pro-inflammatory cytokines. MyD88 forms in pDC signaling complex with IRAK1, IKKα, TRAF3 and IRF7. In response to ligand stimulation, IRF7 is phosphorylated in a IRAK1- and IKKα-dependent manner, forms a dimer, and translocates to the nucleus to regulate expression of type I IFN genes, especially IFN-α. IRF5 and IRF1 also interact with MyD88 and participace in induction of inflammatory cytokines and type I IFN, respectively, in mDCs.

2.5. Type I IFNs

The type I interferon family consists of seven classes: IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, and IFN-τ [100]. In addition, four IFN-like cytokines have been reported: limitin (found only in mice) [101], IL-28A, IL-28B, and IL-29 found in humans and other mammals [102;103]. Whereas the IFN-α gene family consists of many members, coded by 14 IFN-α genes and by 3 IFN-α pseudogenes in the mouse genome [104], there is a single gene encoding IFN-β. Stimulation of many cell types with viruses, bacteria or TLR ligands, such as poly I:C, LPS, CpG and imiquimod, results in the production of IFN-α and IFN-β [105]. Factors involved in the transcription of IFN-β have been well characterized [105] and involve a complex containing the interferon regulatory factor 3 (IRF3). IRF7 after stimulation of pDC is responsible for production of IFN-α. Although the function of type I interferons is most closely associated with their antiviral activities, these cytokines also have diverse effector functions in the development of adaptive immunity that are not restricted to antiviral defense. IFN-α/β enable B cells to undergo isotype switching and differentiation into plasma cells through the activation of DCs [106]. Type I IFN are also activators of NK cells [107]. Finally, IFN-α/β induce DC maturation following stimulation via CpG, poly I:C, or LPS treatment or viral infection [108;109].

3. Adaptive immunity

Innate immune recognition plays a critical role in controlling the activation of adaptive immunity. As noted earlier, there are several classes of PRRs in the mammalian innate immune system. PRRs that signal the presence of infection (e.g., TLRs, RIG-I/MDA-5) not only induce the inflammatory and antimicrobial responses, but also activate the adaptive immune system. Adaptive immune recognition relies on two types of clonotypic antigen receptors: the T cell receptor (TCR) and the B cell receptor (BCR). The specificities of these

receptors are generated by random processes such as gene rearrangement and, therefore, are not predetermined to recognize only pathogen-derived antigens. Receptors of the innate immune system, on the other hand, are specialized for microbial structures and thus their activation can signal the presence of infection. There are two types of lymphocyte that express antigen receptors: conventional lymphocytes and innate-like lymphocytes. In the case of conventional lymphocytes - that is, conventional T cells (mostly αβ T cells) and B cells (also known as B2 cells) - antigen receptors are generated essentially at random. By contrast, for innate-like lymphocytes (B1 cells, natural-killer T cells (NKT) and subsets of γδ T cells) the diversity of antigen receptors is restricted and not entirely random [110]. The differentiation of conventional lymphocytes into a particular effector-cell type and their localization to the site of infection are regulated by the instructions provided by the innate immune system, generally in the form of cytokines and chemokines. There are two types of conventional αβ T cell: T-helper (Th) cells, which are marked by the co-receptor CD4 on the cell surface; and cytotoxic T cells, which express CD8. These cells recognize antigenic peptides bound to major histocompatibility complex (MHC) class II and class I molecules, respectively. The innate-like B cells known as B1 cells reside in the peritoneal and pleural cavities and produce mainly antibodies of the IgM class with specificities skewed towards some common bacterial polysaccharides and some self antigens [111]. The transduction of the innate, nonclonal recognition into signals that activate adaptive immune responses is mediated in part by antigen presenting cells, particularly DCs [59]. Immature DCs are located in peripheral tissues where they are likely to encounter invading pathogens [112]. Interaction of DCs with pathogens leads to the activation of signaling PRRs (e.g. TLRs, Rig-I, NOD etc.). Once activated through PRRs, DCs begin to express high levels of MHC class II and co-stimulatory molecules and migrate to the T cell zone in the draining LNs where they present pathogen derived antigens to T lymphocytes [112-114]. In addition, PRRs induce expression

of DC cytokines that control T cell differentiation into different effector lineages. Thus, TLRs induce production of IL-12 and IL-23 in response to intracellular and extracellular pathogens favoring Th1 and Th17 differentiation, respectively. RIG-I/MDA-5 induces high levels of IFN-α/β, which are important for CD8 T cell activation and differentiation. Thus, different classes of PRRs detect different types of infection and induce the appropriate effector response of the adaptive immune system.

3.1. T cells

3.1.1. CD4+ T cells

Activation of naïve CD4+ T cells requires presentation of antigen by MHC class II molecules in context of the co-stimulatory molecules CD80 and CD86 and the adhesion molecule CD54 [115]. Upon antigen recognition, $CD4^+$ T cells proliferate in response to autocrine IL-2. Proliferation of CD4+ T cells is not as extensive as that of CD8+ T cells. The frequencies of specific CD8+ effector T cells exceed those of CD4+ T cells during most infections. Activation and proliferation of CD4+ T cells is expanded by proinflammatory cytokines such as IL-1, IL-6, and TNF-α, which are produced by cells of the innate immune system in response to infections. The type of activation determines CD4+ T-helper cell polarization to Th1- or Th2-type cells. Virus infections induce both Th1 and Th2 cells. Th1 cells are characterized by secretion of IL-2, IFN-γ and TNF-β; Th2 cells by production of IL-4, IL-5, IL-10, and IL-13. Th1/Th2 polarization of immune response could by also determined by chemokine receptors expression. Th1 cells are characterized by expression of CCR5 and CXCR3; Th2 cells by expression of CCR3, CCR4 and CCR8. Once CD4+ T cells are activated in the T-cell–rich zones of the lymph nodes, they are ready to commence effector functions. Within the lymph nodes, CD4+ T cells are needed for formation of germinal centers at which CD4+ T cells interact with B cells through CD40–CD40L interactions. CD4+ T-cell–derived cytokines are instrumental for isotype switching of B cells. In mice, Th1-cell–

derived IFN-γ promotes switching to IgG2a, whereas Th2-derived IL-4 preferentially induces IgG1 switching. CD4+ T-cell–derived cytokines also drive proliferation of B cells and their differentiation into long-lived antibody-secreting plasma cells. CD4+ T cells help differentiation of naïve CD8+ T cells into effector cells by providing activation signals to DCs and by secretion of growth factors, most notably IL-2, which promotes CD8+ T-cell proliferation and under certain circumstances CD8+ T-cell survival. CD4+ T cells can be sometimes even cytotoxic. They have been shown to protect against a subsequent pulmonary challenge with a lethal dose of influenza A virus. Protection was in part mediated by perforin, suggesting that the T-helper cells eliminated infected cells by direct killing. Protection was also linked to an accelerated antibody response [116]. Other studies showed that selective presence of CD4+ T cells in the absence of CD8+ T cells or neutralizing antibodies increases influenza virus-associated immunopathology and thus mouse mortality following infection [117].

3.1.2. Regulatory T cells

Regulatory T cells represent heterogeneous cell population. Several types of these cells were described but their function, mutual interrelationship and classification have not yet been definitely established. Regulatory T cells (Tregs) are CD4+ CD25+ (α chain of the interleukin 2 receptor) CTLA-4+ (cytotoxic T lymphocyte antigen 4) GITR+ (glucocorticoid-induced tumor necrosis factor receptor) and are characterized by expression of the transcription factor forkhead box (Fox) p3 (FOXP3). They are either induced during thymic development (natural Tregs - nTreg), or in the periphery in the presence of TGF-β, IL-2 and retinoic acid (induced Tregs - iTreg) [118;119]. Mice with genetic deficiencies of TGF-β1, CTLA-4 and FOXP3 all develop a rapidly fatal autoimmune lymphoproliferative syndrome [120;121]. IL-2–deficient mice also develop multiorgan autoimmune disease [122]. Natural Tregs are present in the thymus of TGF-β1 and IL-2-deficient mice, but peripheral CD25-

cells from these mice need both of these cytokines to become FOXP3+ iTregs. Therefore, in peripheral lymphoid organs, both IL-2 and TGF-β are essential for the development of protective FOXP3+ iTreg cells. An exception might be the gut-associated lymphoid tissues (GALT) where retinoic acid can substitute for IL-2 [123]. Natural Tregs respond to self-antigens and are crucial to maintain tolerance. They play a role in infectious diseases by reducing the magnitude of primary immune responses, by limiting effector T-cell functions, and by reducing secondary immune responses. nTreg recognize antigen through less restricted T-cell receptors, and it is assumed that their receptors that respond primarily to self-antigens cross-react with antigens expressed by pathogens. They control adaptive immune responses through secretion of anti-inflammatory cytokines such as IL-10 or TGF-β, through modulation of DCs, and through direct cell-to-cell contact. Tregs express TLRs including TLR7 and TLR8 and are activated by infectious agents. After activation, they migrate to the sites of infection. Mice that lack Treg are better able to control viral infections, but they are also prone to more damaging immune responses.

Most regulatory T-cell clones produce high levels of IL-10 and TGF-β, moderate amounts of IFN-γ and IL-5, but not IL-2 or IL-4. CD4+ T cells generated in this manner have been termed T regulatory 1 (Tr1) cells [124;125]. Although IL-10 was originally described as a product of mouse Th2 cells, Tr1 clones are also capable of regulating Th2 responses, including antigen-specific IgE production [126]. Tr1 cells do not express FOXP3 and can be generated in the absence of FOXP3+ Treg. They appear to be triggered preferentially upon mucosal immunization, presumably to prevent induction of immune responses against antigens present in food or inhaled air. Tr1 cells are induced by DCs and preferentially by mucosal DCs that are not fully matured and secrete IL-10. The role of Tr1 cells during viral infections appears to be similar to that of CD4+ CD25+ Treg. One of the first approaches used for the induction of Treg was the administration of antigen via the oral route. Oral

tolerance takes advantage of the normal physiologic process that is needed to prevent systemic immune responses to ingested proteins. The suppressor cells from orally tolerant mice have been termed Th3 cells and mediate their suppressive effects primarily by secreting TGF-β [127]. Almost all of the recent studies on suppressor/regulatory T cells in mouse or human have focused on CD4+ T cells, a number of recent studies have suggested that potent CD8+ suppressor cells may also exist. A unique subpopulation of CD8+ T cells that expresses high levels of the IL-2R β chain (CD122) has been shown to have immunoregulatory activity [128].

Summary of diversification of CD4+ cells and Tregs is depicted on **Figure 3**.

3.1.3. CD8+ T cells

During a primary virus infection, viral control is achieved predominantly by specific $CD8^+$ T cells that can eliminate virus-infected cells through direct lysis of MHC class I expressing infected cells, and that can further reduce virus replication through the release of cytokines such as IFN-γ. Activation of naïve CD8+ T-cell responses occurs in lymphatic tissues and requires antigen presentation by DCs. Activation occurs rapidly, within hours after peptide-loaded mature DCs reach the T-cell–rich zones of lymphatic tissues. DCs migrate to lymph nodes draining the site of infection, and T cells are stimulated primarily within these draining nodes. Viruses that cause systemic infections are presented in multiple lymph nodes as well as in the spleen. Upon receiving activation signals, CD8+ T cells proliferate extensively in a 6- to 8-hour cycle. Activation of CD8+ T cells by viruses is usually dependent on the help from CD4+ T cells. It has been suggested that CD4+ T cells provide additional differentiation signals to DCs, enabling them to prime CD8+ T cells [129]. Some viruses can apparently induce a similar differentiation pathway of DCs, allowing for activation of CD8+ T cells in the absence of T help. Cytopathic viruses, such as influenza virus, can induce CD8+ effector T cells without CD4+ T help, although the help is required

for efficient formation of memory CD8+ T cells. CD8+ T cells that are deprived of CD4+ T-cell help are commonly short-lived and fail to establish functional memory [130]. During activation, CD8+ T cells undergo a number of phenotypic changes [131]. They reduce expression of CD62L and CCR7, which allows their egress from lymphatic tissues and upregulate expression of CD69 and CD25. Upon activation, CD8+ T cells upregulate expression of CD27 and CD44. A subset of CD8+ effector T cells augments expression of the IL-7 receptor α chain (CD127). CD8+ effector T cells express granzyme and perforin, and they produce cytokines and chemokines after encounter with their cognate antigens, including IFN-γ, TNF-α, IL-2, and MIP-1α/β. Once they are fully activated, CD8+ T cells leave the lymph nodes and migrate to sites of infection. Activated T cells express different chemokine receptors and a higher density of certain types of adhesion molecules (LFA-1 or integrin α4) than naïve T cells, which allows them to leave the blood vessels and enter sites of inflammation [132]. Once activated T cells reach an area of inflammation, their adhesion molecules interact with P- and E-selectins, ICAM and VCAM, which are expressed in increased levels on vascular endothelial cells in the presence of cytokines such as IL-1 or TNF-α. This interaction initially causes a loose attachment of the lymphocytes, followed by their rolling along the vessel walls [133]. Once the lymphocytes reach an area that is rich in chemokines corresponding to their receptors, they bind firmly to the vascular endothelial cells and eventually emigrate out of the vessels into the tissue. Recruitment of activated T cells is thus driven by chemokines and not by the antigen specificity of the T-cell receptors [134]. Once CD8+ T cells reach infected tissue and re-encounter their antigen on MHC class I-positive cells, they commence effector functions by releasing cytokines and mediating lysis of target cells. IFN-γ, which is released by most activated antiviral CD8+ T cells, upregulates the antigen-processing machinery and MHC class I expression, thus facilitating interactions between infected cells and activated CD8+ T cells.

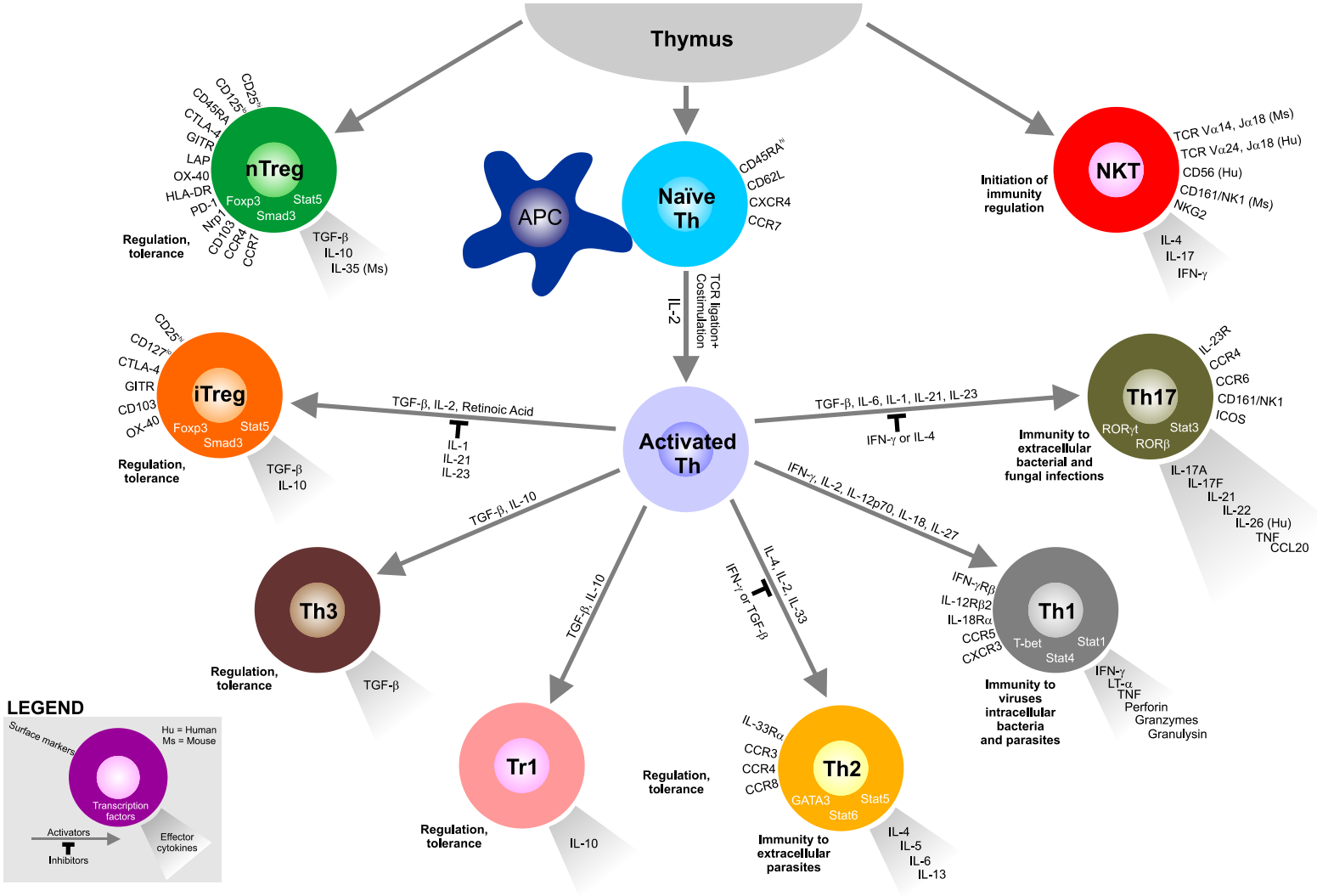


Figure 3. CD4+ T cells differentiation.

CD8+ T cells predominantly lyse their target cells through the release of granzyme and perforin, which form pores in target-cell membranes and cause caspase activation and apoptotic cell death. Another lytic pathway involves interactions between Fas ligand (CD95) expressed by activated T cells and Fas expressed by some types of target cells. Interactions between CD95 and Fas trigger activation of caspases and apoptotic cell death of the target cells [135]. CD8+ T cells are crucial to control acute viral infections caused by cytopathic viruses that kill the infected cells.

3.1.4. Intraepithelial lymphocytes

The major interface between internal organs and the outside environment is the columnar epithelial cell (EC) layer, which covers mucosal tissues. The mucosal epithelial layer includes a population of lymphocytes commonly termed intraepithelial lymphocytes (IELs) [136]. As their name implies, IELs reside between the basolateral surfaces of ECs. It has been estimated that 1 IEL occurs for every 4 to 10 intestinal epithelial cells seen in the small intestine and for every 30 to 50 intestinal epithelial cells found in the large intestine. This shows that large numbers of lymphocytes are situated in the surface regions of intestinal mucosal tissues. The majority of human and murine IELs are classified as T cells because they express the CD3 molecule in association with either of the two forms of T cell receptor (TCR), γδ or αβ. Concerning the expression of CD4 and CD8 by IELs, it has been shown that approximately 80 % of small intestinal IELs belong to the CD8 subset; however, a substantial number of IELs can be grouped as CD4-bearing cells including CD4+CD8- and CD4+CD8+ subsets. The CD8 molecules expressed on IELs consist of either αβ heterodimeric or αα homodimeric chains. CD8αβ+ IELs express Thy-1 and express the αβ TCR. In contrast, CD8αα+ IELs and CD4-CD8- double-negative IELs contain both TCRγδ and TCRαβ fractions [136]. IELs commonly express mucosal specific integrins α4ß7 and αEβ7. The α4ß7 integrin mediates the attachment of T and B cells to MadCAM-1 on HEVs in PPs and

MLNs, facilitating entry of these cells into the mucosal compartment [137;138]. The αEβ7 integrin mediates attachment of IELs to epithelial cells via its association with E-cadherin [139;140]. αEβ7 is also expressed on populations of dendritic cells and mast cells [140]. Greater than 90% of IELs and 45 - 50% of lamina propria T cells found in mouse and human small intestine express the αEβ7 integrin [141]. In contrast, fewer than 5% of human peripheral blood mononuclear cells express the αEβ7 integrin.

3.2. B cells and antibody response

B cells recognize viral antigens through cell surface–expressed receptors, which are membrane-anchored immunoglobulin molecules. Naïve B cells can pick up their antigen through specific cell surface–expressed BCR. The antigen is internalized and degraded within the lysosomes/endosomes into peptides that, provided they have suitable anchoring residues, associate with MHC class II molecules. The peptide/MHC complexes are transported back to the cell surface. Once B cells receive activation signals through BCR, they move to the T/B border of the follicles, where they interact with CD4+ T cells that recognize the MHC/peptide complex on the B cells surface. T-cell help requires direct interactions between T and B cells in which CD40 expressed on B cells binds to CD40 ligands expressed by T-helper cells. A number of other surface molecules on B cells and their corresponding ligands on T cells participate in forming the synapsis between activated T cells and naive B cells. Cytokines secreted by activated CD4+, such as IL-4 and IL-6, are also required for B-cell proliferation and differentiation. B-cell effector functions are mediated by secreted antibodies. B cells can produce five different classes of antibodies: IgM, IgD, IgG, IgA and IgE which express the same antigen-binding site but differ in the composition of their constant regions. IgA, IgG, and IgM contribute to the control of viral infections. IgM is produced first and is then, over time, replaced by IgG upon isotype switching. IgGs are most commonly found in blood, and they are divided into four subtypes. All IgGs with the appropriate specificity can prevent

infection of cells by neutralizing viruses. Antibodies have short half-lives. Nevertheless, after viral infections, specific antibodies can commonly be detected years later.

3.3. Immunoglobulin A

Immunoglobulin A (IgA) is the predominant antibody in the secretions that bathe mucosal surfaces such as the gastrointestinal, respiratory, and genitourinary tracts and in external secretions such as colostrum, milk, tears, and saliva. In addition, IgA is present in serum at concentrations of 2–3 mg/ml, making it the second most prevalent serum immunoglobulin after IgG. As with other Ig heavy-chain constant region genes, each domain is encoded by a separate exon. Unlike some other immunoglobulins, the IgA hinge region is not encoded by a separate exon or exons but is encoded at the 5' end of the Cα2 exon. The Fc region is critical for the effector function of IgA, mediated via interaction with various cell surface receptors. Receptors that bind to the Fc region of IgA include FcαRI (CD89) present on phagocytes such as neutrophils, macrophages, and eosinophils, Fcα/μR on B-cells, and the polymeric immunoglobulin receptor (pIgR) on epithelial cells.

3.3.1. IgA subclasses

Two subclasses of IgA, named IgA1 and IgA2, exist in humans. Two IgA subclasses are also present in most anthropoid apes (chimpanzee, gorilla, and gibbon) [142]. Other mammals have only one IgA isotype, with the exception of the rabbit, which has genes for 13 IgA subclasses, of which 10 or 11 appear to be expressed [143]. The basic difference between the human IgA subclasses is in the hinge region, where an insertion in IgA1 has produced a much more extended hinge than in IgA2. The IgA1 hinge, rich in Ser, Thr, and Pro residues, shares similarities with regions of mucin molecules and generally carries three to five O-linked glycan moieties [144;145]. There are two well-characterized allotypic variants of IgA2, termed IgA2m(1) and IgA2m(2). A third IgA2 variant, IgA2n, has also been reported [146].

The relative contribution of each subclass to the IgA pool varies between different body compartments. In serum, the IgA1 subclass predominates (about 90% IgA1, 10% IgA2). In secretions, the ratio of the subclasses varies depending on the site, but it is generally more evenly balanced than in serum [147;148].

Differences within the hinge regions of IgA1 and IgA2 account for the differential susceptibility of the IgA subclasses to cleavage by a group of highly specific proteolytic enzymes secreted by certain pathogenic bacteria. These enzymes, termed IgA1 proteases, each cleave at a specific Pro-Ser or Pro-Thr bond within the extended hinge region of IgA1. In contrast, IgA2 has a much shorter hinge and remains resistant to cleavage. The bacteria that produce IgA1 proteases (e.g., *Neisseria meningiditis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Streptococcus sanguis)* are responsible for a number of clinically important, and sometimes life-threatening, diseases. Cleavage of IgA1 within the hinge region releases the Fc portion of the antibody; so although the Fab portions might still bind specific antigens on the bacterial surface, no Fc-mediated elimination mechanisms can be triggered. In this way, the bacterium evades the protection that would normally be provided by mucosal immunoglobulins. Increased proportion of IgA2 in intestine could be taken for the compensation of protease sensitivity of IgA1. However, there are pathogenic bacteria producing proteases splitting not only both IgA1 and IgA2 but also other immunologic classes.

3.3.2. J-chain, pIgR receptor and Secretory Component

Of all of the Ig classes, only IgA and IgM share the ability to polymerize through the linkage of several monomer units. IgA predominantly polymerizes into dimers, which are stabilized through covalent interaction with the joining (J) chain (15 – 16 kDa) polypeptide which is also present in pentameric IgM [149]. The J-chain is highly conserved (~70%) between species. Its presence has been demonstrated in a wide range of vertebrate species

ranging from mammals, through birds and reptiles, to fish and amphibians [150]. The J-chain is expressed by antibody-producing cells and is incorporated into polymeric IgA or IgM shortly before or at the time of secretion [151]. Immunoglobin A that is destined for the mucosal secretions is produced locally by organized mucosal-associated lymphoid tissues. It is transported across the epithelium into the mucosal lumen by interaction with the polymeric immunoglobulin receptor (pIgR) [152]. pIgR is expressed basolaterally on epithelial cells and specifically transports polymeric immunoglobulins. Although pIgR is capable of transporting polymeric IgA and IgM at similar rates, the larger size of IgM restricts its diffusion to the receptor through the extracellular matrix and basement membrane so the smaller polymeric IgA molecule is transferred more efficiently [153]. After binding, the pIgR–dimeric IgA complex is internalized and transcytosed through a series of vesicular compartments to the apical plasma membrane. At this point, the extracellular portion of the pIgR, comprising five Ig-like domains, is proteolytically cleaved to form the secretory component (SC). The formation of a disulfide bridge links SC covalently to dimeric IgA, and it is the complex of dimeric IgA and SC, termed SIgA, which is released into the secretions. SC might afford the antibody some protection against proteolytic degradation [154;155], and the carbohydrate residues on SC help to anchor SIgA to the mucus lining of the epithelium, thereby ensuring effective immune protection [154;156].

4. Immune system of respiratory tract

Large numbers of microbes and microparticles enter the airways with every breath and the respiratory tract thus represents a major portal of entry for various viral and bacterial pathogens and allergens of outer environment. In addition to mechanical defenses such as coughing, sneezing, and the action of ciliated epithelia, mucosa-associated lymphoid tissue (MALT) plays a critical role in protection of the upper and lower respiratory tracts against microbial challenge. Although the respiratory tract represents about 25% (80-120 m^2) of the

total 400 m^2 of mucosal surface in the adult human, immune function in the airways are not so intensively studied as that of gastrointestinal tract. Most of our current understanding of mucosal immunity is actually based on information concerning gut, despite the fact that many differences exist between these two mucosal areas. Organized nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) are present in the upper respiratory tract and the importance of these tissues in protection against infection is well accepted but incompletely understood.

4.1. Structure of respiratory tract

The upper respiratory tract includes the nose, nasopharynx and the larynx, which separates the upper and lower respiratory tracts. The upper respiratory tract epithelia are ciliated, pseudostratified columnar cells. The lower respiratory tract includes the trachea, the bronchi, bronchioles and the alveoli. The trachea and the major bronchi are covered by pseudostratified columnar epithelia, whereas the bronchioles are covered with ciliated columnar cells and few goblet cells. Alveoli consist of cells of two types. Most of the alveolar surface area is covered by large, squamous cells called type I pneumocytes. A second epithelial cell type is known as the type II pneumocyte P2. Type I pneumocytes constitute part of the extremely thin gaseous diffusion barrier, whereas type II pneumocytes secrete a surface-active material called surfactant which reduces surface tension within the alveoli, preventing alveolar collapse during expiration. Type II pneumocytes retain the capacity for cell division and can differentiate into type I pneumocytes in response to damage to the alveolar lining. Clara cells of the respiratory bronchioles probably synthesize other components of surfactant.

Air from the trachea enters lung lobes via the two main bronchi, which are each subdivided into smaller bronchi and bronchioles forming the bronchial tree. Each terminal bronchiole ends in several alveolar sacs lined by alveoli where CO_2 and O_2 gas exchange

occurs. Many of the proteins associated with innate immunity of the respiratory tract are found in luminal mucus and the mucin-rich surface layers of the epithelium and the cilia. Mucus moves upwards by mucociliary transfer and through coughing mechanisms.

The lungs contain very few organized lymphoid tissue and the dominant cellular population involved in defense in this organ includes alveolar macrophages rather than lymphocytes. Normal bronchoalveolar lavage fluid (BAL) consists of 90% or more macrophages. Antigen-specific B-cells are induced to isotype switch and undergo somatic mutation in the germinal centers of MALT inductive areas (NALT and BALT) under the influence of cytokines and local microenvironment [157;158]. Transforming growth factor beta (TGF-β) drives switching to IgA, with contributions from IL-4, IL-5, IL-6 and IL-10; these stimulatory conditions were initially demonstrated by studying IgA production in non-specifically stimulated B-cell cultures [159]. It is unclear whether this switching is driven by T-cells or mucosal epithelial cells because both cell types can produce mentioned cytokines, including TGF-β [160].

4.2. Nasopharynx-Associated Lymphoid Tissue (NALT)

Nasal mucosa is one of the first sites for the contact with inhaled antigens. Little research has addressed the local sites at which immune responses are induced in this tissue. In humans and some other species, oropharyngeal lymphoid tissues, including the adenoids, palatine and lingual tonsils (Waldeyer's ring), probably contribute to respiratory and gastrointestinal immunity. However, organized lymphoid tissue in the nasal mucosa have been clearly identified only in rodents [161]. In these species, NALT consists of paired lymphoid structures situated above the soft palate at the entrance to the bifurcated pharygeal duct [162-164]. Each member of the pair is a substantial cylindrical structure, separated by and oriented parallel to the nasal septum. In mice, these structures each measure approximately 0.5 mm - 3 mm and contain 1 - 2 million cells [162]. Rodent NALT is in very

close proximity to the airway, being immediately overlaid by mostly ciliated folliculi-associated epithelial cells (FAE) [164], comprising M cells, alone or in clusters [17;165;166], and a few goblet cells [164;165]. NALT IELs have been noted as well. [167]. The NALT is composed of secondary lymphoid aggregates characterized by follicular B-cell areas and parafollicular T-cell areas [17]. Some plasma cells, principally those secreting IgA, are detectable [162;168]. In mice CD8+ cells are heterodimeric, reported as CD8+αβ and homodimeric CD8+αα [162]. Cytokine mRNA analyses revealed that CD4+ cells are dominantly T-helper 0 (Th0) cells, thus indicating that these cells can differentiate into either Th1 or Th2 cells upon antigen exposure to a particular antigen [17]. APC including DCs and macrophages are also present in the NALT [162;169].

4.3. Bronchus-Associated Lymphoid Tissue (BALT)

Bronchial lymphoid tissue is linked primarily to mucosal defense against inhaled microbes and therefore is one of many parts of integrated mucosal immune system that help to ensure the sterility of the gas exchange apparatus while avoiding sensitization to other inhaled antigens. BALT was originally described as a submucosal lymphoid organ, similar to PPs, found along the bifurcations of the upper bronchi directly beneath the epithelium and generally lying between an artery and a bronchus [170]. Although some species appear to develop BALT independently of antigenic stimulation [171], most normal mice and humans have little evidence of BALT [172]. However, pulmonary infection or inflammation in mice leads to the development of lymphoid follicules that are not restricted to the upper airways and are termed inducible BALT (iBALT) [173]. There is little information about molecular mechanisms that control the formation of iBALT. It has been demonstrated that BALT was induced in mice by inhalation of influenza virus, and further, that strong anti-viral B and T cell primary responses were initiated here. Afferent lymphatics leading to BALT follicles are not seen, and the efferent lymphatics drain to the regional mediastinal lymph nodes. Post

capillary HEVs, sites of lymphocyte immigration into BALT, express substantial levels of VCAM-1 [174] and α4β1 integrin, which underlie selective migration of lymphocytes to BALT. Plasma cells are found only in the periphery of BALT. The majority of B cells in BALT are B cells expressing mostly surface IgA and IgM. BALT is involved in normal immune responses to airborne antigens and infectious agents [175].

5. Influenza virus

Influenza is a serious respiratory illness which can cause complications that lead to hospitalization and death, especially in the elderly. The burden of influenza is currently estimated to be 25 – 50 million cases per year (~ 20% of the population) in the USA alone, leading to 150000 – 200000 hospitalizations and 30000 – 40000 deaths [176]. The risk of serious illness and death is highest among persons aged > 65 years, children aged < 2 years, and persons who have medical conditions that place them at increased risk of complications of influenza. New epidemic influenza A strains arise every 1 to 2 years by the introduction of selected point mutations within two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Therefore, there is no long lasting immunity against the influenza virus, neither after natural infection nor after vaccination, as is the case with smallpox, yellow fever, measles etc. These continuous and usually small changes in the antigenicity of influenza A viruses are termed antigenic drift and are the basis for the regular occurrence of influenza epidemics. In contrast to epidemics, pandemics are rare events that occur every 10 to 50 years. They have been documented since the 16th century, and in the last 400 years, at least 31 pandemics have been recorded [177].

During the twentieth century, three influenza pandemics occurred **(Table 4)**. Their mortality impact ranged from devastating to moderate or mild. The 1918 pandemic was caused by a H1N1 virus of apparently avian origin [178], whereas the subsequent pandemic

strains. H2N2 in 1957 and H3N2 in 1968 were reassortant viruses containing genes from avian viruses: three in 1957 (haemagglutinin, neuraminidase, and RNA polymerase PB1) and two (haemagglutinin and PB1) in 1968 [179]. The major changes in the antigenicity of influenza virus caused by reassortment are called antigenic shift.

Since 1997, when human infections with a highly pathogenic avian influenza A virus subtype H5N1 – previously infecting only birds – were identified in a Hong Kong outbreak, global attention has focused on the potential of this virus to cause the next pandemics [180]. Around 60% of humans known to have been infected with the current Asian strain H5N1 died. Incidence of this infection was low and human to human transfer was not proved. However, H5N1 may further mutate or reassort into a strain capable of efficient human-to-human transmission. **Table 5** represents worldwide summary of cases and deaths in years 2003 - 2009 caused by H5N1 infection.

In March and April 2009, an outbreak of a new strain of influenza commonly referred to as "swine flu" infected many people in Mexico and other parts of the world, causing sometimes severe illness and death – mainly in Mexico. The new strain was identified as a combination of several different strains of influenza virus A, subtype H1N1, including separate strains of this subtype circulating in humans and in pigs.

Influenza pandemics circulate around the globe in successive waves, and there is no way to prevent the spread of a new pandemic virus. The new potentially pandemic viral strain will eventually reach everywhere, and could infect theoretically every human being within a period of a few years.

year	designation	resulting pandemic	death toll
1889	H3N2	moderate	unknown
1918	H1N1 (Spanish flu)	devastating	50-100 million
1957	H2N2 (Asian flu)	moderate	1 million
1968	H3N2 (Hong Kong flu)	mild	1 million
2009	H1N1 (Mexican flu)	?	?

Table 4. Antigenic shifts of influenza virus A and pandemics.

5.1. Structure of influenza virus

Influenza (stemming from Italian/Latin *influentia* meaning epidemic) belongs to the family of *Orthomyxoviridae* It may be argued that members of this family of viruses are essential mucosal pathogens. *Orthomyxoviridae* are enveloped viruses, ranging from small spherical to filamentous in structure, ranging from 80 to 120 nm in diameter **(Figure 3)**. The virus contains a segmented single stranded (ss), negative strand, RNA. The negative strand serves as a template to mRNA synthesis as well as the antigenome positive strand. Unlike in other members of the *Orthomyxoviridae*, influenza RNA transcription and replication takes place in the nucleus of the host cell [181]. Influenza A, B and C viruses (3 different genera, A, B, C) can be distinguished on the basis of their antigenic differences in the nucleoprotein (NP) and matrix (M) proteins. The main surface antigenic determinants of influenza viruses are the haemagglutinin (HA) and the neuraminidase (NA) - transmembrane glycoproteins. On the basis of the antigen differences of these glycoproteins, influenza A viruses currently cluster into 16 HA (HA1 - HA16) [182] and 9 NA (NA1 - NA9) subtypes [183]. Interestingly, each of these subtypes can be isolated from aquatic birds, suggesting avian species are the natural hosts of influenza viruses. Of these viruses, only H1N1, H2N2, H3N2, H5N1, H7N7, and H9N2 subtypes have been isolated from human [184-187], indicating that there is likely to be a host restriction for influenza viruses. HA and NA are responsible of eliciting subtype-specific immune responses which are fully protective within, but only rarely protective across different subtypes.

Influenza B virus is not divided into subtypes but many drift changes take place during its phylogeny which is followed since 30th of the last century. Till now 3 genetically and antigenically different clusters were identified (B/Lee/40, B/Victoria/2/87 and B/Yamagata/16/88) [188]. Striking differences among them will probably better establish the basis for introduction of subtypes even in influenza virus B.

The conventional nomenclature for influenza virus isolates requires connotation of the influenza virus type, the host species (omitted in the case of human origin), the geographical site, serial number, and year of isolation. For influenza virus type A, the haemagglutinin and neuraminidase subtypes are added in brackets [example: A/Puerto Rico/8/34 (H1N1)]. The virus is enveloped by a lipid bilayer covering the M1 (matrix protein). The lipid bilayer and M1 are traversed by the M2 protein, which forms ion channels. Nucleocapsid contains 8 segments of ssRNA (composed of 890 to 2,341 nucleotides) in the form of helical ribonucleoproteins (RNP). Transcriptase and polymerase complexes are associated with the RNPs. **Figure 4** depicted the structure of influenza virus including 8 segments (A), electron photograph of influenza virus (B) and color electron photograph of influenza virus (C). Influenza A envelope is derived from the host's cell membrane during the budding process. The HA spikes are rod-shaped, whereas the NA spikes are mushroom-shaped. The HA binds to sialic acid receptors on respiratory tract epithelium, and mediates the fusion of the virus membrane with the endosomal membrane of the host cell in low pH. NA exists as a homo-tetramer and has enzymatic activity which cleaves the α-ketosidic linkage between D-galactose or D-galactoseamine residues. While NA does not appear to be necessary for viral infectivity, it is believed to be important in releasing new synthesized virions from infected cells. Avian influenza virus strains preferentially bind to sialic acids attached to galactose via α-2,3 linkage. This is the major sialic acid on epithelial cells of the duck gut. In contrast, human influenza virus strains preferentially attach to sialic acids attached to galactose by α-2,6 linkage. This is the major type of sialic acid present on human respiratory epithelial cells. α-2,3 linked sialic acids are found on ciliated epithelial cells, which are a minor population within the human respiratory tract, and also on some epithelial cells in the lower respiratory tract. Despite current knowledge mentioned above, it was recently demonstrated that avian H5N1 influenza strains can bind *ex vivo* cultures of human nasopharyngeal adenom and

tonsilar tissues, despite the lack of expression of α-2,3-sialic acid receptors [189]. Summarizing information about the structure and binding of influenza virus is available at the following link: http://www.sumanasinc.com/webcontent/animations/content/influenza.html

5.2. Haemagglutinin

Haemagglutinin (HA) is a glycoprotein containing either 2 of 3 glycosylation sites and having molecular weight of approximately 76 kDa. It spans the lipid membrane so that the major part, which contains at least 5 antigenic determinants, is exposed on the outer surface. HA serves as a receptor for binding to sialic acid (N-acetylneuraminic acid) and induces virus penetration into the cell and release of the interior content of the virus particle to cytoplasm by fusion with endosomal membrane. Haemagglutinin is the main influenza virus antigen. The antigenic sites are localized on the head of the molecule, while the feet are embedded in the lipid layer. The body of the HA molecule contains the stalk region and the fusiogenic domain which is responsible for membrane fusion with the host cell. At low pH in endosome, the fusion peptide is turned to an interior position. The HA forms trimers and several trimers form a fusion pore. Prominent mutations in the dominant antigenic sites reduce or inhibit the binding of neutralising antibodies, thereby allowing a new subtype to spread within a non-immune population. This phenomenon is called antigenic drift. The mutations that cause the antigenic drift represent the molecular basis for the seasonal influenza epidemics during winter time in temperate climatic zones. The immune response to the HA antigenic sites leads to the production of protective neutralising antibodies, which can prevent infection. Anti-HA neutralizing antibodies of elderly individuals can provide sometimes partial cross-protection against newly emerging pandemic strains. Antigenic shift - also termed genome reassortment or just reassortment – arises when the HA gene segments are exchanged between to virus subtypes, for example HA1 replaced by HA5 resulting in the formation of a mosaic virus.

Country	2003		2004		2005		2006		2007		2008		2009		Total	
	cases	**deaths**	**cases**	**deaths**	**cases**	**deaths**	**cases**	**deaths**	**cases**	**deaths**	**cases**	**deaths**	**cases**	**deaths**	**cases**	**deaths**
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	**8**	**5**
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	**1**	**0**
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	0	0	**8**	**7**
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	**38**	**25**
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	**1**	**0**
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	17	0	**68**	**23**
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	0	0	**141**	**115**
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	**3**	**2**
Lao People's Democratic Republic	0	0	0	0	0	0	0	0	2	2	0	0	0	0	**2**	**2**
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	**1**	**0**
Nigeria	0	0	0	0	0	0	0	0	1	1	0	0	0	0	**1**	**1**
Pakistan	0	0	0	0	0	0	0	0	3	1	0	0	0	0	**3**	**1**
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	**25**	**17**
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	**12**	**4**
Viet Nam	3	3	29	20	61	19	0	0	8	5	6	5	4	4	**111**	**56**
Total	**4**	**4**	**46**	**32**	**98**	**43**	**115**	**79**	**88**	**59**	**44**	**33**	**28**	**8**	**423**	**258**

Table 5. Cumulative number of confirmed human cases of avian influenza A (H5N1) reported to World Health Organization (WHO). Total number of cases includes number of deaths. WHO reports only laboratory confirmed cases. All dates refer to onset of illness (www.who.int).

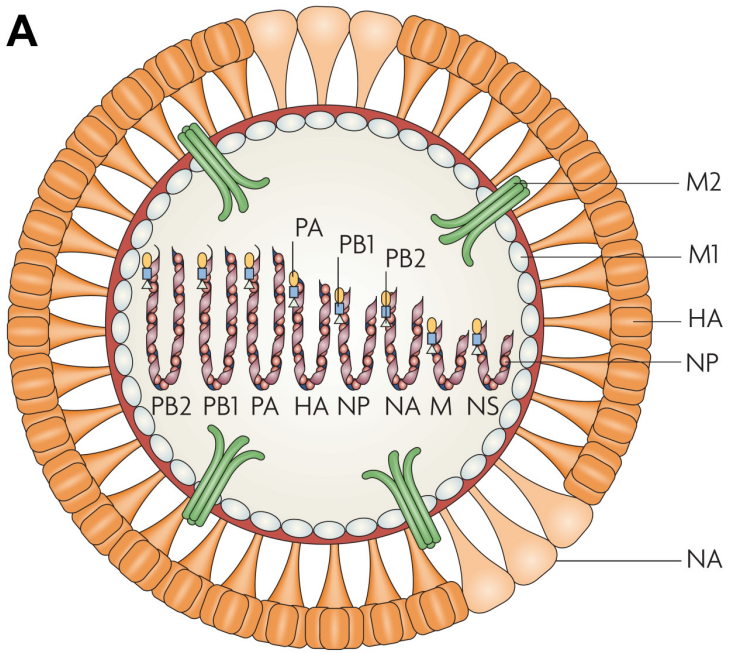
This may happen when one cell is infected by 2 different influenza viruses and their genome segments are exchanged during replication. Influenza virus infects a wide variety of warm-blooded animals, including birds and mammals. In aquatic birds, normal influenza replication takes place in the intestinal tract and tends not to cause symptoms. In mammals like humans and swine, influenza replication is limited to epithelial cells of the upper and lower respiratory tract. This tissue tropism is controlled to some extent by the limited expression of the appropriate protease for viral activation. In mammals, the suspected protease in the respiratory tract is tryptase Clara, a serine protease produced by nonciliated Clara cells of the bronchial and bronchiolar epithelia [190].

5.3. Neuraminidase

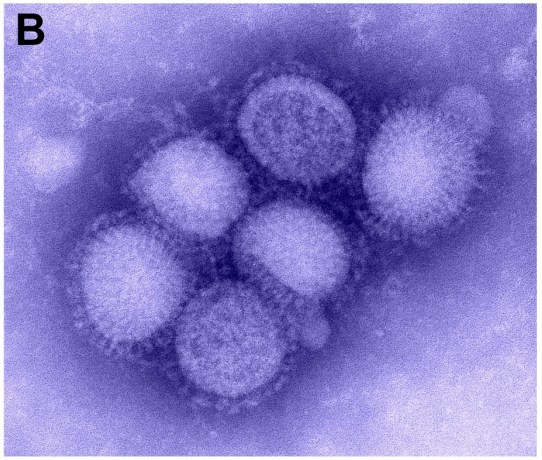
Neuraminidase (NA) is a second antigenic glycoprotein, localized on the viral surface. It forms a tetrameric structure with an average molecular weight of 220 kDa. In influenza infected cell, The NA molecule presents its main part on the outer surface of the cell, spans cell membrane and has a small cytoplasmic tail. NA acts as an enzyme releasing HA molecule of budding virus from host cell receptors, from other NA molecules and from glycoproteins and glycolipids on the cell surface. It also serves as an important antigen, and in addition, seems to be necessary for the penetration of the virus through the mucin layer to the respiratory epithelium. Antigenic drift can occur in the NA similarly as in HA [191].

5.4. Innate immune responses against influenza virus

The respiratory tract keeps at disposal numerous innate defense mechanisms comprising large antimicrobial proteins such as lysozyme and lactoferrin as well as small antibacterial cationic peptides, mainly defensins [192]. Salivary agglutinin and lung scavenger receptor glycoprotein 340 display anti-infleunza activities [193]. Human neutrophil peptide 1, a member of the alpha-defensins, inhibit influenza virus infections *in vitro* [194].



segment 1: Polymerase B2 protein (PB2)
segment 2: Polymerase B1 protein (PB1)
segment 3: Polymerase A protein (PA)
segment 4: Haemagglutinin (HA)
segment 5: Nucleocapsid protein (NP)
segment 6: Neuraminidase (NA)
segment 7: Matrix protein M1 and M2
segment 8: NS1 and NS2 (found only in infected cells)



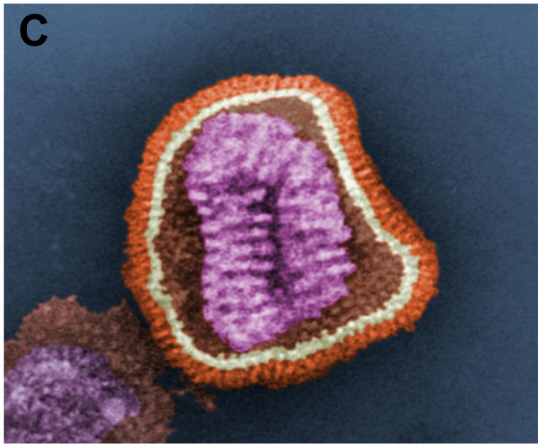


Figure 4. Structure of influenza virus. Description of influenza virus including list of 8 segments (A). Electron photograph of influenza virus (B) and color electron photograph of influenza virus (C). (Source of B and C: www.cdc.gov).

A family of host defense lectins, called collectins, including surfactant-associated proteins SP-A and SP-D opsonize for phagocytosis and enhance killing of microorganisms (including influenza virus) [195]. Specifically, SP-A and SP-D have been shown to inhibit the neuraminidase activity of the virus [196]. Human and animal sera contain various inhibitors that can neutralize the infectivity or inhibit the haemagglutination of influenza virus [197]. Three classes of such inhibitors have been reported. Two, α and γ inhibitors, are sialylated glycoproteins that inhibit viral haemagglutination by behaving like receptor analogues. The third, so-called β inhibitors, which are not receptor analogues, do not contain sialic acid. Mouse mannan-binding lectin (MBL) was found to be β inhibitors because they inhibited the infectivity and haemagglutinating activity of the H1 and H3 subtypes of influenza viruses [198-200]. Human MBL inhibits influenza A virus infection by two mechanisms. First, it blocks viral attachment to host cells, and second, it prevents viral spreading to contiguous cells by interfering with the budding process and viral release [201]. Complement components, [e.g. CD59a - sole membrane regulator of the membrane attack complex (MAC)] have also been suggested to have a role in the defense against influenza. The lack of this protein was shown to exacerbate influenza-induced lung inflammation through complement dependent pathways [202]. TLR7/TLR8 appears to play an important role in protection following intranasal influenza challenge of rats. This effect was dependent on IFN-α and TNF-α production [203]. TLR7 recognizes influenza virus RNA [203;204]. Infection of murine alveolar epithelial cells with influenza virus induced the release of monocyte chemoattractants CCL2 and CCL5 followed by monocyte transepithelial migration which was dependent on expression of CCR2 but not CCR5 [205]. Exposure of human monocyte derived DC to influenza induced the expression of a number of genes measured by microarray analysis. These included IL-8, TNF-α, IL-1β, IL-6, type I interferons, CD86, ICAM-1, IL-15R, IL-7R, IL-3R, CD83, and NF-κB p50, p52 and p65 [206]. Neutrophils and

macrophages accumulate in the lungs following influenza challenge and their TLR4 may also play a role in viral pathogenesis. TLR4 could play an important role in the host defense against influenza through activating innate immunity, including the phagocytic elimination of infected cells, in a ligand-dependent manner. It is unlikely that influenza virus-infected cells express LPS, a ligand for TLR4, and the mechanism by which TLR4 protects animals from infection with influenza virus is not known at present [207]. TLR-3 has also been implicated in the inflammatory responses following influenza infection of lung epithelial cells [208]. As HA binds to sialic acid receptors on NK cells, these cells are activated by influenza [209]. The role of NK cells in the defense against influenza was demonstrated in some studies in mice demonstrating that in the absence of an NK receptor (Ncr1 in mice and NKpg46 in humans), influenza infection was lethal [210;211]. IL-18 may augment the NK-mediated cytotoxicity against influenza-infected cells [212]. The monocyte chemotactic protein 1 (MCP-1) has been found to be important for immunity against influenza. Wild type mice have increased levels of MCP-1 in their lungs after influenza challenge, the MCP-1 deficient mice are more sensitive to influenza infection (enhanced weight-loss, elevated viral loads, reduced leukocyte (mainly macrophages and granulocytes) recruitment into lungs and reduced pulmonary IgA levels)) [213]. Activation of TLR3 on respiratory epithelial cells by influenza virus may mediate signaling through TRIF (but not MyD88) which leads to NF-κB activation and ultimately secretion of IL-8, IL-6, RANTES and IFN-β as well as upregulation of ICAM-1 [214]. Anti-influenza innate humoral responses were shown to be induced by direct stimulation of TLR on B cells and subsequent production of IFN-α [215]. Moreover, IFN-α production by lung B cells occurred within 48 h post influenza infection, causing an arrest in clonal expansion of B cells in regional lymph nodes [216]. Because Th-deficient mice controlled influenza infection better than combined Th/B cell deficient mice, a Th-independent role of B cells in influenza virus clearance has been suggested [217]. Natural antibody, defined as IgM antibodies that

bind to antigens not previously met by the host together with complement can mediate neutralization of influenza virus [218]. Type I IFN receptors on B cells stimulate early B cell responses in lungs through upregulation of the activation molecules CD69 and CD86 [219]. Recently, it was shown that IL-18, but not IL-12, is important for the development of anti-influenza CD8+ T cells [220]. IL-12 was reported to have an important role in the NK cell dependent IFN-γ production [221].

5.5. Adaptive immune responses against influenza virus

The adaptive immune responses against influenza include antibodies, function of Th cells and cytotoxicity exerted by both CD4+ and CD8+ T cells. It is believed that CD8+ T cells or B cells can each independently control influenza infections. Thus, mice lacking either CD8+ T cells or B cells can survive influenza challenge, while mice lacking both CD8+ and B cells do not survived infection. Presence of serum haemagglutination inhibition activity against influenza viruses strongly correlate with protection against disease. Anti-haemagglutinin antibodies are subtype specific. These antibodies can be sometimes cross-protective against diverse drift variant of the virus. Intersubtypic cross-protection is extremely rare and is more probable after infection than after vaccination. Many murine and human studies support the importance of mucosal IgA responses in protection against influenza infection. In both murine and human studies, mucosal IgA induced by intranasal immunization, was proved to protected against multiple strains of influenza virus in contrast to serum IgG induced by systemic immunization [222-231]. Therefore, recent efforts have focused on intranasal immunization strategies that induce both local IgA and systemic IgG responses [224;225].

5.5.1. CD4+ T cell-mediated responses

Influenza-specific CD4+ effector T cells protect against influenza by both perforin-mediated cytotoxicity and promotion of antibody-mediated responses [232]. Influenza-

specific IFN-γ expressing CD4+ T cells in the lungs lack expression of the chemokine receptor CCR7, and thus corresponding chemokine is not required for their homing [233]. Collagen, which is abundant and accounts for about 15% of dry weight of lungs expresses both of the collagen binding integrins α1β1 (CD49a) and α2β1 (CD49b). While CD4+ T cells express CD49b, CD8+ T cells express CD49a and thus these two T cell subsets differ in their binding to the interstitial environment of the lungs [234]. Although influenza neuraminidase-primed CD4+ T cells may traffic to the lungs, they do not proliferate in the absence of antigen [235]. In the absence of B cells, CD4+ T cells are unable to clear an influenza infection [236]. Interestingly, CD4-independent influenza-specific IgG responses can occur during primary infection of CD40-deficient or MHC II deficient mice [237]. Curiously, in MHC-II deficient or CD40-deficient mice, early during influenza infection, a CD4-dependent IgA response is generated which does not depend on cognate B cell-T cell interactions. However, in support of the previous findings, in these mice, influenza-specific IgM or IgG responses were not generated [238]. Generation and maintenance of influenza-specific long-term CD4+ memory T cell responses depends on the antigen dose, duration of repeated interactions with antigen-presenting cells, and the microenvironment of inflammatory and growth cytokines around the CD4+ T cells [239].

5.5.2. CD8+ T cell mediated responses

CD8+ cytotoxic T cell responses against influenza are generally induced against conserved, internal proteins, such as polymerase and nucleoproteins [240]. Acute heterosubtypic CTL responses in spleen, cervical lymph nodes (CLN) and MLN were proved in mice following pulmonary priming with nonpathogenic influenza strain Udorn (H3N2), and challenge with the mouse adapted pathogenic PR/8/34 strain (H1N1), while mucosal memory CTL responses were highly dependent on mucosal route of priming [241]. While presence of influenza-derived antigens is required to activate CD8+ T cells in the lungs,

subsequent localization of influenza-specific cells in the lungs is not antigen driven [242]. The magnitude of the recall response against influenza is strongly CD4+ T cell dependent [243]. The specificity of CD8+ T cell responses can substantially vary in primary vs. secondary influenza infections, a phenomenon that depends on the participation of both DC and non-DC cells in antigen-presentation during the primary response and mostly DC cells during the secondary response [244]. The size of the pre-existing antigen-specific CD8+ T cell pool as well as the amount of antigen and mode of antigen-presentation can all affect the size of the endogenous recall CD8+ T cell responses [245]. Lung CD8+ T cells specific for influenza virus nucleocapsid and acid polymerase-derived epitope produced IL-2, TNFα and IFN-γ [246]. In vaccinated humans, higher numbers of IFN-γ producing NK and T cells were detected in peripheral blood following vaccination with live attenuated virus compared to inactivated influenza virus [247].

5.5.3. Antibodies against influenza virus

Three different classes of antibodies participate in protection against influenza. These include IgM, IgG and IgA. It is generally accepted that influenza-specific IgG antibody responses are CD4 and CD40-dependent, but on the other hand, it was also described that mice lacking CD40 or CD4 developed anti-influenza IgG responses and recovered from primary influenza infection similarly as intact mice. However, mice lacking B cells were not protected against primary influenza infection [237]. SIgA plays its role at the portal of entrance of influenza on mucosal surfaces and is particularly important in memory type responses. Moreover, it is thought that induction of local IgA through intranasal vaccinations may induce better cross-reactivity with heterologous influenza strains.

6. Influenza vaccines

Vaccination is the primary method for the prevention of influenza and its complications. The continual genetic and antigenic variation that influenza viruses undergo requires constant global surveillance to identify and select new variants with epidemic potential or novel viruses with pandemic potential for new vaccine design. Two general types of influenza vaccines, inactivated or live attenuated vaccines, both grown in embryonated hen's eggs, are currently licensed for use. Inactivated vaccines induce immunity to infection in 70-90% of healthy adults <65 years of age when there is a good antigenic match between vaccine and circulating virus strain [248;249], but they are generally less effective in older adults. New strategies for influenza vaccines include altering the dose, site of application, or method of delivery of inactivated vaccines, the use of adjuvants or immunomodulators to enhance immune response, or targeting viral proteins that may promote broader cross-protective responses. Current influenza vaccines are designed primarily to induce antibodies directed against the HA since mainly these antibodies are believed to prevent the infection. Transmembrane viral M2 protein is highly conserved among human influenza A virus subtypes, and is therefore considered a suitable candidate to elicit broad immunity against multiple influenza A subtypes [250].

6.1. Inactivated influenza vaccines

Inactivated influenza A and B virus intramuscular vaccines are licensed for administration in humans. The vaccine is reformulated each year to include the strains thought to be prevailing in coming season. The choice of seed virus is made by the WHO and the US Centers for Disease Control (CDC). The vaccine virus is currently grown in embryonated eggs but because many human influenza virus isolates do not grow to high yield in eggs, a reassortment virus is made using the high egg-yielding PR/8/34 genetic backbone and the HA and NA genes of the candidate virus. Most influenza vaccines are split-vaccines produced

from detergent-treated purified influenza virus, or surface-antigen (subunit) vaccines containing purified HA and NA proteins [251;252]. Whole virus vaccines are nowadays used infrequently in annual influenza vaccines as they are associated with increased adverse reactions, especially in children. Annual influenza vaccines are trivalent containing 15 □g of each of two influenza A subtypes (H1N1 and H3N2) and one influenza B strain and are delivered by the intramuscular route. The serum antibody induced by inactivated vaccine is strain-specific and antigenic differences between the vaccine and circulating strain may reduce the efficacy of the vaccination. Universally accepted correlate of protection is serum hemagglutination inhibition assay titer ≥ 1:40. Inactivated vaccines have not convincingly shown efficacy in generating long-lasting immunity, particularly in the elderly, and may not be sufficiently cross-reactive to protect against antigenic variants [253;254]. Although the intramuscularly injected vaccines are known to induce serum immunoglobulin G (IgG) antibodies, they are poor stimulators of secretory IgA (SIgA) at respiratory mucosal sites and cause sporadic CD8+ cytotoxic T-lymphocyte (CTL) activation [255-257]. Efforts are currently under way to develop influenza vaccines that generate significant SIgA, as well as high serum IgG titers, by exploiting mucosal immunization [258-261].

6.2. Live influenza vaccines

A second type of vaccine, a live attenuated influenza vaccine (LAIV), was licensed in the U.S. and is approved for use in healthy persons aged 5-49 years [224;225]. LAIVs have been widely used in the former Soviet Union and Russia in influenza vaccine prevention programs in children and adults [262]. LAIVs are based on the concept of cold-adaptation (growth of influenza viruses at less than optimal temperatures, resulting in attenuation of donor strain). This strategy has been used by both U.S. and Russian investigators to generate highly stable master strains of influenza A and B viruses with cold adapted and temperature sensitive phenotypes. A live attenuated vaccine (Flumist) has been licensed [263]. The

vaccine virus is based on the genetic backbone of a cold-adapted virus A/Ann Arbor/6/60. Five mutations in the PB2, PB1 and NP genes were associated with the temperature sensitive phenotype of cold-adapted A/Ann Arbor/6/60 [264;265], the master donor strain was used to produce influenza A virus antigens of the U.S. licensed vaccine. These mutations enable efficient replication of the master donor strain at lower temperatures (33 °C) in the nose but essentially prevent replication at higher temperatures (39 °C) in lower airways [266]. A similar set of temperature-sensitive attenuating mutations are found in the A/Leningrad/137/57 cold-adapted master donor strain used to generate the influenza A components of Russian cold-adapted vaccines [267]. Recombinant vaccine viruses that possess 6 internal genes from the master donor cold-adapted strains and the HA and NA genes from the wildtype epidemic strain are generated by traditional reassortment techniques. Live vaccines delivered intranasally replicate to a limited extent in the upper respiratory tract and thus induce immunity more similar to that induced by natural infection then that induced by parenteral inactivated vaccines. LAIV are likely to induce mucosal antibody responses and cellular responses, which may contribute to high protective efficacy even against a variant epidemic strain that is antigenically distinct from the vaccine strain [268]. Nevertheless, like the current inactivated vaccines, there is a need to continually update LAIV with the HA and NA genes of currently circulating influenza strains.

6.3. Adjuvanted subunit vaccines

Vaccination with LAIV was shown to be effective in the induction of a protective response. Nevertheless, the use of a living viral vaccine is never without any risk. Unfortunately, inactivated viruses are usually not effective enough in mucosal immunization. Therefore, for optimal immunization, effective and safe mucosal adjuvants are required. A variety of immunopotentiating adjuvants and delivery systems have been used in animal experiments and clinical trials to enhance the efficacy of mucosal and systemic influenza

vaccines. These include cholera toxin (CT) and its mutated derivatives, heat labile enterotoxin from enterotoxigenic *Escherichia coli* (LT) and its mutants, oil in water emulsions, proteosomes, immunostimulating complexes (ISCOMS), liposomes, CpG oligonucleotides, plasmid DNA and RNA (e.g. alphavirus-based replicon particles).

CT and LT are powerful mucosal adjuvants when co-administered with soluble antigens. CT and LT have high homology (80% identity) in their primary structure [269;270] and superimposable tertiary structures [271]. Both toxins have an AB5 structure: the A subunit is an enzyme with ADP-ribosylating activity that is responsible for the toxicity, whereas the B subunit is a pentameric oligomer that binds the receptors located on the surface of eukaryotic cells. However, their use in humans is complicated by their extremely high toxicity. During the past few years, site-directed mutagenesis has permitted the generation of LT and CT mutant's fully non toxic or with dramatically reduced toxicity, which still retain their strong adjuvanticity at the mucosal level. Among these mutants, are e.g. LTK63 (serine-to-lysine substitution at position 63 in the A subunit) and LTR72 (alanine-to-arginine substitution at position 72 in the A subunit). A potentially safer detoxified mutant of LT (LTK63) has been tested in a Phase I intranasal influenza vaccine trial [272]. LTK63 is an effective mucosal adjuvant with no detectable toxic ADP-ribosyltransferase activity, and holds promise as a mucosal adjuvant [272].

After aluminium, emulsions are the most frequently used adjuvants in humans and animals; however, only oil in water emulsion (MF59) has been licensed for intramuscular vaccination of human till now. MF59 is an emulsion composed of 5% v/v squalene, 0.5% v/v polysorbate 80 and 0.5% v/v sorbitan trioleate, emulsified under high pressure to produce uniform droplets which is licensed in some European countries [273]. MF59 adjuvanted vaccine was shown to significantly enhance serum hemagglutination-inhibition antibody responses to influenza subtype H3N2 and the B vaccine component particularly in elderly

individuals with chronic diseases [274]. Although local reactions were more frequent in individuals who received the MF59-adjuvanted vaccine compared with non-adjuvanted vaccine they were predominantly mild and transient [275]. Another modification of the subunit vaccine approach is the virosomal influenza vaccine.

Virosomes consist of influenza virus surface glycoproteins HA and NA released from virus by detergent disruption and reconstituted into phosholipid bilayer to form liposomes [276;277]. This approach is thought to present the HA and NA proteins to the hosts in a manner similar to that of the intact virus, resulting in augmented cellular response [278]. The trivalent parenterally administered virosomal vaccine has been licensed in som European countries since 1996 [279]. An inactivated virosomal-subunit influenza vaccine licensed in Switzerland (Nasalflu, Berna Biotech) was available for the 2000–2001 influenza season; it contained a mutant of *Escherichia coli* heat-labile toxin as a mucosal adjuvant (commercial name - Escherigen Berna®). However, after licensure and use in Switzerland, 46 cases of Bell's palsy (temporary partial facial paralysis) were reported over a 7 month period and the vaccine was withdrawn from the market. A case-control study provided evidence to suggest a strong association between the inactivated intranasal influenza vaccine and Bell's palsy with a peak incidence occurring 31-60 days post vaccination [280]. It has since been shown that intranasally given CT (and possibly LT) can access CNS via olfactory bulb and bind to neuronal cells, hence causing Bell's palsy either directly or through activation of dormant viruses such as herpes virus.

Proteosomes are hydrophobic, membranous, multimolecular preparations of meningococcal outer membrane proteins that form nanoparticles of size 20-800 nm in aqueous solutions [281]. Porin B from *Neisseria meningitidis* B strains, a major component of proteosomes, has been shown to activate APC by interacting with TLR2 and up-regulating the

expression of the costimulatory molecule CD86 on the surface of B cells and other APC [282].

Immunostimulating complexes (ISCOMs) contains the saponin Quil A from the tree *Quillaja saponaria Molina* which has adjuvant properties. The components are incorporated into icosahedral particles of about 40 nm in size by hydrophobic interaction during assembly of the complex. The immunogenicity enhancing activity of ISCOMs may correlate with enhanced expression of class II MHC and costimulatory molecules on APC. Moreover, ISCOMs deliver antigens for class I and class II presentation, induce predominant Th1 or balanced Th1/Th2 response, and induce CTL [283].

ODN containing unmethylated CpG motifs have been shown to bind to TLR9 on pDC and B cells [69;284]. Phase I clinical trials with intramuscularly given CpG ODN adjuvanted trivalent influenza vaccines containing 10 times reduced antigen dose of A/Beijing/262/95, A/Sydney/5/97 and B/Harbin/7/94 were well tolerated and induced similar levels of serum HAI antibodies as the unadjuvanted full dose vaccine [285].

DNA vaccines have attracted much attention since they were reported to induce protective immune responses in experimental animals (mice, chicken or ferrets) [286;287]. DNA vaccines are non-infectious, non-replicating *E. coli* derived plasmids containing transcription machinery that encodes only protein(s) of interest. Influenza virus DNA based vaccines induced protective long-lasting HAI antibodies in rodents, ferrets and non-human primates. DNA vaccines against conserved internal proteins such as NP generated CTL response and conferred heterosubtypic immunity against lethal challenge [288].

Chitosan is the carbohydrate biopolymer derivative of chitin, found in the exoskeletons of crustaceans and insects and in mushrooms. It has also been evaluated as an adjuvant for influenza vaccine delivery. Chitosan have been shown to activate peritoneal macrophages *in vitro*, induce NO production and chemotaxis of phagocytic cells and suppresses tumor growth

in mice. An influenza virus surface antigen vaccine formulated with chitosan and delivered intranasally to mice induced strong local and systemic antibody responses [289].

Bacteria generally are immunologically very active and many of their constituents and products exert immunostimulatory properties and represent potential adjuvants for induction of specific antibody response. Various biological properties of bacteria were studied in our institute in the past. During this study strong immunopotentiating activity of *Bacillus firmus* was found.

7. Bacillus firmus

Bacillus firmus (BF) is a nontoxic and nonpathogenic G+ bacterium of external environment. Immunomodulatory properties of BF were revealed by Mára et al. [290] in a large screening comprising many species and strains of G+, G- and acid-fast bacteria. BF exceeded strongly all other G+ bacteria tested in increasing the resistance of mice against experimental listerial infection. Several other immunostimulatory properties were proved in our further studies: polyclonal activation of human lymphocytes [291;292], macrophage activation in mice [293;294] and the increase of antitumor resistance in rats [295]. We compared the immunostimulatory effect of BF on Ig production by human lymphocytes *in vitro* with that of several other bacilli and found the activity of BF to be the highest [291]. BF is a quite harmless bacterium and both live and killed bacteria in high doses are very well tolerated by mice after mucosal or parenteral (intraperitoneal) application. This is a very important prerequisite for future possible practical use of BF as adjuvant. All these results directed us to the study of the effect of BF on specific immune response and to its possible use as an adjuvant. Strong stimulatory effect on immunoglobulin synthesis, including the synthesis of IgA, led us to the application of this adjuvant for mucosal immunization. Mlckova et al. reported pronounced immunostimulatory effect of BF after intranasal and intratracheal immunization of mice by BF in combination with model antigen ovalbumin

(OVA) [296]. Immunization via respiratory tract with mixture BF+OVA had a clearly stimulatory impact on antigen specific humoral response. A significant increase was observed in anti-OVA IgG specific antibodies in serum, BAL and intestinal lavages. High levels of anti-OVA IgA specific antibodies were demonstrated in BAL after intratracheal and in intestinal lavages after intranasal immunization [296]. Functions of T cells were determined after intranasal and intratracheal immunization of mice with OVA and BF as adjuvant [297]. The way of T cell involvement was unclear. No OVA-specific proliferation or cytokine production was proved in splenocytes derived from either OVA or OVA + BF exposed mice but analysis of anti-OVA IgG subclasses demonstrated that both Th1 and Th2 subpopulations were probably stimulated. Adjuvant activities of BF and its delipidated form DBF were compared and DBF was found to be more active [296;297]. The effect on stimulation of mouse peritoneal cells *in vitro* was evaluated by testing nitric-oxide-synthesis induction and cytokine formation [298]. Results listed above led us to the use of BF as adjuvant in the study of immune response to practically important antigen and epidemiologically important infection agent – influenza virus.

AIMS

The main aim of this thesis was to elucidate the effect of bacterial adjuvant - *Bacillus firmus* on tuning of innate and adaptive immunity after intranasal or intratracheal immunization of mice with inactivated influenza virus type A and B.

Particular aims:

1. **To explore the effect of *Bacillus firmus* as adjuvant in mucosal immunization of mice with inactivated influenza virus. Impact on systemic and mucosal immunity.**

2. **To test the potential of *Bacillus firmus* to induce intrasubtypic and intersubtypic cross-protection against influenza infection.**

3. **To characterize the mechanism of adjuvant effect of *Bacillus firmus.* Influence on activation of innate immunity (gene expression of TLRs and IFN type I). Influence on tuning of adaptive immunity (gene expression of Th1 and Th2 cytokines).**

RESULTS

Influenza virus infections cause considerable morbidity and mortality in the world. Current immunization against influenza is provided using parenterally given influenza vaccines. These vaccines can induce good systemic immunity but they fail to induce a protective mucosal immunity. When the mucosal immunity is insufficient, the virus replicates in the airways and can easily spread within the population. Systemic immunity can stop the infection only after the virus penetrates mucosa of the respiratory tract. Good mucosal immunity can be induced only by mucosal immunization. Mucosal immunization should be the optimal way for vaccination against influenza. Vaccination with live virus is rather risky and inactivated virus is not efficient enough in mucosal immunization. Suitable mucosal adjuvants are being sought to increase immunization efficiency. BF or delipidated form of BF (DBF) were used in all experiments as adjuvants in the effort to increase a potency of inactivated influenza virus type A and type B in immunization via respiratory tract.

⌘

Immune response after adjuvant mucosal immunization of mice with inactivated influenza virus

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In the first study, we investigated the effect of BF on the production of antibodies after intratracheal immunization of mice with inactivated influenza virus type B which is included in trivalent influenza vaccine and which is supposed to be less immunogenic than influenza A virus. We tested the possibility to increase the immune response against inactivated influenza virus by BF or DBF as adjuvant. Production of specific anti-influenza antibodies was tested by ELISA method. Lymphocyte proliferation was tested on splenocytes from immunized animals after stimulation with influenza virus (HT 1:5) via incorporation of ^{3}H thymidine.

Cytokines were determined in culture supernatants by ELISA method after 24 h (IL-2), 3-day (IL-4 and IL-10) and 5-day cultivation (IFN-γ). Expression of selected genes: IL-2, IL-4, IL-10, IFN-γ and iNOS were tested by RT-PCR in lungs. Changes in morphology of lungs after intratracheal immunization were examined histologically.

Production of specific anti-influenza antibodies after intratracheal immunization

Production of specific anti-influenza antibodies was measured by ELISA method after intratracheal immunization in serum, BAL and intestinal washings. Immunization with inactivated virus alone evoked only a mild systemic response and no mucosal response. After immunization with virus suspension plus bacteria, the anti-influenza antibodies increase markedly, the adjuvant effect of DBF being higher than that of BF. The levels of serum IgM antibodies do not change during immunization, and represent naturally present antibodies. The largest rise in the levels of serum antibodies appears in the IgG class, which reflects best the status of systemic immunity. A milder rise in serum antibodies occurs in IgA. A rise of the antibody activity of all three classes, especially in IgG and IgA, is evident after adjuvant immunization in BAL, which reflects the state of antibody immunity in the respiratory tract. BAL antibodies mainly that of IgA class are locally produced but IgG antibodies originate mainly from serum and enter respiratory system by transudation in lungs. Another part of the mucosal immune system, the intestine, exhibits after adjuvant intratracheal immunization a marked increase of IgG antibodies and only a weak increase of IgA antibodies.

Antibodies against *Bacillus firmus*

Even non-immunized organisms contain serum antibodies against BF, which have the character of natural antibodies or specific or cross-reacting antibodies against the ubiquitous bacterium. The serum contains high levels of IgM class antibodies and very low, limiting

values of IgG and IgA class antibodies. The levels of IgM and IgA antibodies hardly change after immunization with BF while the levels of IgG antibodies rise conspicuously. Antibodies against BF in BAL are below the detection limit, a marginal rise of IgM antibodies and marked rise of IgG and IgA is observed after immunization. IgG and IgM antibodies in the intestine are below the detection limit while the levels of IgA antibodies are perceptible. Following immunization, IgM and IgA antibodies do not change whereas IgG antibodies increase slightly.

Stimulation of T lymphocytes

Stimulation of splenocytes of immunized animals *in vitro* by inactivated influenza B virus of the same drift variant as that used for immunization causes a 5-10 fold lowering of blastic transformation as compared with non-stimulated splenocytes (stimulation index - SI 0.1 – 0.2). Production of cytokines characteristic for Th1 and Th2 (IL-2, IFN-γ and IL-4 or IL-10) was tested by ELISA in supernatants of splenocyte cultures after stimulation with influenza virus. In this case, the stimulation was detected but was only very slight relative to non-immunized controls. IL-2 was stimulated to the same extent in animals immunized with the virus alone and in those immunized with virus+BF. The production of IL-4, IL-10 and IFN-γ by splenocytes from mice immunized with virus+BF was higher than in mice immunized with the virus alone. Immunization with virus+DBF did not affect the production of cytokines in the culture after specific stimulation. Splenocytes from nonimmunized controls and from mice immunized with bakteria alone exhibited, after *in vitro* stimulation with influenza virus, a lowering of production of the above cytokines relative to the spontaneous synthesis in non-stimulated cultures.

Local production of cytokines in the lungs

RT-PCR technique was used to estimate the expression of IL-2, IFN-γ, IL-4 and IL-10 in lungs. Only traces of mRNA for these cytokines were detected in the lungs of non-immunized animals. Immunization with the virus alone led to an increase in mRNA for all cytokines under study, a still greater mRNA production for all cytokines being found after immunization with BF alone or virus+BF. The results indicate that systemic and local production of cytokines is increased without a perceptible Th1 or Th2 polarization.

Local activation of macrophages in the lung

RT-PCR proved the presence of mRNA for inducible NO syntheses (iNOS), which can serve as a marker of macrophage activation. All types of immunization induced an increased production of mRNA for iNOS relative to non-immunized controls, although even in the controls the production was quite sizable.

Lung histology after immunization

Even control mice that aspirated only PBS exhibit a marginal mononuclear cell infiltration; a similar picture is seen after aspiration of bacterial suspension alone (DBF). Immunization with the virus, though inactivated, brings about a fairly pronounced inflammatory infiltration with occasional haemorrhagies. The infiltration is much less pronounced after immunization with virus+DBF; this indicates that a simultaneous administration of DBF alleviates the inflammatory effect of the virus.

Immunization with the inactivated influenza virus B alone did not produce a sufficient immune response, while the use of BF or DBF as adjuvants evoked remarkable antibody response, both systemic and mucosal. Intratracheal immunization gave rise to high

levels of mucosal antibodies, in particular in the respiratory tract. The immunization increased the local cytokine production in the lungs without any marked Th1 – Th2 polarization. The use of adjuvant lowers inflammatory changes in lungs accompanying intratracheal immunization. Activation of lung macrophages after immunization was proved by production of iNOS.

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Adjuvant effect of *Bacillus firmus* in intranasal immunization of guinea pigs with inactivated type B influenza virus

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Adjuvant effect of BF after intranasal immunization of guinea pigs was tested in this study. Inactivated influenza virus type B + BF as adjuvant in comparison with the virus alone, can stimulate higher titers of serum hemagglutination-inhibiting antibodies and virus-neutralizing antibodies. This phenomenon could be exploited for preparation of immune sera for *in vitro* diagnosis.

Intranasal immunization

Guinea pigs were immunized with inactivated influenza B/Lee/1/40 virus alone (16 animals - standard immunization) and inactivated virus plus BF as an adjuvant (25 animals - adjuvant immunization). 15 animals were immunized with BF suspension alone and 15 non-immunized animals were used as the controls. Detection of antiviral antibodies was performed by hemagglutination inhibition test (HIT and TW/E-HIT), virus neutralization test (tested on MDCK cells) and complement-fixation reaction (CFR). Sera of non-immunized controls and guinea pigs immunized with BF alone showed complete negativity in all tests. Markedly

higher titers of anti-influenza antibodies were obtained after intranasal immunization with virus + BF as adjuvant than with immunization by virus alone.

High level of neutralizing antibodies after adjuvant immunization of guinea-pigs with inactivated virus demonstrates that possibility of safety preparation of diagnostic sera against highly pathogenic strains with respect to standard biosafety guidelines.

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Protective and cross-protective mucosal immunization of mice by influenza virus type A with bacterial adjuvant

Zanvit Peter, Havlíčková Martina, Táčner Jaroslav, Novotná Olga, Jirkovská Marie, Čechová Dana, Julák Jaroslav, Šterzl Ivan, Prokešová Ludmila *[Immunology Letters 115 (2008) 144–152]*

Because of persisting threat of new highly pathogenic influenza A subtypes development, a vaccination inducing intersubtypic cross-protection is desirable. In the next study, we tried to reach this goal by mucosal immunization of mice using delipidated *Bacillus firmus* (DBF) as adjuvant. BALB/c mice were immunized intratracheally with inactivated influenza A H1N1 and H3N2 viruses. The production of antibodies in sera and secretions was determined by the ELISA. The protective and cross-protective effect against infection was tested in *in vivo* experiments after infection with influenza virus A H1N1. The local situation in the lungs was assessed histologically and by testing the cytokine expression.

Production of antiviral antibodies detected by ELISA

The type A virus A/PR/8/34 was found to be much more immunogenic than type B viruses. Intratracheal immunization with inactivated virus alone induced perceptible antibody

response in contrast to type B viruses used for immunization in the same amount [299]. Apparently due to this perceptible response to immunization by the virus alone, the effect of the adjuvant was not as marked as with type B viruses. The efficiency of the immunization was verified in protective infectious experiment *in vivo*.

The levels of virus-specific antibodies were detected after intratracheal immunization in serum, BAL and intestinal washings. The levels of IgG and IgA in serum of control mice immunized with PBS were low and so were also the levels detected in the group immunized with the adjuvant alone. The group immunized with A/PR/8/34 virus alone exhibited a more conspicuous production of virus-specific antibodies of IgG and IgA classes. The highest IgG and IgA levels were detected in the group of mice immunized with a virus+DBF mixture; this indicates a strong induction of systemic immunity. In BAL the lowest levels of virus specific antibodies were detected in mice immunized with PBS or with the adjuvant alone. Increased levels of IgG and IgA were detected in the group immunized with the virus alone. The highest levels of both IgG and IgA were again detected in the group immunized with the virus+DBF mixture. The relatively high IgG level attests to a close connection of the systemic and mucosal immunity in the lungs. This immunization therefore induced good systemic as well as mucosal immunity in the airways. The testing of levels of virus-specific antibodies in intestinal washings showed that mice immunized with PBS, and also mice immunized with the adjuvant alone exhibited low levels of IgG while the IgA levels were about equal in all immunization groups. The group immunized with the virus alone displayed slightly increased levels of IgG while the highest levels of virus specific IgG class antibodies were detected in mice immunized with the mixture virus+DBF.

Local production of cytokines in the lungs

Testing of cellular immunity in the lungs after immunization was performed by real-time PCR determining the local expression of cytokines characteristic for Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) response. The groups immunized with virus alone or adjuvant alone exhibited a down-regulated expression of cytokines IFN-γ, IL-2 and IL-4 while the expression of IL-10 was up-regulated in comparison with the control group. The group immunized with virus+DBF displayed a many-times up-regulated expression of IL-10 and of all other cytokines. – IFN-γ, IL-2 and IL-4 which obviously constitute a compensation of the suppression caused by IL-10 and represent a stimulation of cellular response after adjuvant immunization.

Protective experiment *in vivo*

The protective character of immunization was tested in an *in vivo* experiment. Among others, we concentrated on the possibility of induction of cross-protection against influenza A virus of another subtype. The mice were immunized with the virus A/PR/8/34 (H1N1) and the heterologous type A/California/7/04 (H3N2). Two various concentrations of adjuvant were tested – 50 and 500 μg of DBF. The infection was induced 10 days after the second immunization dose by using a live, mouse-adapted strain A/PR/8/34 (H1N1), and the progress of infection was monitored for 14 days by following body weight loss and mortality. The local situation in the lungs after the protective experiment was tested histologically.

Control mice and mice immunized with helerologous type H3N2 were not protected against live H1N1 influenza challenge and, surprisingly, also mice immunized with homologous type H1N1 were not well protected. In contrast, mice immunized with combination of H1N1+DBF or H3N2+DBF in lower and higher concentrations of adjuvant were well protected against challenge with live influenza virus H1N1. The challenge with

homologous or heterologous virus subtype proved cross-protective effect of adjuvant immunization. Mice given only adjuvant in lower or higher dose were protected against death but not against disease. Mice immunized with lower dose had higher weight loss then those immunized with higher dose of adjuvant. In both groups immunized with adjuvant alone, lungs were more afflicted than when mice were immunized by virus+adjuvant.

The protective effect of both standard and adjuvant immunization was confirmed in the infection experiment: immunized mice exhibited lower weight loss and lower mortality. The lethal effect of the virus was completely eliminated and the weight loss was minimized after the adjuvant immunization. We succeeded in showing a marked cross-protection between heterologous virus A subtypes H1N1 vs. H3N2, which was reflected in lower weight loss and zero mortality (100% survival), and was also demonstrated by the histological picture of the lungs after infection.

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Stimulation of protective and cross-protective immunity against influenza B virus after adjuvant mucosal immunization of mice

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Vaccinations against influenza currently consist of a single annual parenterally given cocktail of purified HA protein from egg-derived influenza viruses based on the three WHO recommended strains - two influenza A strains and one strain of influenza B. Usually concomitant circulation of two or more antigenic variants of type B in one season make the choice of one vaccination strain difficult - the solution of this problem could be either the inclusion of two topical B strains into the vaccine or construction of the vaccine inducing

cross-protection. In this study we tested the protective and cross-protective effect of BF after immunization of mice with two different and phylogenetically distant strains of influenza B (B/Lee and B/Yamanashi) and subsequent infection of mice with lethal influenza B/Lee/1/40.

Protective experiment *in vivo*

Mice were pre-immunized either by influenza virus strain B/Yamanashi, B/Lee, DBF alone as adjuvant and by combination of virus+adjuvant (B/Lee+DBF and B/Yamanashi+DBF). 10 days after the 2nd immunization dose, mice were infected by strain Lee which is lethal for mice. Infection was monitored for 14 days by following body weight loss and mortality. All control mice and mice given DBF alone died, that with DBF with certain delay. The death delay was probably caused by stimulation if innate immunity by DBF. The majority of mice immunized with heterologous strain B/Yamanashi died, mice immunized with homologous strain B/Lee were fully protected both from death and disease (no weight loss). These results prove the marked difference between two strains of virus type B. On the other hand, adjuvant immunization protected mice immunized by either strain from death. Mice immunized by homologous strain were fully protected. Mice immunized by heterologous strain fell ill (pronounced weight loss) but almost all mice survived and recovered.

Intratracheal immunization of mice with inactivated influenza B virus and DBF as adjuvant increases protection of mice against infection with the homologous virus strain and induces cross-protection: mice immunized by influenza virus B/Yamanashi 166/98 were protected even against phylogenetically distant virus drift variant B/Lee/40 lethal for mice.

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Adjuvant effect of *Bacillus firmus* on the expression of cytokines and toll-like receptors in mice nasopharynx-associated lymphoid tissue (NALT) after intranasal immunization with inactivated influenza virus type A

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The aim of this study was the elucidation of the mechanism of adjuvant effect of DBF by studying the changes in the gene expression in NALT after intranasal immunization of mice. In rodents, NALT represents a paired lymphoid organ formed by an aggregation of lymphoid cells in the upper airways, and is considered to be the only well-organized lymphoid tissue of the respiratory tract. To evaluate the effects of adjuvant immunization we studied the expression of genes important for the reaction of both innate and adaptive immunity by real-time PCR: toll-like receptors recognizing antigens of G+ and G- bacteria and microbial nucleic acids (TLR2, TLR3, TLR7, TLR9), type I interferons (IFN-α4, IFN-α11, IFN-α12, IFN-β), Th1 and Th2 type cytokines (IL-2, IFN-γ, IL-4, IL-6, IL-10) and some other genes (CCR7 and iNOS) at time points of 3, 6, 12, 24, 48, 72 and 168 hours post immunization. Data obtained from real-time PCR were further analyzed by relative quantification (RQ) and principal component analysis (PCA).

Relative quantification

RQ data imply that the main changes in gene expression occur during the first 24 h after immunization. Groups immunized with DBF alone or with virus+DBF display very fast changes peaking at 3 and 6 h post immunization. Significant changes after immunization with virus alone were detected later - 12 and 24 h after the treatment. Immunization with the virus

alone increases mainly the expression of TLR7, IFN-γ and type I interferons. DBF increases significantly the expression of TLR2, IL-6, iNOS, IFN-γ, and CCR7 and lowers the expression of IL-4. Relative to the virus alone, the combination virus+DBF accelerates the expression of TLR2, TLR7, IL-6 and type I interferons; when compared with DBF alone it increases the expression of TLR7, CCR7 and iNOS. Immunization with the combination virus+DBF has a faster and longer effect (3 - 72 h) on the expression increase of type I interferons (particularly IFN-α4 and IFN-β1) than treatment with DBF alone.

Principal component analysis (PCA)

The results of PCA imply that the largest changes in the group of genes characteristic for pDC were detected in mice immunized with virus+DBF. These exhibit a similarity between the expression of genes of the functional group for pDC and the functional group of genes for type I interferons. Mice immunized with virus alone evince a „probable" activation of pDC only later at 12 to 24 h post immunization, in contrast to the group immunized with virus+adjuvant, in which a marked change in the expression of genes typical for pDC occurred very early at 3 h post immunization – and the increased expression persisted until 24 h. Immunization with DBF alone caused only mild differences in expression of genes characteristic for both pDC and type I interferons. Mice immunized with the virus alone showed a Th1 polarization of the immune response 3 – 12 h post immunization; 24 h after immunization, the response had rather mixed Th1/Th2 character. In the groups immunized with DBF or virus+DBF, the type of immune responses was mixed (Th1/Th2) in the intervals of 3 – 12 h post immunization it was slightly polarized towards Th1 12 h after immunization and it was Th2-skewed at the interval of 24 h. The most efficient in terms of protection of the organism against virus infection appears to be immunization with virus+DBF, which leads to

a fast antiviral immune response based on pDC activation, production of interferons and activation of a mixed Th1/Th2 immune response.

Expression of transcription factor IRF7

In addition to the results described in above mentioned manuscript, the expression of transcription factor IRF7 was tested. It is known that increased gene expression of TLR7 and TLR9 activate MyD88-dependent signaling pathway and trigger expression of IRF7. IRF7 regulates the production of type I interferons and could be involved in the process of pDC activation after immunization with virus+DBF. We have found revealed a pronounced differences in expression of genes for TLR7, TLR9 and CCR7 by PCA analysis. These changes are characteristic for pDC activation. In a group of mice immunized with virus+DBF, a significant difference has been confirmed in IRF7 expression after 6 h ($p=0.0451$), 12 h ($p=0.0009$) and 24 h ($p=0.0062$) after immunization by relative quantification and cluster heatmap analysis (**Figure 5**). It is apparent that in these time intervals the changes are the most relevant for the pDC activation process. We found the biggest increase of the IRF7 expression between the 3 and 6 hours after immunization. The increase remains stable from 6 to 24 hours.

Intranasal immunization with inactivated influenza virus in combination with our bacterial adjuvant increases the expression of a number of genes encoding the components of both innate and adaptive immunity. Acceleration, increas and extension of expression of genes for type I interferons, could be caused by activation of pDC. Adjuvant immunization supports the development of Th1 type immune response indispensable for the development of cellular immunity important in antiviral defense.

Bacterial adjuvant supports the expression of inflammation mediators that can positively affect antigen presentation and a specific immune response.

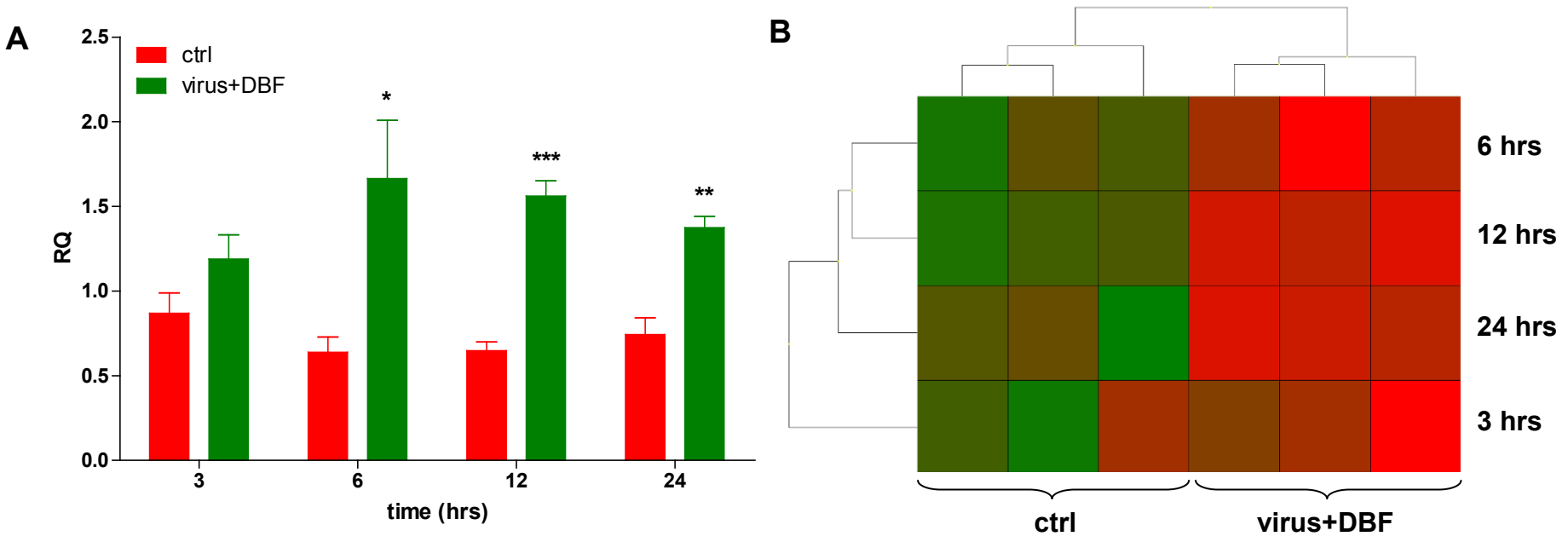

Figure 5. Expression of IRF7 after immunization of mice with the mixture virus+DBF.

A) Relative quantification of IRF7 in interval of 3, 6, 12 and 24 hours in group of mice immunized with virus+DBF versus mice immunized with PBS (controls). The statistical significance of difference between control and virus+DBF is expressed by asterisks (* $p<0.05$, ** $p<0.001$, *** $p<0.0001$). **B)** Cluster heatmap analysis. The group ctrl is presented by 3 mice immunized with PBS, the next group by 3 mice immunized with virus+DBF. Green color describes low gene expression and the red color means up-regulated expression. On the top of heatmap two clusters are visible – the first one representing mice immunized with PBS and the second one mice immunized with virus+DBF. On the left side of the heatmap, cluster expressing the relationship among 4 different time points could be seen.

DISCUSSION

Influenza virus remains important viral pathogen of significant medical importance causing each year pronounced morbidity and considerable mortality in population [176]. Influenza epidemics continue to infect large numbers of people worldwide, despite the availability of vaccines. These are derived from the current circulating viral strains and continuously modified according to actual epidemiological situation, because of frequent natural variation of the hemagglutinin (HA) and neuraminidase (NA) surface proteins of the virus. This variation allows to the virus to escape neutralization by preexisting circulating antibodies in the blood, present as a result of either previous natural infection or immunization. The current vaccination against influenza succeeded in achieving a considerable lowering of morbidity and mortality of this disease. However, the hitherto predominantly parenteral mode of vaccination cannot be considered optimal. This mode of vaccination induces immunity mostly against a homologous strain of the virus used for vaccination. Although systemic immunity is reached, mucosal immunity is not stimulated efficiently enough. In contrast, mucosal vaccination can cause good mucosal as well as systemic immunity [300]. Mucosal stimulation is generally much more efficient with live than with inactivated virus. The use of live viruses is always connected with certain risk. However, various types of mucosal adjuvants can enhance the stimulatory effect of inactivated virus in mucosal immunization. The attention is focused on vaccines that are able to induce cross-protection and could be effective also in the case of a sudden appearance of a new virus variant. Cross-protection was studied mainly on the experimental mouse model [227]. In addition to the protection against a homologous virus, mice infected with type A influenza virus could display also protection against the drift virus variants under certain conditions. The probability to induce intrasubtype-specific or heterosubtypic cross-protective immunity is much higher after a natural infection than after vaccination [301]. Efforts are therefore made

to make the vaccination as close to the natural infection as possible. Vaccines containing live viruses (LAIV) that have been used in Russia and the USA seems to be well protective [263-268]. These vaccines are administered intranasally what corresponds to the natural entry point of the influenza infection into the organism. Intranasal application induces excellent mucosal immunity, activates not only antibody but also cellular response, and can induce cross-protection [301]. The handicap of these vaccines is the risk of application to immuno-compromised patients (young children, old and immunodeficient individuals). In these recipients, the attenuated live virus could induce infection, though milder than that evoked by a wild type virus [302]. Another risk is the large variability of the influenza virus A and the ensuing potential risk of reversion of the cold-adapted mutant. The vaccine used in the USA has considerable indication limitations (5-49 years of age) that exclude population groups that are at the highest risk of influenza [262-265]. It is necessary to admit that an ideal anti-influenza vaccine is not available. At any rate it is important to continue in the effort to design optimal, preferably inactivated, vaccines which can raise good systemic as well as mucosal immunity [224;225]. Inactivated influenza viruses are known to be often insufficiently effective when used for mucosal immunization and for induction of cross-protection. The drawbacks of vaccination with inactivated virus can be overcome by using a suitable adjuvant.

Our studies of immunomodulating properties of microorganisms revealed that the G+ bacterium of outer environment *Bacillus firmus* has pronounced immunostimulatory properties and, live or inactivated, it is harmless to mice *in vivo*; in addition, even high doses of inactivated bacterium do not suppress the viability of cells in culture [290-294;303].

Excellent adjuvant properties of this bacterium, which were proved in immunization of mice with ovalbumin via the respiratory tract [297], were also seen after intranasal immunization of guinea pigs with inactivated type B influenza virus (B/Lee/1/40). High levels

of anti-influenza neutralizing antibodies were detected after adjuvant immunization with virus+BF in contrast to immunization with virus alone. Production of anti-influenza diagnostic sera is often performed by mucosal immunization of guinea pigs or ferrets and this mode of immunization is usually efficient when live viruses are used. High level of neutralizing antibodies after adjuvant immunization of guinea-pigs with inactivated virus demonstrates the possibility of preparation of diagnostic sera against highly pathogenic strains with respect to standard biosafety guidelines [304]. Adjuvant properties of BF were further tested in mouse model after intratracheal immunization of mice with inactivated influenza virus both of type B [305] and type A [306]. Influenza type B has a relatively low immunogenicity and, in the immunization with the virus alone, the level of specific antibodies in the serum and in secretions does not differ much from antibodies in non-immunized control animals. In contrast to influenza type B, influenza type A was found to be much more immunogenic. Intratracheal immunization with inactivated influenza virus type A (A/PR/8/34) alone induced a perceptible antibody response in contrast to influenza type B. Intratracheal immunization with virus (influenza type A or type B) + adjuvants (BF or DBF), strongly stimulates the production of systemic antibodies of IgG class detectable in serum and the production of mucosal IgA antibodies in BAL. Apparently due to the higher immunogenicity of influenza type A after intratracheal immunization with virus alone, the effect of the adjuvant (DBF) was not as evident as with type B virus. Intratracheal immunization by both influenza type A or type B in combination with adjuvants (BF or DBF) had a weak (type B) or no effect (type A) on IgA antibody induction in the intestine. This implies that the choice of mucosal site for immunization is important because of subcompartmentalization of mucosal immune system. In contrast, adjuvant immunization with influenza type B also affected production of anti-influenza antibodies of IgG class in BAL as well as in intestine. Immunization of mice with influenza type A virus+DBF led to

induction of high levels of IgG in BAL, but only low levels of anti-influenza IgG antibodies in intestine. The increased levels of IgG antibodies in BAL are mainly caused by serum transudation in lungs. So that BAL antibodies reflect both mucosal and systemic immunity. In regard to intestinal antibodies, some transudation from serum could be supposed as well. Stimulation of cellular immunity in the lungs after intratracheal immunization was examined by determining the local expression of cytokines characteristic for Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) responses by RT-PCR after immunization with influenza virus type B [307] and by real-time PCR after immunization with influenza virus type A [306]. The results indicate that expression of cytokines was increased without a perceptible Th1 or Th2 polarization after adjuvant immunization with influenza virus type B. Adjuvant immunization of mice with influenza virus type A resulted in a mixed Th1/Th2 response as well. The predominant Th2 response was recorded after immunization with the virus alone. The increased expression of cytokines, mainly those of Th1 type (especially of IFN-γ) points to an increased activation of cellular immune response after adjuvant immunization. The protective effect of a standard and an adjuvant intratracheal immunization was confirmed in protective *in vivo* experiment after challenge of mice with live influenza A/PR/8/34 highly pathogenic for mice. After the adjuvant immunization, the lethal effect of the virus was completely eliminated and the weight loss signalizing disease was minimized. We succeeded in showing a marked intersubtypic cross-protection between heterologous virus A subtypes H1N1 vs. H3N2, which was reflected in lower weight loss and zero mortality (100 % survival) after infection with heterologous virus. The protective effect was also demonstrated by the histological picture of the lungs after infection. It is noticeable that, despite the large increase in total antivirus antibodies and the excellent protective effect, no perceptible increase has been detected in the levels of virus-neutralizing antibodies. This indicates that the role of antibodies acting against conserved internal antigens or the role of cellular immunity against internal antigens in the

protection against influenza infection may be more pronounced than generally thought. DBF exerted conspicuous protective effect even in infection of mice immunized with the adjuvant alone; this is in concordance with its stimulatory effect on innate immunity.

The main effort is currently exerted on the induction of protective immunity against influenza A which is more significant from the point of view of public health. Much less attention is paid to vaccination against influenza B. The choice of a suitable virus B strain for vaccination can be difficult. Influenza virus B is not split in various subtypes, nevertheless it exist in many antigenically different variants which developed during phylogeny of the virus and can be clustered into three main developmental groups with very limited cross-reactivity: early strains (1940-1970) including strain B/Lee/40, lineage B/Yamagata-like (1972-2005) including strain B/Yamanashi 166/98 and lineage B/Victoria-like (1975-2007) [188]. We were able to induce cross-protection of mice against lethal influenza B/Lee/40 by adjuvant immunization with phylogenetically and antigenically distant strain B/Yamanashi (B/Yamagata-like). Immunization with virus alone did not have cross-protective effect. The study of cross protection among various strains of both influenza A and B viruses reveal the potency of our adjuvant to support the induction of immune response against cross-reactive epitopes of influenza viruses.

The effect of BF and DBF was compared in our first experiments. Our data confirm slightly stronger stimulatory potencial of DBF than BF. Therefore, DBF was used in majority of further experiments.

The mechanism of adjuvant effect of DBF was followed by studying the changes in the gene expression in the NALT after intranasal immunization of mice. The first defense line against influenza is an innate immunity with its essential component, type I interferons [100;102-104], which are mainly produced by pDC [47;48;70;71]. This function is closely connected to their ability to express TLR7 and TLR9 in early endosomes, which enable them

to recognize foreign viral or bacterial nucleic acids. To evaluate the effects of adjuvant immunization, we studied the expression of genes important for the reaction of both innate and adaptive immunity by qPCR: genes for toll-like receptors recognizing antigens of G+ bacteria and microbial nucleic acids (TLR2, TLR3, TLR7, TLR9), type I interferons (IFN-α4, IFN-α11, IFN-α12, IFN-β), type Th1 and Th2 cytokines (IL-2, IFN-γ and IL-4, IL-6, IL-10, respectively) and some other genes (CCR7 and iNOS) at different time points (3, 6, 12, 24, 48, 72 and 168 hours) post immunization. For evaluation of data, relative quantification method (RQ) and principal component analysis (PCA) were used. PCA involves a mathematical procedure that transforms a number of variables (expression values of various genes) into a smaller number of uncorrelated variables called principal components. In this way, the dimensionality of the data is reduced to a number of dimensions that can be plotted in a scatter plot in two dimensions [307]. Our results showed that immunization with DBF alone had a very fast effect; it has markedly influenced the gene expression already 3 h after immunization. This effect decreased at later time points. This is in agreement with the fact that bacterial adjuvants support mainly innate immunity. Affecting innate immunity then influences adaptive immunity. DBF causes a highly significant early increase in expression of IFN-γ, which can considerably support the immune response of Th1 type, what is important in the defense against viral infection. Marked change in type Th2 cytokines (IL-4, IL-10 and IL-6) after immunization with DBF was evident from PCA analysis as well. However, this cannot be ascribed to stimulation of Th2 response because the expression of IL-4 was not significantly increased at any time point – in fact, at the first time points of 3 and 6 h the expression of IL-4 was even significantly lowered. The large changes in PCA are apparently due to a markedly increased expression of IL-6 which, along with the concomitantly increased expression of iNOS, is mainly caused by the inflammatory effect of DBF. DBF alone increased the expression of IL-10 as well. An environment with increased concentrations of

IL-6 and IL-10 is known to support the production of IgA. In contrast to the group immunized with DBF, mice immunized with virus alone exhibited delayed and short-term changes in expression of genes followed. A significant increase in expression of both IL-4 and IFN-γ was demonstrated only 24 h after immunization. Comparison of the results of RQ and PCA indicates that the onset of the Th1 type response occurs 12 h after immunization. It therefore appears to be a mixed Th1/Th2 response with a slight Th1 accent. After adjuvant immunization with virus+DBF, the response was fast, protracted and has a mixed Th1/Th2 character, the Th1 response being the strongest after 3 and 6 hours while the Th2 one after 12 and 24 hours. The DBF effect on stimulation of innate immunity was tested by TLRs expression. TLR2 recognizes different bacterial components such as lipoproteins, lipopeptides and peptidoglycans and is the principal receptor for recognition of G+ bacteria [308]. TLR2 is thus important for recognition of DBF obtained from G+ *Bacillus firmus.* The results point to a significantly increased expression of TLR2 at the time interval of 3 - 6 h after immunization either with DBF alone or with the combination virus+DBF. TLR3 and TLR7 participate in the recognition of influenza virus in certain cell populations. A strong production of type I interferons set in after the recognition of the influenza virus by TLR7 present in early endosomes of pDC. [309]. TLR9, which is also expressed in pDC, is able to recognize nonmethylated CpG regions in viral and bacterial ssDNA [310;311]; this receptor could thus also participate in the recognition of the bacterial adjuvant. Mature pDC are further characterized by an increased expression of the chemokine receptor CCR7, which plays a key role in the migration of pDC to lymph nodes. Our data demonstrate strong activation of genes characteristic for pDC (TLR7, TLR9, CCR7), mainly in the group immunized with virus+DBF, at early time points after immunization. DBF alone does not cause any marked increase in TLR7 expression. The group immunized with the virus alone evinced only a non-significant increase in TLR7 expression 3 and 12 h after immunization whereas the increase

after 24 h was already significant. These data imply that immunization with the virus alone and with virus+DBF activates pDC, the immunization with virus+DBF causing a stronger, faster and longer activation of genes typical for pDC. The increase in TLR3 (recognizing viral dsRNA) expression was at the limit of significance especially in the group immunized with virus+DBF in the interval of 24-72 hours whereas groups immunized with the virus alone or with DBF exhibited a relatively weaker increase. Plasmacytoid dendritic cells are the main producers of type I interferons, which ensure an early innate protection against viral infection. The mouse genome contains 14 known IFN-α genes and 3 IFN-α pseudogenes. The highest anti-proliferation and antiviral activity relative to IFN-α1 is exhibited especially by IFN-α4, IFN-α11, INF-α12 and IFN-β [312]. For instance, the activity of IFN-α4 is 5-10-fold higher than that of IFN-α1 [313;314]. A two-step mechanism of expression has been described in interferons [315;316]. Transcription of genes encoding IFN-α4 and IFN-β takes place very early after viral infection and is governed by the transcription factor IRF-3. Transcription of further genes of interferons is then controlled by the transcription factor IRF-7. Viral infection first activates the expression of IFN-α4 and IFN-β; this activation is then followed by an increase in the expression of other interferon types. Our data indicate that the increase in expression of interferons IFN-α4, IFN-α11, IFN-α12 and IFN-β begins early after immunization with virus+DBF and can be detected at all time points from 3 to 72 hours. Type I interferons released by pDC not only prevent viral infection but also activate NK cells, myeloid dendritic cells (mDC) and also B and T lymphocytes, and participate therefore in the regulation of both innate and adaptive parts of immunity. We have found a pronounced difference in expression of genes TLR7, TLR9 and CCR7 by PCA analysis in group of mice immunized with virus+DBF when compared to other treatments (virus alone or DBF alone). I has been reported that increased gene expression of TLR7 and TLR9 activate MyD88-dependent signaling pathway and trigger expression of IRF7 [75;76]. This change is

characteristic for pDC activation. We confirmed significant difference in IRF7 expression after 6, 12 and 24 hours in group immunized with virus+DBF **(Figure 5)**. The up-regulated expression of IRF7 is in concordance with the increased expression of the type I interferons. These results indicate that immunization with inactivated influenza virus type A together with adjuvants trigger the activation of pDC.

Bacillus firmus has been shown to be very efficient adjuvant with strong effect on activation of both mucosal (induction of high levels of IgA in secretions) and systemic (induction of high levels of IgG in serum) antibody responses. Adjuvant immunization protected mice against lethal infection by both homologous and heterologous strains of influenza virus (intrasubtypic and intersubtypic cross-protection).

CONCLUSIONS

In relation to the aims of this work we tested impact of adjuvant effect of *Bacillus firmus* on stimulation of mucosal and systemic immunity after immunization of mice via respiratory tract. Intratracheal immunization gave rise to high levels of mucosal antibodies, in particular in the respiratory tract, whereas intranasal immunization enhanced the local production of antibodies both in the respiratory tract and in the intestine. Both types of immunization stimulate systemic immunity as well. Inactivated influenza virus type B alone was less immunogenic alone but high levels of mucosal and systemic antibodies were detected after adjuvant immunization. In contrast to inactivated influenza type B, inactivated influenza virus type A alone induced a satisfactory systemic and mucosal antibody response which was still increased by adjuvant.

Currently used influenza vaccines are strictly homotypic and they fail in induction of cross-protection. Our *in vivo* experiments documented a pronounced protective effect of the adjuvant immunization against homologous virus and a conspicuous cross-protection was observed in protective experiments (protection against H1N1 after immunization with H3N2 and protection against B/Lee after immunization with B/Yamanashi). In addition, a distinct protective effect against infection was observed even after preimmunization with BF alone. Mice given only delipidated BF (DBF) were protected against death but not against disease. Their lungs were more afflicted than in mice immunized by virus+adjuvant. Partial protective effect of DBF was probably caused by stimulation of innate immunity.

On the basis of the previous results we tried to characterize the mechanism of action of BF in NALT after intranasal immunization. Intranasally given DBF and mainly mixture virus+DBF induced elevated expression of cytokines characteristic for Th1 immune response (IFN-γ and

IL-2). Expression of typically Th2 cytokine IL-4 was lowered in contrast to increased expression of IL-6, and IL-10. Th1 polarization of immune response after immunization with DBF alone or virus+DBF is important in the defense against viral infection. Increased concentrations of IL-6 and IL-10 are important for production of IgA. Differences in expression of TLR7, TLR9, CCR7 and type I IFN followed by PCA analysis leads us to the idea of pDC activation. IRF7 regulates the production of type I interferons and its increase after adjuvant immunization could be involved in the process of pDC (IFN-α4, IFN-α11 and IFN-α12) and mDC (IFN-β) activation. Adjuvant immunization had also influenced inflammation in respiratory lymphatic tissue which was evident from the increased iNOS expression in NALT. Increased gene expression found is in accordance with stimulation of both innate and adaptive immunity and elucidates adjuvant effect of *Bacillus firmus.*

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