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Milk Derived Peptides with Immune Stimulating Antiviral Properties

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1. Introduction

Milk is thought to be the main source of biologically active compounds for infants, providing antibacterial and antiviral activities, facilitating nutrient absorption, promoting bone growth, enhancing immunological protection and supporting the development of host immune competence. In milk, the main categories of compounds related to antiviral activity through immune stimulation and suppression of host immune inflammation are the casein proteins, whey proteins and their derived peptides [1-3].

Casein proteins, as well as casein fragments, function as antiviral and immune regulatory factors by regulating the innate immune response both through up-regulation to enhance killing of viruses, and down-regulation to reduce detrimental conditions such as sepsis [1, 3-7]. Additionally, caseins link the innate immune system to the adaptive immune system by activating and/or enhancing B- and T-cell mediated functions. The whey protein lactoferrin, and pepsin derived peptide fragments of this protein (e.g. lactoferricin) have been studied extensively for its antiviral properties [8-10] i.e. its direct interaction with the virus particle, interaction with cellular receptors on the target cells, and lately more complex antiviral mechanisms involving stimulation and regulation of the immune system have been discovered [2, 11-16]. Similarly, peptides tailored on specific protein fragments of casein and α -lactalbumin have also been investigated for their antiviral and immunomodulatory properties. Many of these studies have identified biologically active peptides that can prevent a viral infection, as well as regulate the immune status of the host [1, 2, 17-21]. Currently, some of these peptides are being investigated in clinical trials, like human lactoferrin fragment 1-11 (AM Pharma, Bunnvik, The Netherlands) [22] and LTX-302 (Lytix Biopharma, Tromsø, Norway) [20]. Moreover, another promising class of synthetic peptides with therapeutic potential is a group of innate defence regulator peptides, which exhibit immune protection by enhancing or suppressing the host immune response [23-26]. It is

tremendously encouraging that many of these proteins and peptides have pharmaceutical potential within antiviral and anticancer therapy, as vaccine adjuvants, as immunosuppressants for the treatment of autoimmune diseases and in conjunction with organ transplantation, etc. [20, 23-25, 27-29].

Studies have demonstrated that active milk protein and peptide compounds can be extracted from a variety of species including humans, bovine, porcine, mice and camel. The main focus in this paper is the antiviral and immune regulating properties (Table 1) of milk proteins (Table 2) and their peptide derived fragments (Table 3). The vast majority of the discussed studies deal with proteins and peptides of bovine origin, and these will be referenced with their protein names, while proteins and peptides from other origins will be explicitly specified with the species name.

Virus	Protein or peptide	Model of antiviral function	Reference
Enveloped Virus			
Herpes simplex virus 1	human and bovine lactoferrin and lactoferricin, lactoperoxidase chemically modified milk proteins e.g. serum albumin, α -lactalbumin, β -lactoglobulin	Binding to both virus particle and cellular receptors (heparan sulphate) to prevent viral adsorption and entry; Interference with intracellular replication events or synthesis of progeny viral components	[9, 35, 55, 77-79, 81-83] [89, 93, 99, 100]
Herpes simplex virus 2	human and bovine lactoferrin β -lactoglobuline	Binding to virus receptor of non-GAG nature Binding to virus particle	[10] [93]
Hepatitis C virus	lactoferrin	Binding to viral envelope protein E1 and E2	[52, 63, 71, 220]
Hepatitis B virus	iron- or zinc-saturated lactoferrin	Binding to cellular molecules interfering with viral attachment/entry	[47, 59]
Hepatitis G virus	lactoferrin	Unknown	[63]
Respiratory syncytial virus	lactoferrin, lactoperoxidase	Binding to F1 subunit of RSV F protein to inhibit viral absorption	[35, 44-46]
Human immunodeficiency virus	human and bovine lactoferrin, lactoperoxidase chemically modified milk proteins like serum albumin, α -lactalbumin, β -lactoglobulin	Binding to cellular receptor to inhibit viral absorption and replication	[8, 18, 19, 34, 48, 49, 62, 68, 70] [9, 89-92]
Influenza virus (H3N2, H1N1 and H5N1)	lactoferrin, κ -casein, glycomacropeptide, lactoperoxidase modified human serum	Binding to hemagglutinin of virus	[36, 53, 135] [94, 101-103]

Virus	Protein or peptide	Model of antiviral function	Reference
Human cytomegalovirus	albumin and β -lactoglobulin, α -lactalbumin, lactoferrin and lactoferricin	Interfere with virus target cells; up-regulation of killer cells; synergistic antiviral effect with cidofovir	[12-16]
	chemically modified milk proteins like serum albumin, α -lactalbumin, β -lactoglobulin	Binding to virus particle	[12, 93, 96, 221]
Feline herpes virus 1	human and bovine lactoferrin	Binding to cellular molecules	[54]
Canine herpes virus	human and bovine lactoferrin (apo- and holo-)	Binding to virus particle and cellular receptor on target cell	[74]
Hantavirus	lactoferrin	Binding to cellular molecules; synergistic effect with Ribavirin on inhibiting viral replication	[72, 122]
Vesicular stomatitis virus	lactoferrin	Induction interferon- α/β expression to inhibit viral replication	[11]
Friend virus complex	human lactoferrin	Regulation on the myelopoiesis; synergistic effect with interferon- γ	[11, 56, 57, 64]
Human papillomavirus	human and bovine lactoferrin, human and bovine lactoferricin	Binding to heparan sulphate cell receptor	[65, 121]
Alphavirus heparan sulphate- adapted sindbis virus and semliki forest virus	human lactoferrin, charge-modified human serum albumin	Binding to heparan sulphate cell receptor	[73]
Severe acute respiratory syndrome coronavirus	lactoferrin	Binding to heparan sulphate cell receptor	[120]
Non-enveloped virus			
Rotavirus	human lactoferrin (apo-/holo-), α -lactalbumin, β -lactoglobulin	Binding to viral particles to prevent both rotavirus haemagglutination and viral binding to receptors on susceptible cells	[30, 40]
	human lactadherin	Binding to structural protein of rotavirus and inhibits virus replication	[37, 38]

Virus	Protein or peptide	Model of antiviral function	Reference
	high molecular glycoprotein (e.g. mucin)	Inhibitor for viral-cell binding to prevent productive virus infection	[107, 108]
	immune globulin	<i>In vivo</i> effect on inhibition of viral replication	[107]
Poliovirus	lactoferrin, modified bovine β -lactoglobulin	Binding to viral receptor on target cell	[50, 58, 104]
Coxsackie virus	modified bovine β -lactoglobulin	Binding to viral receptor on target cell	[104]
Adenovirus	lactoferrin	Binding to viral protein III and IIIa; competition with virus for common membrane receptors	[60, 61, 69]
Enterovirus (71, echovirus 6)	lactoferrin	Binding to both cellular receptors and the viral surface protein VP1	[51, 66, 67]
Felin calicivirus	lactoferrin	Binding to cell receptor	[50]
Echovirus	lactoferrin, lactoperoxidase	Binding to cell receptor and viral structural proteins	[35, 75, 95]

Table 1. Models of antiviral proteins & peptides from milk proteins

2. Protein composition of milk and their antiviral activity

There are in general two groups of proteins found in milk, casein and whey. The casein family accounts for approximately 80% of the protein mass and includes several types of casein, e.g. α s1, α s2, β and κ , which form micelle complexes in the water phase of milk. The whey proteins account for the remaining 20%, and include β -lactoglobulin (not present in human milk), α -lactalbumin, serum albumin, immunoglobulins, lactoferrin, transferring, and many minor proteins. Most of the whey proteins have been demonstrated to effectively prevent viral infection. For example, milk derived proteins including α -lactalbumin, β -lactoglobulin, apo-lactoferrin (iron free), and homo-lactoferrin (Fe^{3+} carrying), were able to inhibit rotavirus attachment to cellular receptors by binding to the viral particle [30]. Among these proteins, apo-lactoferrin was proven to be the most active. Studies also showed that immunoglobulins of raw milk from non-immunized cows and camels, as well as from a commercially available bovine macromolecular whey protein fraction, have specific antibodies against human rotavirus, which are capable of inhibiting replication of rotaviruses in tissue culture and protect mice from infection in a murine model of rotavirus infection [31-33]. Lactoperoxidase, a haem-containing glycoprotein of the mammalian peroxidase family, is an important enzyme in the whey fraction of milk. In combination with its physiological substrates hydrogen peroxide and thiocyanate, lactoperoxidase manifests a wide spectrum of virucidal activities against human immunodeficiency virus, herpes simplex virus 1, respiratory syncytial virus and echovirus [34, 35]. Oral administration of lactoperoxidase also attenuate pneumonia in influenza virus infected mice through suppression of infiltration of the inflammatory cells in the lungs [36]. Furthermore, the 46kD

glycoprotein termed lactadherin, also known as milk fat globule-EGF factor 8 protein, inhibited rotavirus binding to cellular receptors (acetylneuraminic acid and/or integrin) on target and/or specifically interacting with viral structural glycoprotein VP4 of rotavirus, blocking host-pathogen interaction [37-40].

Lactoferrin, first isolated in 1960 from both human [41, 42] and bovine milk [43], has been demonstrated to exhibit antiviral activity against many viruses [8, 10-16, 18, 19, 40, 44-74] (Table 2). Most studies indicate that lactoferrin and its derived peptides are likely to interfere in the virus host cell interaction (Figure 1). For example it has been demonstrated that lactoferrin is able to bind both viral receptor and the viral surface protein VP1 on enterovirus (enterovirus 71 and echovirus 6), thus interfering with viral entry [51, 66, 67, 75, 76]. Similarly, both apo- and holo-lactoferrin has been demonstrated to interact both with canine herpes virus and surface receptors on the Madin-Darby canine kidney cells, thus inhibiting canine herpes virus infection [74]. With regard to the anti-herpes simplex virus 1 ability of lactoferrin, both bovine and human lactoferrin and lactoferricin have demonstrated the ability to block viral entry and also inhibit viral cell-to-cell spread in a dose dependent manner [55, 77-79], through interaction with negatively charged glycosaminoglycans like heparan sulphate on the cell surface [55, 80-83] and elements of the viral particle [55]. Differently from herpes simplex virus 1, Marchetti *et. al.* found that lactoferrin inhibited herpes simplex virus 2 plaque forming activity also in cells without glycosaminoglycans suggesting that lactoferrin might block one of the specific herpes simplex virus 2 entry receptors [10].

Many of the traditional entry blocking effects observed by lactoferrin involve electrostatic interaction with anionic heparan sulphate molecules on the host cell surface [82]. The ability to interact with anionic heparan sulphate is maybe not that surprising, when evaluating the three dimensional structural composition of lactoferrin, demonstrating a rather striking cationic patch on the N-terminal lobe of the molecule [84] (Figure 1). Similarly, other highly cationic peptides have also been demonstrated to effectively interfere with herpes simplex virus attachment and entry [80, 85].

Conversely, several other milk proteins i.e. β -lactoglobulin [86], α -lactalbumin [87] are described with anionic patches on their surfaces, while the casein homologues like α s2-casein [88] have both specific anionic and cationic patches on the surface (Figure 1). Thus, charge modification of milk proteins may increase their ability to interfere with virus host cell interactions. 3-hydroxyphthalic anhydride modification of human and bovine serum albumin, and bovine β -lactoglobulin, increased the proteins negative charges in addition to their ability to prevent interaction between human immunodeficiency virus 1 envelope glycoprotein gp120 and the CD4 host cell receptor, by direct interaction and blocking of the CD4 receptor [89, 90]. Similar effects have also been observed for 3-hydroxyphthalic anhydride modified α -lactalbumin and α s2-casein, as well as for maleylated- and succinylated-human serum albumin, indicating that human immunodeficiency virus inhibition was a general property of negatively charged polypeptides [9, 91, 92]. Among the inhibitory proteins, 3-hydroxyphthalic anhydride β -lactoglobulin also demonstrated a broad

spectrum activity affecting herpes simplex virus 1 and 2 in addition to human cytomegalovirus by binding to the virus particles, inhibiting particularly the binding of monoclonal antibodies towards glycoprotein E and glycoprotein C [93]. Comparative results have been shown for anionic-modified human serum albumin and β -lactoglobulin which prevents influenza virus membrane fusion with the host cell membrane, a process mediated by the viral glycoprotein hemagglutinin [94]. Interestingly, this anti-influenza effect has not been observed for other milk proteins carrying negative charges, like succinylated bovine serum albumin, lactalbumin, lactoferrin, lysozyme and transferrin [94]. It is said that inhibition of viral fusion demonstrates a certain degree of specificity for negative charged proteins. However, addition of net negative charges to lactoferrin by acylation with either succinic- or acetic anhydride abolished its anti-poliovirus and anti-feline calicivirus activity, which may be attributed to the obliterate binding of acylated lactoferrin to the surfaces of susceptible cells [95]. Also, when negatively charged groups were added to lactoferrin by succinylation, the antiviral effect on human immunodeficiency virus 1 was increased, but the antiviral potency against human cytomegalovirus was mostly decreased [96], illustrating the proteins different modes of action. Similar results were also obtained by Florisa *et. al.* which demonstrated a stronger antiviral effect against human immunodeficiency virus by developing poly-anionic milk proteins, while stronger effects could be obtained against human cytomegalovirus by creating poly-cationic milk proteins [12].

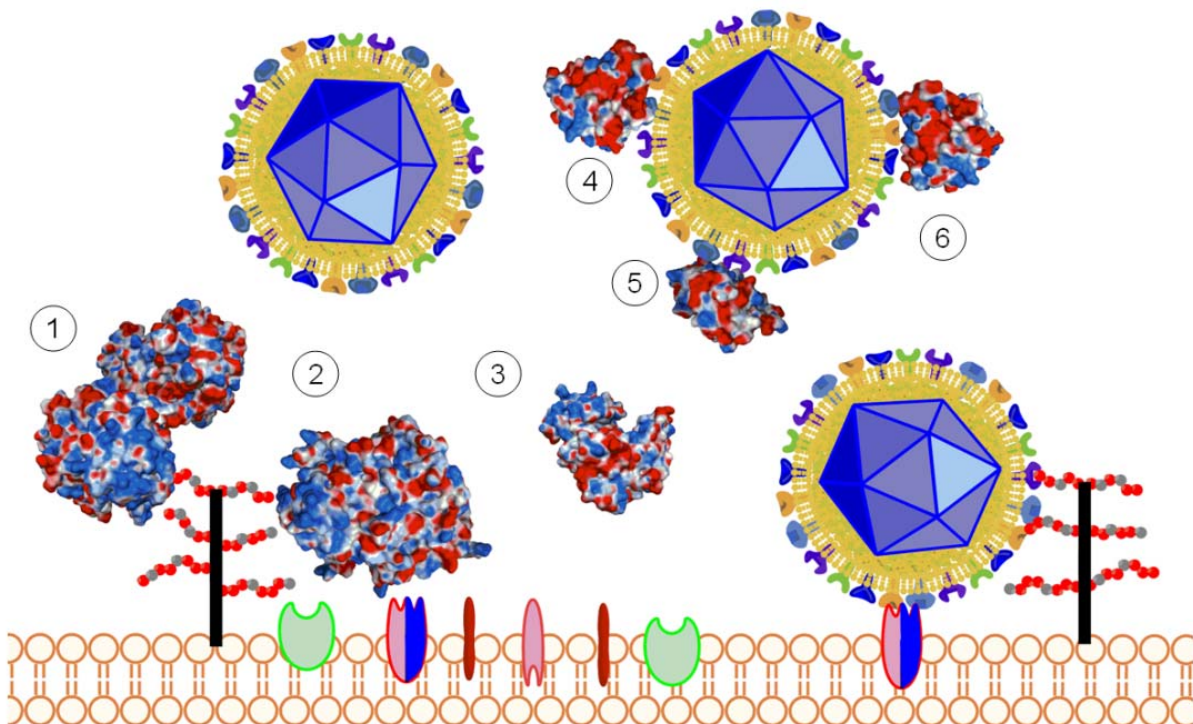


Figure 1. The traditional direct antiviral mechanisms of selected milk proteins. Several proteins are characterized to interact directly with cell surface heparan sulphate, like lactoferrin (1) and lactoperoxidase. Casein species like γ -casein (3) and α s2-casein (4) are despite high cationic character on their surface not described to interact with heparan sulphate, and the latter in stead been demonstrated to interact with the virus particle. Anionic milk proteins like α -lactalbumine (5) and β -lactoglobuline (6) are also illustrated to interact directly with the virus particle, thus preventing host receptor interaction.

Source	Protein	% of whey protein	% of casein	Molecular size (kDa)	Nature	PDB code
Whey	β -lactoglobulin	50-55	NA	~18.4	Apolipoprotein	1DV9
	α -lactalbumin	20-25	NA	~14.1	Albumin	1A4V
	Immunoglobulins	10-15	NA	~150	Glycoprotein	---
	Lactoferrin	1-2	NA	~80	Glycoprotein	1BLF
	Lactoperoxidase	0.5	NA	~70	Glycoprotein	3GC1
	Serum albumin	5-10	NA	~66	Albumin	---
	Glycomacropeptide	10-15	NA	---	Phosphoprotein	---
Casein	α s1-casein	NA	40-50	~23	Phosphoprotein	---
	α s2-casein	NA	10-15	~23	Phosphoprotein	1NA7
	β -casein	NA	30-35	~24	Phosphoprotein	---
	κ -casein	NA	10-15	~19	Phosphoprotein	---
	γ -casein	NA	5-10	~75-100	Phosphoprotein	2CHL

Note. The PDB extension codes are for crystal structure files for the respective milk proteins. The structures have been used when generating the graphic illustration on figure 1.

Table 2. Bioactive proteins from bovine milk.

The strong antiviral activity of poly-cationic compounds is generally explained by the compounds ability to interact with anionic heparan sulphate on the host cell surface, which works as a broad spectrum attachment receptor for several viruses [97, 98]. Thus, it is not surprising that methylated or ethylated α -lactalbumin and β -lactoglobulin demonstrate antiviral activity against the bacteriophage M13 through the inhibition of the phage DNA replication, as well as against herpes simplex virus 1 replication, with increasing activity proportional to the extent of esterification or increased basicity of the modified proteins [99, 100]. The net positive charge-modified human serum albumin had a similar antiviral effect as lactoferrin, against heparan sulphate adapted sindbis virus and semliki forest virus, by blocking the virus receptor on the cell surface, indicating that the antiviral activity of lactoferrin mainly is related to its net positive charge [73]. Methylated α -lactalbumin, β -lactoglobulin and lactoferrin also demonstrate enhanced antiviral activity against human influenza virus A subtype H3N2 and subtype H1N1 [101, 102], and lethal avian influenza A (H5N1) [103]. This effect is most likely linked to the disruption of the electrostatic interactions within hemagglutinin, by the esterified whey proteins, thus affecting the proteins stability and capacity to trigger envelope fusion with the host cell. Furthermore, methylation of β -lactoglobulin does also enhance the proteins antiviral activity against coxsackie virus and poliovirus type 1 in a dose dependent manner [104]. This illustrates that chemically modified whey proteins with added negative or positive charges can exert increased antiviral effect against a diverse group of viruses, through different antiviral mechanisms. The virucidal activity of the modified milk proteins, with additional negative charges, may attribute to a stronger interaction of these proteins with the viral envelope proteins. Esterification of whey proteins with methanol or ethanol would increase their cationic charge, thus increasing their affinity for negatively charged macromolecules such as host cell receptors and viral DNA or RNA, thus inhibiting viral attachment to cellular

membranes or inhibiting viral replication and transcription, respectively. The structural differences between enveloped and non-enveloped viruses in addition to the unique protein composition in milk from different species preclude a generalized conclusion of the milk proteins potential. Thus, further studies should be carried out to identify the underlying molecular interactions involved, and the true therapeutic potential of these milk derived molecules.

3. Traditional antiviral mechanisms of milk-derived proteins

The life cycle of a virus comprises several phases such as binding to the host cell surface, entry or fusion, replication of the viral genome, viral protein synthesis, virus progeny assembly and release. All these steps may be targeted by antiviral agents or milk derived proteins.

Binding to structural virus proteins prevent virus host cell interaction. For the non-enveloped viruses, structural proteins on the surface of the virion protruding as spikes, such as glycoprotein VP4 of rotavirus [105] or fibers associated with each penton base of the capsid on for example adenovirus [106]. These proteins recognize host cell surface receptors, and are involved in facilitating the initial virus to host cell attachment. Enveloped viruses, meaning the viral capsid is coated with a lipid membrane known as the viral envelop, infect host cells via the interaction between envelop proteins and cellular receptors. The envelop proteins include E1 and E2 of hepatitis C virus, F protein of respiratory syncytial virus, hemagglutinin of influenza viruses, etc.

Many of the antiviral milk proteins can bind to structural proteins of the virion in order to prevent binding of the virus to the target cell and subsequently inhibit entry of the viral genome into the host cell. Human lactoferrin (apo- or Fe^{3+}), α -lactalbumin, β -lactoglobulin, human lactadherin, mucin, and immunoglobulin from milk could prevent rotavirus infection through the binding to structural viral protein VP4 [30, 37, 38, 40, 107, 108]. Also, the antiviral activity of lactoferrin against adenovirus has been attributed to the interaction of the milk protein with viral capsid proteins [60, 61, 69].

Furthermore, Ikeda *et. al.* has also demonstrated that lactoferrin effectively protect against hepatitis C virus infection in hepatocytes and lymphocytes by neutralizing the virus, while a basic N-terminal loop of lactoferrin named lactoferricin exhibited no antiviral properties in the same experiments [63]. Lactoferrin has also been demonstrated to inhibit the absorption and growth of respiratory syncytial virus in cell culture through direct interaction with the F(1) subunit of the viral F protein, which is the most important surface glycoprotein participating in viral penetration [44, 45]. Blocking of viral entry like this, leads to down-regulation of respiratory syncytial virus induced interleukin-8 secretion from the HEp-2 cells, which consequently leads to a dampening of the immune response as the low levels of interleukine-8 is inadequate to recruit neutrophils to phagocytose the viral antigen [46].

Hemagglutinin is an antigenic glycoprotein found on the surface of influenza viruses. The glycoprotein has two main functions; recognition of target cells through the binding of sialic

acid-containing receptors and facilitating entry of the viral genome into the target cells by initiating fusion of host endosomal membrane with the viral membrane. Thus, targeting the hemagglutination activity of hemagglutinin could be a robust mechanism in fighting influenza virus infections. Influenza hemagglutinin has also successfully been targeted by both human and bovine lactoferrin (apo- and holo-), as well as κ -casein glycomacropeptide, reduce viral hemagglutination [30, 53]. Moreover, the addition of methylated β -lactoglobulin in the medium of Madin-Darby canine kidney cell lines infected with influenza virus H1N1 reduced hemagglutination in a concentration-dependent manner [101].

Interference with viral entry, through virus and/or cell surface interaction. Viruses recognize and conjugate to specific host cell receptors. These receptor molecules are mainly of protein nature, including glycoprotein, lipoprotein and glycolipid-protein. Hence, host cell specificity or preference is guided by the level of expression of these individual receptor molecules on the different cells. For example, the main goal of human immunodeficiency virus is to infect CD4⁺ T-lymphocytes and initiate replication of a large number of progeny virions. However, the initial infection with this virus is usually of epithelial dendritic cells, which then are used for transport to the lymph nodes. Human immunodeficiency virus attachment to for example emigrating dendritic cells, is mediated by the successive interactions of the viral envelop glycoprotein gp120 with CD4 (a glycoprotein known as cluster of differentiation 4) and a co-receptor, CXCR4 (C-X-C chemokine receptor type 4, also known as fusin or CD184) or CCR5 (C-C chemokine receptor type 5, also known as CD195) [109-111]. However, in cells like macrophages and skin dendritic cells that are lacking or weakly expressing CD4, many other cell surface molecules such as heparan sulphate proteoglycans [112, 113], mannose receptor [48, 114], or dendritic cells-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) [62, 109] can play a key role in the initial multistep interaction between the virus and host cell surface. Consequently, one might hypothesize that human immunodeficiency virus entry into the host cell might be efficiently inhibited via the interaction between antiviral milk proteins from bovine or human sources and some of the receptors described above. This has also been demonstrated to be true, for lactoferrin which effectively can bind heparan sulphate as well as mannose receptor like nucleolin, both which will inhibit virus attachment [18, 48]. Other studies have also indicated that a peptide fragment (hLF1-33) of human lactoferrin (residue 1-33) constituting the glycosaminoglycan recognizing site of the human lactoferrin, exhibit inhibitory effect on human immunodeficiency virus 1 attachment to epithelial cells, though its activity was lower as compared to the native protein [19]. Interestingly however, hLF1-33 had no inhibitory effect on transferring human immunodeficiency virus 1 from immature dendritic cells to CD4 T-lymphocytes, but enhancing virus transmission in contrast to human lactoferrin. This may suggest that the hLF1-33 exposed domain is not involved in human lactoferrin associated inhibition of human immunodeficiency virus 1 transfer to CD4 T-cells [19]. Moreover, bovine lactoferrin could bind strongly to DC-SIGN to prevent human immunodeficiency virus 1 capture and subsequent transmission on dendritic cells, and bovine lactoferrin was a much more efficient inhibitor than human lactoferrin on blocking not only dendritic cell mediated human immunodeficiency virus 1 transmission to - but also replication in CD4 T-cells [49].

Although it has been identified that a metallopeptidase, angiotensin-converting enzyme 2, is a functional receptor for severe acute respiratory syndrome coronavirus infection [115], other reports have demonstrated that DC-SIGN, L-SIGN (also called CD209L, specific for liver/lymph node) [116-119], and heparan sulphate [120] also are involved in the virus pathogenesis. Thus, there are reasons to believe that lactoferrin could prevent severe acute respiratory syndrome coronavirus spread in the host through the same mechanism as described for human immunodeficiency virus, by interacting with DC-SIGN or heparan sulphate receptors. Recently, Lang *et. al.* also described that lactoferrin curtailed the entry of severe acute respiratory syndrome coronavirus into HEK293E/ angiotensin-converting enzyme 2-Myc cells by binding to heparan sulphate [120].

Human papillomavirus can also use heparan sulphate on the target cell surface as a receptor. Thus, by incubating HaCaT cells and papillomavirus 16 virus like particles with human and bovine lactoferrin Drobni *et. al.* have confirmed that human papillomavirus entry can be inhibited by lactoferrin in a dose-dependent fashion [65]. Subsequently, they also demonstrated that bovine lactoferrin peptide (bLF17-33) region 17-33 was a more potent inhibitor of both human papillomavirus 5 and 16 pseudovirus infection than the native protein, while human lactoferricin (hLF1-49) region 1-49 from human lactoferrin, showed modest antiviral activity against the same viruses and bLF17-42 prevented only papillomavirus 5 pseudovirus infection. With regard to the viral attachment, only hLF1-49 and bLF17-42 exhibited antiviral effect [121].

In a classical pre-incubation study on Vero E6 cells, it was demonstrated that lactoferrin had enhanced antiviral activity against hantavirus infection when added prior to infection. However, this boost in activity could be removed if the cells were subsequently washed with phosphate buffered saline prior to infection [72, 122]. These results might be explained by the weak interaction between lactoferrin and other cellular molecules rather than heparan sulphate, as the interaction between lactoferrin and heparan sulphate should withstand phosphate buffered saline washing [80]. Further research should be developed to identify whether $\beta 3$ integrin and/or $\beta 1$ integrin molecules are binding to lactoferrin [123-125].

Similarly, using indirect immunofluorescence, McCann *et. al.* found that bovine lactoferrin could bind to Crandell-Reese feline kidney cells used for propagation of feline calicivirus, as well as Monkey Embryo kidney cells used with poliovirus, indicating that the interference of viral infection might be attributed to lactoferrin binding to the cellular receptor on the respective cells, though the related cell receptors for feline calicivirus and poliovirus have not yet been identified [50]. Contradicting this, it was demonstrated that lactoferricin decreased feline calicivirus but not poliovirus infection. Moreover, feline herpes virus-1 replication could be prevented by exposing cultured Crandell-Reese feline kidney cells to lactoferrin prior to or during viral adsorption, but not following viral adsorption, suggesting that the inhibitory effect on feline herpes virus 1 adsorption to the cell surface and/or viral penetration into the cell might be related to the interaction between lactoferrin and cellular receptor on the Crandell-Reese feline kidney cells [54].

Interference with certain viral enzymes required for virus replication. The process of viral replication will involve a myriad of enzymes, such as DNA- or RNA-polymerases, reverse

transcriptase, integrase, etc. The necessity of viral enzymes for viral replication means that interference with any of them potentially could result in a selective antiviral mode of action. Ng *et. al.* has assayed the inhibitory effect of proteins from bovine milk on the crucial enzymes for the human immunodeficiency virus type 1 life cycle [126]. They demonstrated that lactoferrin strongly inhibited reverse transcriptase but only slightly inhibited the viral protease and integrase. In parallel, α -lactalbumin, β -lactoglobulin and casein were demonstrated to affect human immunodeficiency virus protease and integrase, while not affecting the reverse transcriptase [126].

4. Modulation of innate immune responses - A novel antiviral strategy

The immune system consists of the innate and the adaptive branch, which exerts its functions through recognition of foreign pathogen resulting in a series of responses to eliminate the infectious material. Both innate leukocytes (including macrophages, dendritic cells, and natural killer cells) and adaptive immune cells (B-cells and T-cells) are involved in host immune protection and bridging these two pathways is a variety of traditional signal molecules (cytokines and chemokines). Recently it has also been documented that natural occurring host defence peptides (and proteins) are involved in the orchestration of a well balanced and effective immune response [127-129]. Lactoferrin is one such host defence protein, and it has been demonstrated that lactoferrin can increase the cytotoxic functions of natural killer cells and lymphokine-activated killer cells especially in infants, which normally have low activity in these cell populations [130]. Lactoferrin can also enhance the mobility of polymorphonuclear leucocytes and increase the production of superoxide [131], activate macrophages and stimulate the release of both pro- and anti-inflammatory cytokines, i.e. interleukin-1,-6,-8,-18, interferon- γ and tumor necrosis factor- α [132]. The antiviral effect of lactoferrin on cytomegalovirus in a murine infection model has been demonstrated to be a result of augmentation of natural killer cell activity rather than of the cytolytic T-lymphocytes [14]. Similarly, human lactoferrin has also been proven to have an effect on natural killer cell cytotoxicity against haematopoietic and epithelial tumor cells [133].

Furthermore, the antiviral activity of lactoferrin against vesicular stomatitis virus has been related to its capacity of up-regulating the accumulation of interferon- β in peritoneal macrophages from mice [11]. Another experiment with interperitoneal administration of lactoferrin to CBA mice demonstrated enhanced production of tumor necrosis factor- α and interleukin-12. Similar results were also reported after *in vitro* stimulation of J774A.1 murine macrophages by lactoferrin [134]. Increased expression of interleukin-12, in addition to interferon- β and NOD2, were also observed in mice that were administered lactoferrin orally after being infected with influenza virus, thus suggesting that lactoferrin potentially can promote systemic host immunity [135]. As an important inducer of interferon- γ production in T-cells and natural killer cells, interleukin-12 exhibited a marked synergism with interferon- γ in activating monocytes and macrophages, promoting the differentiation of B-cells and T-cells, and increasing the induction of major histocompatibility complex I and II molecules by up-regulating expression of the interleukin-18 receptor on cells producing interferon- γ [136-138].

With regard to modulation of the adaptive immune system, lactoferrin could exert higher growth stimulatory activity on lymphocytes than transferrin [139], induce phenotypic changes of immature B- and T-cells from newborn or chromosome X-linked immunodeficient mice, as well as enable B-cells to present antigen to an antigen-specific T-helper type 2 cell line [140, 141]. Immature B-cells cultured with lactoferrin will also increase their ability to promote antigen-specific T-cell proliferation, indirectly indicating enhanced B-cell antigen presentation [140].

In summary, the effects of lactoferrin on the activation, maturation, migration and antigen presentation of the innate and adaptive immune cells, suggest that lactoferrin have the potential to associate the cellular functions and responses of the innate and adaptive immune cells, respectively. The modulating effects of lactoferrin on cytokine levels, especially of interleukin-12 and interleukin-18 illuminates the milk proteins role in connecting the innate and adaptive immune response.

5. Milk derived peptides as immune modulators

There is a great quantity of milk proteins and peptides other than lactoferrin that can lead to immune regulation, involving in both up- and down-regulation of the immune system. Peptides from casein [4, 6, 142], β -lactoglobulin [143, 144], and α -lactalbumin [7] also enhance and/or suppress immune cell function (Table 3).

Precursor protein	Fragment	Peptide sequence	Name	Function	Reference
α s1-casein	23-27	FFVAP	α -casokinin-5	ACE-inhibition	[222, 223]
	28-34	FPEVFGK	α -casokinin-7	ACE-inhibition	[222]
	23-34	FFVAP FPEVFGK		ACE-inhibition	[224]
	104-109	YKVPQL		ACE-inhibition	[225]
	158-162	YVPFP	α s1-casomorphin	Opioid agonist, immunomodulation	[159, 226]
	169-193	LGTQYTDAPSFSDIPNPIGSENSEK		ACE-inhibition	[227]
	194-199	TTMPLW	α -casokinin-6	ACE-inhibition, immunostimulatory activity	[5, 228]
	201-212	IGSENSEKTTMP		ACE-inhibition	[229]
α s2-casein	94-103	QKALNEINQF		ACE-inhibition	[166]
	163-176	TKKTKLTEEEKNRL		ACE-inhibition	[166]
β -casein	1-25	RELEELNVPGEIVES (P)LS(P)S(P)S(P)EESITR	casein phosphopeptide	Immunostimulatory activity	[3]
	54-59	VEPIPY		Immunostimulatory activity	[6, 149]
	60-66	YFPGPPI	β -casomorphin-7	ACE-inhibition, immunomodulation activity	[1, 230, 231]
	63-68	PGPIP		Immunomodulation	[6, 232]
	60-70	YFPGPPIP	β -casomorphin-11	Immunostimulatory activity, opioid and ACE-inhibitory activities	[233]

Precursor protein	Fragment	Peptide sequence	Name	Function	Reference
	73-89	NIPPLTQTPVVVPPFIQ	β -casochemotide-1	ACE-inhibition	[229]
	114-118	YPVEP		Promote innate host immune response	[142]
	124-133	MPFPKYPVEP		ACE-inhibition	[229]
	169-175	KVLPVPQ		ACE-inhibition	[225, 229]
	177-183	AVPYPQR	β -casokinin-7	ACE-inhibition	[223]
	191-193	LLY	β -casokinin-10	Immunomodulation activity	[6, 234]
	193-202	YQQPVLGPVR		ACE-inhibition, immunostimulatory activity	[1]
	210-221	EPVLGPVRGPF		ACE-inhibition	[229]
κ -casein	108-110	IPP		ACE-inhibition	[235]
	106-116	MAIPPCKKNQDK	casoplatelin	Antithrombotic activity	[236]
		YIPIQYVLSR	Casoxin C	Opioid agonist	[237]
		YPSY	Casoxin 4	Opioid agonist	[238]
α -lactalbumin	18-20	YGG	α -lactorphin	Immunomodulation	[1]
	50-53	YGLF		ACE-inhibition	[143, 144]
	99-108	LDDDLTDDI		ACE-inhibition	[239]
	104-108	LTDDI		ACE-inhibition	[239]
β -lactoglobulin	22-25	TMKG	β -lactorphin	ACE-inhibition	[239]
	32-40	AGTWYSLAM		ACE-inhibition	[239]
	94-100	IPAVFKI		ACE-inhibition	[239]
	106-111	NKVLVL		ACE-inhibition	[239]
	102-105	YLLF		ACE-inhibition	[143, 144]
	142-148	ALPMHIR		ACE-inhibition	[240]
Bovine serum albumin	399-404	YGFQNA	serorphin	Opioid	[241]
	208-216	ALKAWSVAR	albutensin A	ACE-inhibition	[242]
Bovine lactoferrin	17-41	FKCRRWQWRMCKLGAPSITCVRRAF	lactoferricin	Anti-herpes simplex virus activity, ACE-inhibition, immunomodulation activity	[2, 80]
	17-26	FKCRRWQWRW		immunomodulation activity	[21]
Human lactoferrin	1-49	GRRRRSVQWCAVSQPEATKCFQWQR	lactoferricin	Anti-herpes simplex virus activity	[80]
	1-32	NMRKVRGPPVSCIKRDSPIQCI	LF-33 (human)	Anti-human immunodeficiency virus activity	[18, 19]
		GRRRRSVQWCAVSQPEATKCFQWQR			
	222-230;	ADRDQYELL;	Lactoferrampin	Inhibit herpes simplex virus 1 infection	[243]
	264-268	EDLIWK			
	268-284	KWNLLRQAQEKFGKDKS		Immunomodulation activity	[148]
	318-323	YLGSGY	Lactoferroxin A	Opioid agonist	[244]
	536-540	RYYGY	Lactoferroxin B	Opioid agonist	[244]
	673-679	KYLG PQY	Lactoferroxin C	Opioid agonist	[244]

Note: ACE-inhibition, Angiotensin-converting enzyme-inhibition.

Table 3. Milk proteins-derived peptides with antiviral and immunomodulatory activity

Up-regulation of immune system. Bovine and human lactoferricin, from N-terminal end of bovine and human lactoferrin, respectively, are known for their ability to improve and modulate the function of host immune system [145-147]. Additionally, deletion fragments of lactoferrin like the peptide sequence FKRRWQWRW, corresponding to N-terminal fragment 17-26 of bovine lactoferrin, has demonstrated potent activation of polymorphonuclear leukocytes [21]. Another lactoferrin derived peptide termed lactoferrampin, containing residues 268-284 from the N-terminal domain of lactoferrin, is located in close proximity to the cationic lactoferricin sequence, in the three dimensional structure. Lactoferrampin has been shown to exhibit antimicrobial activity and can improve immune function and gut health in the presence of lactoferricin. Dietary supplementation of piglets with an expressed fusion peptide composed of bovine lactoferricin linked to lactoferrampin demonstrated the ability to increase serum levels of IgA, IgG, and IgM, while decreasing the incidence of diarrhea in the piglets [148].

Immunomodulating casein peptides have been found to stimulate the proliferation of human lymphocytes and the phagocytic activities of macrophages [4]. Casein phosphopeptides from fermented milk products, such as plain yogurts and cheeses, has shown beneficial effects on the immune system including the mitogenic effect and IgA enhancing effect in mouse spleen cell cultures [3]. According to the results of other studies, human β -casein fraction 54-59 has demonstrated to enhance the phagocytic activity of macrophages both in mice and humans and increase resistance against certain bacteria in mice [6, 149].

Chemotactic factors in the tissue do also play an essential role in host defence against microbial infection by inducing leukocyte infiltration. A pentapeptide (β -casochemotide-1) with amino acid sequence (YPVEP) matching an actinase E digest peptide from bovine β -casein (corresponding to fraction 114-118), has been tested and demonstrated to both chemoattract and activate, human and mouse monocytes and macrophages by using a unique G-protein coupled receptors [142].

In addition, Colostrinin, also known as PRP, is a naturally occurring mixture of proline-rich polypeptides derived from colostrums and it can stimulate the immune response in animal and *in vitro* studies by causing differentiation of murine thymocytes into functionally active T-cells [150], as well as inhibit autoimmune disorders. Subsequent studies have shown that Colostrinin largely consists of the peptides derived from proteolytic processing of the milk proteins β -casein and β -casein homolog's [151]. Among the Colostrinin digestion peptides are an active nona-peptide fragment VESYVPLFP demonstrating a full spectrum of biological activities [150]. Furthermore, fermentation of milk by *Lactobacillus helveticus* has also proven to generate novel peptide fragments, i.e. three derived from β -casein and one peptide from α -lactalbumin, and the peptides have demonstrated the ability to stimulate the production of tumor necrosis factor- α and modulate macrophage activity [7].

Down-regulation of immune responses. It has been demonstrated that a modified whey protein concentrate, developed as a by-product from commercial manufacturing of cheese, not only suppress B- and T-lymphocyte proliferating responses to mitogens in a dose-dependent fashion, but also suppress alloantigen-induced lymphocyte proliferation during a mixed

leukocyte reaction. Moreover, the modified whey protein concentrate could also have been demonstrated to suppress other indices of lymphocyte activation, e.g. cytokine secretion and the formation of activated (CD25+) T-cell blasts, showing that the mechanism of suppression may be related to an inhibition of the lymphocyte activation process. However, the interleukin-2 cytokine-mediated response was not affected by the presence of the modified whey protein concentrate in culture [152]. Similarly, intact κ -casein and κ -caseinoglycopeptide (fragment 106-169), which have been prepared from κ -casein digested with rennin, in addition to a commercial whey protein concentrate, all significantly inhibit the mitogen-induced proliferative response of mouse spleen lymphocytes and Peyer's patch cells [153-155]. As a result of this it has been proposed that κ -caseinoglycopeptide fragment 106-169 can inhibit the phytohaemagglutinin induced proliferation of mouse splenocytes via at least two different models; production of an inhibitory component that reacts with the anti-interleukin-1 antibody or through suppression of interleukin-2 receptor expression on CD4+ T-cells [156].

The opioid system plays a major role in immune modulation, both through classical opioid receptor, but also through other mechanisms. For example, opioid peptides have been demonstrated to inhibit phagocytosis [157], decrease natural killer cell number and activity and decrease cell-mediated hypersensitivity [158]. Also, α s1-casomorphin, an opioid agonist, can modulate antibody and cytokine secretion by multiple myeloma cells in a cell line-dependent and opioid receptor-independent manner, but it was shown to decrease the antibody secretion by normal B-lymphocytes and the proliferation rate of multiple myeloma cells through opioid receptor activation [159]. In other words, there might be two different opioid mechanisms, mediated by parallel signalling pathways, i.e. one early non-opioid receptor related effect modulating the constitutive secretion of immunoglobulin and cytokine, as well as a second long lasting receptor-mediated action of cell growth. Thus, opioids might be employed in controlling the humoral immunity.

Furthermore, the rennin-angiotensin-aldosterone system is not only a major regulator of blood pressure; it also plays a key role in autoimmunity. The angiotensin peptide (AII), is one component of the rennin-angiotensin-aldosterone system, and has direct activity on T-cell function, including activation, expression level of tissue-homing markers and production of tumor necrosis factor- α [160]. Inhibitors of angiotensin-converting enzyme will dampen the proteolytic process of the larger angiotensin peptides (AI) to the active AII. Thus, inhibition of T-cell angiotensin-converting enzyme blocks production of tumor necrosis factor- α , which modulates the proliferation of human immunodeficiency virus [161] and regulates the helper activity in B-cell activation [162]. This will also suppress the auto-reactive T-helper 1 and T-helper 17 cells and promotes antigen-specific CD4+FoxP3+ regulatory T-cells through inhibition of the canonical NF- κ B1 transcription factor complex and activation of the alternative NF- κ B2 pathway [163]. Moreover, angiotensin-converting enzyme inhibitors play a pivotal role in immune defence by decreasing the degradation of bradykinin and enkephalin [4, 164].

A variety of angiotensin-converting enzyme inhibitory peptides have been found in the hydrolysates of milk using different enzymes; the bovine α s1-casein (fragment) f24-47, f104-

109, f169-193, f194-199 and f201-212, α s2- casein f94-103 and f163-176, β -casein f60-66, f60-70, f169-175, f177-183 and f193-202, α -lactalbumin f18-20 and f50-53; β -lactoglobulin f102-105 and f142-148, bovine serum albumin f208-216, lactoferrin f17-41 (Table 3). Among all these angiotensin-converting enzyme inhibitors, it should be emphasised that peptides α s1-casein f194-199, β -casein f60-66 and f193-202 have shown to have both angiotensin-converting enzyme inhibitory activities and immune stimulatory effect.

Moreover, recombinant human α s1-casein expressed in *Escherichia coli* has been purified and digested with trypsin in an attempt to find peptides with angiotensin-I-converting enzyme inhibitory activity. Three novel angiotensin-converting enzyme inhibitory peptides, A-II, B-II and C, have been isolated and their amino acid sequences identified as YPER (residues 8-11), YYPQIMQY (residues 136-143) and NNVMLQW (residues 164-170), respectively [165]. Two other sequences QKALNEINQF and TKKTKLTREEKNRL from bovine milk α s2-casein have even stronger inhibitory effects on the angiotensin-converting enzyme [166]. Regardless, no structure-function relationship study for milk-derived peptides in respect to their angiotensin-converting enzyme inhibitory effect has yet been described. However, it has been suggested that peptides with angiotensin-converting enzyme inhibitory function show some common features. First, the interaction between different inhibitory peptides and the angiotensin-converting enzyme is strongly influenced by the C-terminal tripeptide residues of the substrate, which interacts with the active sites of the enzyme [167]. The inhibitory potency of the peptides is further attributed by the hydrophobic (e.g. proline) as well as the positive charged (e.g. arginine and lysine) amino acids in the C-terminal end [168]. Additionally, the model of angiotensin-converting enzyme inhibition involves interaction, not only to the active site but also to the anionic inhibitor binding sites, which are different from the catalytic sites of the enzyme.

6. Synergy between milk proteins and conventional antiviral drugs

A combination of human lactoferrin with recombinant murine interferon- γ resulted in synergistic suppressive effects on disease progression in friend virus complex infected mice [56]. The experiment concluded that natural killer cell activity decreased by friend virus complex, and that the cellular activity returned to normal levels and survival rates increased upon treatment with lactoferrin and interferon- γ . Another study also supporting immune cell regulation by lactoferrin was performed by Spadaro *et. al.* In this study the anticancer activity of a recombinant form of human lactoferrin, talactoferrin-alfa (Agennix, Houston, TX) was evaluated. Talactoferrin-alfa was administered orally to BALB/c mice and the results showed an increase in the intestinal mucosal interferon- γ production, CD8+ T-cell cytotoxicity and the Peyer's patch cellularity which included expansion of CD8+ T lymphocytes and nature killer T-cells, whereas no such phenomena were showed in interferon- γ knockout mice [169]. Thus, the inhibition of friend virus complex infection and tumor growth by lactoferrin/talactoferrin-alfa seems to be mediated by an interferon- γ -dependent enhancement of CD8+ T-cells and natural killer T-cell activity, leading to diversified functions like antiviral defence, immune activation and cell growth regulation [170-173].

Also, combined pre-infection administration of lactoferrin with post-infection administration of Ribavirin on Vero E6 cells could completely inhibit focus formation during hantavirus infection (similar to the traditional plaque formation). This combination therapy also demonstrated significantly increased survival rates in an *in vivo* mice model, not particularly surprising as lactoferrin inhibits viral adsorption to cells and Ribavirin interferes with viral RNA synthesis [122]. Moreover, the antiviral synergy of lactoferrin/lactoferricin with Cidofovir, Ribavirin, Zidovudine, and Acyclovir as all been well documented against human cytomegalovirus, hepatitis C virus, human immunodeficiency virus 1 and herpes simplex virus, respectively [13, 174-176].

Although it is known that lactoferrin has been used to inhibit initial viral infection by interfering with viral attachment and/or entry, the mode of antiviral activity against lots of viruses needs to be clarified in the future, e.g. the infection with hepatitis G virus in human MT-2C T-cells was prevented by bovine lactoferrin with no clear mechanism [63]. With regard to friend virus complex infection, most researchers have confirmed that human lactoferrin has anti-friend virus complex activity in a mouse leukemia model [57, 64], but have no direct effect on friend virus complex infection *in vitro*, indicating a mechanism involving immune regulation rather than direct viral affects. They discovered that human lactoferrin prolonged survival rates and decreased viral titres in the spleen of infected mice by administering human lactoferrin intraperitoneally in the early phase of friend virus complex infection. Probably, the anti-friend virus complex mechanism of human lactoferrin related to the regulation on the myelopoiesis [64], which should be verified thoroughly.

7. Commercial potential of milk derived proteins and peptides

Immune regulation. There is emerging evidence that the utility of many immune mediators originating from milk represents novel therapeutic approaches depending on their activity of immune stimulation, immune suppression and induction of immunological tolerance. Hence, milk-derived proteins and peptides with immune modulating activity are claimed to be a health enhancing nutritional dietary supplement in functional food and pharmaceutical preparations. For instance, Colostrinin from bovine colostrum have demonstrated possible efficacy against various illnesses including viral infections, and ailments characterized by an overactive immune system, such as allergies, autoimmune diseases, neurodegenerative diseases like Alzheimer's disease, etc. Capsules or chewable tablets containing Colostrinin are sold as an over the counter dietary supplement and are available in many countries in the world under names like Colostrinin, MemoryAid, Cognisure, Cognase, Cognate and Dyna (ReGen Therapeutics Limited, London, England) [151]. Moreover, whey proteins are used as common ingredients in various products including infant formulas, specialized enteral and clinical protein supplements, sports nutrition products, and specific weight management- and mood control products.

Additionally, synthetic peptides derivatives tailored on natural milk proteins or fragments there of, may be another powerful way for design of immune regulating pharmaceutical candidates. For example, synthetic peptides tailored from milk proteins have been shown to

enhance proliferation of human peripheral blood lymphocytes. In particular, two fragments (YG and YGG), of bovine α -lactalbumin (fraction 18-19 and 18-20) can significantly stimulate the lymphocyte proliferation, while β -casomorphin-7 and β -casokinin-10, corresponding to fragments 60-66 and 193-202 of bovine β -casein, respectively, suppresses lymphocyte proliferation at low concentrations while enhanced proliferation at high concentrations [1] (Table 3).

Recent studies have shown that synthetic innate defence regulator peptides offer protection by enhancing innate immune defenses of the host while suppressing potentially harmful excessive inflammatory response triggered by the invading pathogen. For example, innate defence regulator peptide 1 was chemotactic for T-helper cells type 1 [23], monocytes [27] and neutrophil response [28], acting in a mitogen-activated protein kinase-dependent manner, while reducing pro-inflammatory cytokine responses. Another peptide, innate defence regulator 1002, induces chemokines in human peripheral blood mononuclear cells [24] which prevents the production of interleukin-1 β -induced matrix metalloproteinase 3 and monocyte chemotactic protein-1 and selectively suppresses the inflammatory response [25].

With the aid of computational molecular modeling technologies, theoretical prediction of immune regulatory peptides has become available and practical. For example, RDP58, a novel d-amino acid decapeptide (r-(nle)₃-r-(nle)₃-gy-CONH₂), which was developed by computer-aided rational based design on human leukocyte antigen-derived peptides [177], has been discovered to suppress the T-helper 1 cytokine profile, decrease production of inflammatory cytokines including tumor necrosis factor- α , interferon- γ , interleukin-2 and interleukin-12 in both cell lines and animal models [26, 178, 179]. Several clinical trials on human including phase I safety in normal volunteers, phase II mild-moderate active ulcerative colitis, phase II moderate active ulcerative colitis and phase II Crohn's disease had been completed (Genzyme Corporation; Sanofi, Bridgewater, NJ). Moreover, quantitative structure-activity relationship analysis has been done for peptide design and optimization in developing novel antimicrobial drugs [180-183], and the numerical improvements of quantitative structure-activity relationship studies has been exemplified recently [184], though there are limitations to the predictive ability of the models [185, 186] this technology clearly accelerates lead peptide discovery.

Suppression of immunological functions by milk derived proteins is thought to be important in the ontogeny of the neonatal gastrointestinal immune system, specifically by ensuring a state of tolerance with respect to food proteins. Kulkarni and Karlsson has demonstrated the essential role of milk-derived immunosuppressive factors (i.e. growth factor- β) during early development, and that neonatal mice deficient in transforming growth factor- β remain viable only as long as they receive maternal milk containing this same growth factor [187].

Also, it is envisaged that most of the potential immunosuppressive activity of milk-derived peptides would be effective on chronic inflammatory diseases and organ transplant patients by decreasing allergy, autoimmunity, and organ rejection. For example, lactoferrin could

enhance the production of anti-inflammatory factors, like interleukin-11, not only in a hepatitis mouse model, but also in human intestinal myofibroblasts [188]. Additionally, hydrolysis of caseins with *L. casei* GG-derived enzymes has generated molecules with suppressive effects on lymphocyte proliferation and benefited the intestinal bacteria in the down-regulation of hypersensitivity reactions to ingested proteins in patients with food allergy [189]. Furthermore, two synthesized analogs of the hexapeptide of human β -casein (fraction 54-59) with modification at the N-terminal region not only showed inhibition in alloantigen inducing lymphocyte proliferation and production of interferon- γ in a SRBC mice model, but also demonstrated increased production of interleukin-4 and improved the skin graft survival. Thus, these peptides might serve as good templates for development of safe and effective immunosuppressant drugs [17]. Similarly, two other synthetic β -casein peptides HLPLP and WSVQPK, have demonstrated potent antioxidant activity and inhibitory activity of angiotensin-converting enzyme [190]. Accordingly, proteins and peptides from milk could potentially be used in production of immune stimulating- and immunosuppressant agents for both prophylaxis and treatment of infectious diseases and immune related illnesses.

Antiviral therapy. Lactoferrin might be a useful addition to conventional antiretroviral therapy, as traditional therapy supplemented with lactoferrin has demonstrated a more effective increase in CD4⁺ cell count than either treatments alone [191]. A potential oral vaccine resulting in expression of enterovirus 71 VP1 capsid protein in a transgenic animal system, under the control of an α -lactalbumin promoter and an α -casein leader sequence has demonstrated protection against enterovirus 71 [192]. Also, the combined treatment with human lactoferrin and recombinant murine interferon- γ on feline calicivirus infection might be of significance as a potential therapy for patients with leukemia and those infected with retroviruses [56].

Most of the proteins and peptides with antiviral potential has also demonstrated synergy with conventional antiviral drugs, reducing the dose of the antiviral drugs, and limiting the development of drug-resistant viruses on account of the selective targeting of the host rather than infectious pathogens. At the present time, many peptides with immune regulating effects have been approved for clinical use against virus infection, such as Zadaxin, IM862, SCV-07 and so on [193]. Similarly, two peptide inhibitors of interleukin-10 may be applied to increase anti-hepatitis C virus immune response by restoring the immune stimulatory capabilities of dendritic cells, which have been suppressed by high levels of interleukin-10 [194]. Moreover, candidacidal activities of a synthetic peptide from human lactoferrin fraction 1-11 and 21-31 have been investigated for killing of multidrug-resistant pathogens [195-199]. Present research results, such as phase I safety and tolerability trials of human lactoferrin by AM Pharma [22], indicate that human lactoferrin 1-11 acts by selectively stimulating the innate immune system [200]. Thus, human lactoferrin 1-11 is more likely to be an interesting candidate for further exploration in various clinical tests, such as coating for dental or bone implants, in biosensing applications or in radiopharmaceutical therapy [199].

Vaccine adjuvant. Vaccine adjuvants, such as an immune potentiator or immunomodulator, have been used for decades to improve the immune response to vaccine antigens. This

involves presentation of the antigen to the immune system, regulation of both quantitative and qualitative aspects of the immune responses, targeting of specific cells, etc. Many adjuvants had been developed in the past, but were never accepted for routine vaccination because of safety concerns, such as acute toxicity and the possibility of delayed side effects. Thus, novel vaccine adjuvants without side effects should be proposed. Despite numerous publications on milk proteins and milk derived peptides with immune regulating activity, there are scarce reports of their adjuvant potential to vaccine. Lactoferrin could function as an effective adjuvant as it has been documented to enhance efficacy of the Bacillus Calmette-Guerin, the current vaccine for tuberculosis disease by promoting host protection and decreasing disease manifestation [201, 202]. Additionally, recombinant porcine lactoferrin significantly increased serum IgA, IgG and infectious bursal disease virus-specific antibody, as well as enhanced interferon- γ and interleukin-12 expressed in chicken T-lymphocytes, suggesting that porcine lactoferrin could enhance cell-mediated immunity and strengthen the ability of vaccinating against infectious bursal disease infection [203, 204].

An innate defence regulator peptide, HH2, has shown synergy with oligonucleotides containing CpG motifs, when used as an immunoadjuvant to enhance the immune response through stimulation of T-helper 1 and T-helper 2 responses in newborn piglets which were vaccinated with a pseudorabies attenuated virus vaccine [29]. Recently, Brown *et. al.* found that the combination of oligonucleotides contain CpG motifs and HH2 displayed robust adjuvant effects on induction of T-helper 1 cellular immune response in mice by formulating with a booster recombinant Chlamydia antigen subunit vaccine [205]. Another synthetic peptide, WKYMVm, originally identified as a peptide that stimulated the activity of monocytes, neutrophils and dendritic cells [206-210], has demonstrated to selectively enhance the vaccine-induced CD8⁺ T-cell responses in a dose-dependent manner, in terms of interferon- γ secretion and cytolytic activity when it was co-delivered with human immunodeficiency virus, hepatitis B virus and influenza virus vaccines [211]. It is indicated that WKYMVm may function as a novel adjuvant for DNA vaccine.

Cancer inhibition. More recently, a widely-read article focused on the amazing cases in which milk proteins and derived peptides were used in the treatment of different kinds of cancers. Whey protein is superior to other dietary proteins for suppression of tumour development in animal models usually for colon and mammary tumorigenesis [212]. Furthermore, lactoferrin and its peptides, for example, lactoferricin [212-214], both possess anticancer activity by inducing apoptosis; inhibit angiogenesis, modulating the carcinogen metabolizing enzymes, and so on. Casein and casein derived peptides have antimutagenic properties, and other whey protein components, such as β -lactoglobulin, α -lactalbumin and serum albumin, have also demonstrated anticancer potential [212, 215-217]. Moreover, a recombinant adenovirus containing the human lactoferrin cDNA has been constructed and its effects against tumor growth have been investigated in mice bearing EMT6 breast cancer. The results showed that recombinant delivery of human lactoferrin cDNA could induce apoptosis of the tumor cells by triggering the mitochondrial-dependent pathway and activation of caspase 3, suggesting that this recombinant cDNA delivery might be a promising drug strategy for breast cancer gene therapy [218].

In addition, the synthetic peptide, P60 (RDFQSFRKMWPFFAM) [219], has demonstrated potential of inhibiting the immunosuppressive activity of murine and human derived regulatory T-cells and enhances the effector T-cell stimulation *in vitro* by binding to regulatory T-cells specific forkhead or winged helix transcription factor 3. Thus, P60 can improve the immunogenicity of cancer and viral vaccines against CT26 tumor challenge and hepatitis C virus infection. Also, the *in vivo* antitumoral effects of LTX-302, a 9-mer peptide derived from bovine lactoferricin, have been examined by intratumoral injection. The results showed that LTX-302 induced tumor necrosis and infiltration of inflammatory cells followed by complete regression of the tumors, as it results in long term and specific cellular immunity against the A20 B-cell lymphoma that is CD4+ and CD8+ T-cells dependent [20].

8. Conclusion

Most of the milk proteins and peptides that have been identified with antiviral properties are broad spectrum components targeting general features and mechanisms involved in a viral infection cycle. Hence, many of these milk proteins do also demonstrate synergy with conventional antiviral drugs. Recently, the diverse immunomodulatory activities of milk proteins/peptides have illustrated these molecules interesting potential as antiviral therapeutics, though the precise mechanisms of immune regulation needs to be thoroughly described. Although the synthetic peptides usually are shorter than natural proteins, the antiviral immune regulating properties of many of these synthetic derivatives appear to be similar as for the entire proteins. Thus we would argue that milk proteins and peptides, have great potential to serve as templates for design of more potent antiviral drugs. With proper scientific effort these molecules may have great therapeutic potential as supplements for current antiviral and anticancer therapy, as novel vaccine adjuvants for both human and far animals, and as immunosuppressants for autoimmune diseases and allergy treatment.

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