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THE INVOLVEMENT OF CHEMOKINES TO NORMAL AND PATHOLOGICAL IMMUNE RESPONSES

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Summary

Chemokines (CMK) belong to a complex biological superfamily including structurally and functionally related proteins. They are elaborated, constitutively or inducible, by various populations of cells. CMK are basic polypeptides deprived of glucidic residues and exist in many biologically active forms (monomers, dimers, trimers and tetramers). CMK superfamily includes four classes: alpha, beta, gamma and delta chemokines, differentiated by the amino acids sequences and by the cellular types they act upon. Signals represented by CMK become active by interactions with membrane receptors. Their functions are characterized by redundancy and robustness. Chemokines exert multiple actions upon various cell populations (stimulation of the migration, expressing the activity of specific cellular granules, modification of cellular adhesiveness, amplification of NK cellular activity, enhancing of bactericidal activity of neutrophils, etc.). They play a major part in many immune and pathological mechanisms (angiogenesis, hematopoiesis, acute and chronic inflammations, infectious processes, neoplasis etc.).

CMK belong to a superfamily of structurally and functionally related soluble proteins. The chemokines name comes from their property to induce mechanisms of direct chemotaxis onto cells that are sensitive to their actions and in the proximity of which they activate, being **chemo**tactic cyto**kines**. Their primary structure includes 67-127 amino acids (*Mosser and William*, 2007) and has molecular weights which vary between 8-10 kDa, in case of monomer forms. The genes sequences who code CMK and their amino acids sequences, included in four classes, have homology in proportions that vary between 20 and 50%. Molecules belonging to the chemokines' superfamily share some structural characteristics: reduced dimensions, the presence of four cystein residues at some conserved sites and tridimensional conformation. They play major roles in many immune mechanisms, which include the guided cellular migration, cellular activation, citotoxicity, maturation of dendritic cells, T and B cells, chronic and acute inflammations and other pathological processes as well as in angiogenesis, hematopoiesis, trauma healing etc. Those activities are being expressed especially through stimulation and regulation of cellular migration. Following their selective migration on the pathological or reparatory sites, different types of cells are accumulated, for example: T lymphocytes, neutrophilic granulocytes or monocytes.

Generally, chemokines may realize beneficially or damaging actions, through stimulation of appropriate immune responses to the microbial invasions or by mediating the destruction of different tissues as part of the evolution of different pathological processes (autoimmune diseases, atherosclerosis, allograft rejection,

neoplasic processes). Also, CMK have essential functions within the regulation of normal traffic of lymphocytes in the primary and secondary lymphoid organs. They also regulate the localization and the proliferation of haematopoietic stem cells (*Christopherson and Hromas*, 2001).

Some recent studies show that CMK and some receptor molecules for CMK are involved in the pathogenesis of human multiple sclerosis (MS). CMK have been detected in the demielinizating lesions, and have also been detected in high concentrations in the cerebrospinal fluid of patients with MS. These data suggest that the interference with the chemokines' system may constitute a beneficial therapeutically approach (*Weber et al*, 2004, *Szczucinski and Losy*, 2007).

Receptors for chemokines are also involved in other pathological mechanisms including allergies, psoriasis, atherosclerosis and malaria (*Murdoch and Finn*, 2000).

In the last few years the development of bioinformatics and the use of some performant molecular biology methods, contributed to the identification of many chemokines and of their receptors. Increased attention has been given to studies regarding chemokines after it has been discovered that receptors for chemokines are co-receptors for the human immunodeficiency virus (*Rossi and Zlotnik*, 2000).

The production of chemokines

The production of chemokines is realized on the basis of two mechanisms (*Mantovani*, 1999 and others):

(1) Constitutive synthesis; it is a permanent property of some specialized cells (monocytes, macrophages, dendritic cells, thymocytes and other cells from the lymphoid organs) who produce, for instance, chemokines belonging to the MDC group (for abbreviations see the section regarding chemokines' classification)

(2) Inducible synthesis, the mechanism used for the production of the majority of chemokines, involving cell activation by different endogen and exogenous stimuli, such as pro-inflammatory factors (LPS, IL-1, TNF-α, traumatic and microbial agents) and activators of some immune mechanisms (antigens, mitogens, IgE). Inducing factors develop a quick action; therefore the elaboration of CMK starts 1-2 hours after stimuli intervention.

Production and secretion of chemokines are realized by almost all cell types belonging to different tissues, with differences regarding chemokine classes. Alpha-chemokines are mainly produced by mononuclear phagocytes as well as by fibroblasts, endothelial cells, megakariocytes (precursor of trombocytes, which contain stored chemokines). The main source of beta-chemokines is represented by activated T cells. In case of MCP-1 beta-chemokines, to those cells are also added monocytes stimulated with PHA or LPS as well as cells belonging to other cell types (*Leonard and Yoshimura*, 1990).

Activated cells belonging to some populations have the capacity to synthesize, in a redundant manner, many types of chemokines. This property, known as "polyspeirism", represents a characteristic of some cells such as

mononuclear phagocytes and endothelial cells exposed to LPS action or to some cytokines such as IL-1 and TNF.

During the process of chemokines' production an important part is designated to cellular context and to some cytokines which determine the directions of CMK production by the macrophages and endothelial cells. IL-4, IL-10 and IL-13 have different, even divergent effects, regarding the synthesis of CMK. IFN-gamma amplifies the production of MCP (elaborated by macrophages) and inhibits the production of IL-8 and GRO (elaborated by endothelial cells). Other molecules act upon macrophages and endothelial cells (LPS, IL-1, TNF, thrombin) or just upon endothelial cells (IL-6) and can determine a decrease in the intensity of CMK elaboration.

Structural elements and classification of chemokines

Chemokines are basic polypeptides, generally deprived of glucidic residues. They exist in many biologically active forms: monomers, dimers, trimers and tetramers. Chemokines are active in small concentrations of 10^{-8} to 10^{-10}.

There have been described approximately 50 CMK. Based on their primary structure they are classified in four subfamilies (classes) (*Ward and Westwik,* 1998 and others), using as defining criteria the presence or absence of an amino acid residue among the first two from the four conserved cystein residues and the alignment of Cys NH_2-terminal residues. Schematic aspects of the basic structure of CMK are shown in fig.1 and fig.2. Fig. 2 shows some characteristic elements from the structure of CMK belonging to the CX3C class: the presence of a hydrophobic and a mucine-type domain.

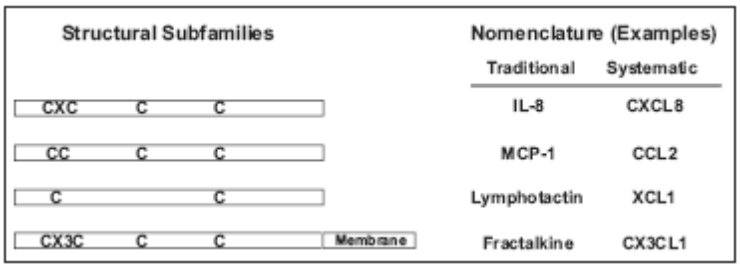

Fig.1 Chemokine subfamilies: structure and examples
(Traditional and systematic nomenclature)

The chemokines classes described so far are:

(1) **CXC chemokines** (α-chemokines) have a structure that includes four conserved cysteins. Among the first two there is an interposed amino acid (X) (the sequence is written as …C-X-C….C....C….). They are also called cis-X-cis chemokines. From all of them, the most important are: NAP-1 (from neutrophil-activating peptide 1 or neutrophil attractant/activation protein-1, another name for

IL-8), NAP-2, NAP-4, GRO-alpha (growth related oncogene, also called MGSA, from melanoma growth stimulatory activity), GRO-beta (MIP-2α, macrophages inflammatory protein 2-α), GCP-2 (granulocyte chemotactic protein 2), PF-4 (platelet factor 4), PBP (platelet basic protein), γ-IP-10 (γ-IFN inducible protein 10);

(2) **CC chemokines** (β-chemokines or cis-cis chemokines) in which's structure is included the same motif of four cystein residues but without the interposition of an amino acid between the first two (the basic structure is C-C….C….C.). In a short way, this family is noted as CC. To this family belong: MIP-1α (macrophage inflammatory protein alpha), MIP-1 beta, RANTES (regulated upon activation, normal T cells expressed and secreted), MCP-1 (macrophage chemoattractant protein 1), MCP-2, MCP-3, MDC (macrophage-derived chemokine). Also, the C-C family includes eotaxines. Cis-X-cis chemokines and cis-cis chemokines bind to the heparin from the structure of heparin sulphate proteoglycans at the surface of endothelial cells and to the components of the extra-cellular matrix, therefore stimulating the migration of leucocytes which attach themselves to the vascular endothelium and to the matrix.

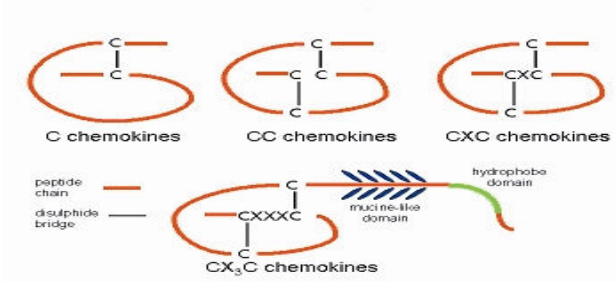

Fig. 2. Basic structure of the chemokines belonging to the four subfamilies

(3) C chemokines (γ-chemokines) who have only two conserved cystein residues, those in the second and fourth position, so the sequence is ….C….C….This class includes less molecules, among them Ltn (lymphotactin) and ATAC (activation- induced, T cell-derived and chemokine-related molecule).

(4) CX3C chemokines (δ-chemokines) which include so far only one member (fractalkyn or CX3CL1) which acts as a chemoattractant and as an adhesive molecule.

All chemokines have similar tridimensional structure, stabilized by disulphide bonds located between preserved cystein residues (the classic aspect of chemokines is represented in fig. 3; inside the molecule there has been noticed the presence of alpha- helix sequences and beta-antiparalel sheets).

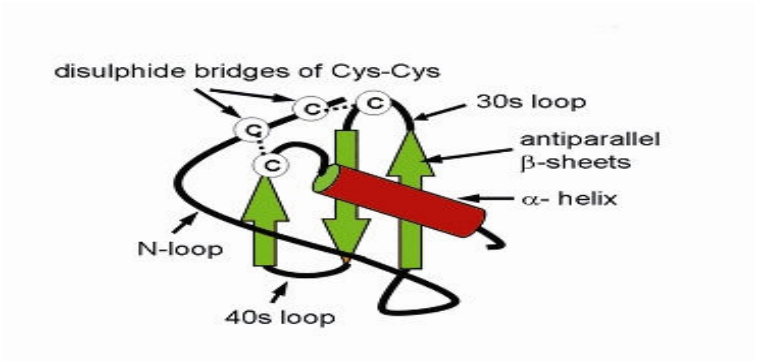

Fig. 3 Tridimensional structure of chemokines

Chemokine receptors

These are molecules included in the cellular membranes and belonging to the superfamily of receptors for rodhopsine and serpentine (*Taub,* 1998, *Moser*, 2003, and others). In their structure seven characteristic transmembranar regions bound with G protein are included (GPCR, from G protein-coupled receptor).

From the structure of CMK receptors the NH_2-terminal region and three extracellular domains are involved in binding chemokines; the COOH-terminal sequence and three intracellular domains are involved in the signals transduction mediated by protein G (fig. 4, *Moser*, 2003).

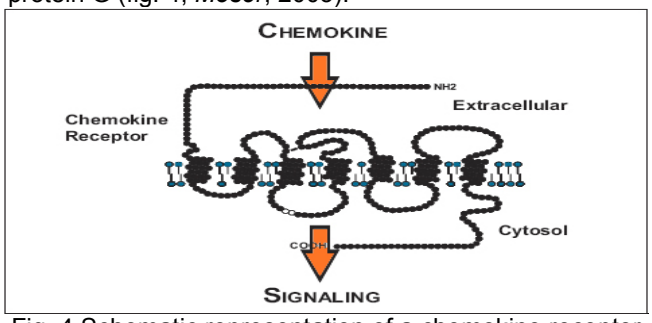

Fig. 4 Schematic representation of a chemokine receptor

Between chemokine receptors exist different homology levels.

Examples of receptor molecules for CMK are: CXCR1 (receptor for IL-8 and GPC-2), present in neuthrophils; CX3CR (receptor for IP-10, MIG, I-TAC) described in T-helper and citotoxic cells (T_H1 and T_C1); CCR1 (receptor of MIP-1 alpha, RANTES, MCP-3, MCP-4 chemokines) from the surface of monocytes, T cells, NK cells, immature dendritic cells (iDC), neuthrophilic granulocytes; CCR3 (receptor for eotaxine, MCP-3, RANTES) identified in eosinophilic and basophilic

granulocytes, as well as at the T_H2 cells; CCR4 identified at the surface of T_H2, T_C2, NK and iDC cells; CCR5 is identified in monocytes, helper and cytotoxic T cells (T_H1, T_C1), NK cells and immature dendritic cells. Mentioned nomenclature indicates CMK families which interact with certain receptors (for example, CXCR is a receptor for a molecule that belongs to the CXC chemokine family).

The expression of cell surface receptors is essential for the activity as well as for the differential selection of cells they act upon. Therefore, T_H1/T_C1 cells on one side and T_H2/T_C2 cells, on the other side, have different receptors and react to different chemokines. Following the binding of a CMK to its receptor, there are induced immediate cellular responses: modification of shape, activation of the integrins, degranulation, and secretion of enzymes, oxidative mechanisms and, especially, the initiation of cell migration due to the chemotactic action of chemokines.

The receptors for chemokines have an essential role in recruiting T lymphocytes and in the induction of their functions (*Word and Westwick*, 1998, *Schutyser et al*, 2005 and others). These cells represent a source of CMK, expressing, in the same time, membrane receptors for chemokine belonging to the CC and CXC families (CXCR4, CCR5 and others). CCR5 is a co-receptor for HIV-1 strains; the intrusion of this virus inside the cytoplasm of the T cells is mediated by RANTES, MIP-1alpha and MIP-1 beta chemokines and by their receptors. CXCR4 is a major co-receptor for other HIV strains whose intrusion in the target cells is inhibited by the SDF-1 chemokine.

Chemokine receptors have also important functions in the biology of natural killer (NK) cells. According *Robertson* (2002), inactive Cd^{dim} $CD16^{+}$ NK cells express to the surface CXCR1, CXCR2, CXCR3, CXCR4 and CX3CR1 molecular receptors .These cells migrate rapidly and intensely as a response to their interaction with CXC12 and CXC3L1 chemokines. Chemotaxis of $NKCD56^{bright}$ $CD16^{-}$ NK cells is stimulated mainly by CCL19, CCL21, CXCL10, CXCL11 and CXCL12. NK cells may migrate as a result of the interaction between other chemokines belonging to the CC and CXC families and their receptors. Others CMK have the property to amplify the cytolytic activity of these cells. NK cells produce numerous chemokines that can recruit other effectors cells within cellular responses. CCL3 and CCL4 chemokines released by NK cells inhibit the *in vitro* replication of HIV. Also, as a result of their interaction with membrane receptors, CMK mediates responses through NK against tumoral cells.

Hansel et al (2006) have noticed the presence in mammals of some atypical receptors for chemokines, receptors which do not perform the already known role of these molecules or do they participate in the signaling mechanisms following interactions with chemokines. Those receptors are specialized in sequestrating chemokines, acting as regulators of CMK biodisposition and influencing the responses which involve typical receptors.

Main functions of the chemokines

The most important cellular properties and mechanisms involved in chemokines activity (*Wilkinson* 1998, and others) are:

1. Stimulating the locomotion capacity, a necessary property in order to realize the migration, which belongs to some cell populations found in certain stages of their development; so, the precursors of myeloid cells have no receptors for CMK and are immobile; on the other hand neuthrophil granulocytes and mature monocytes are mobile.

2. Chemokynesis, representing chemokines' property to stimulate leukocyte migration; this function is expressed into two forms: ortokynesis (frequency and speed of migration, including the speed modification, the main CMK function) and klinokinesis (changing the direction of migration, an action performed especially on bacteria and, at a less extent, on leucocytes).

3. Chemotaxis, indicating the property to stimulate organized movement of leucocytes, determined by the direction and intensity of CMK activity; the stimuli are represented by the chemokines concentrations.

4. Guidance through contact, a mechanism that determines the direction of migration according to the physical properties of the environment where the leukocytes move.

5. Haptotaxy, indicating the oriented movement over a surface that presents a modified adhesion gradient; this mechanism appears when crossing the vascular endothelium and when leucocytes pass through conjunctive tissues.

Fig. 5 (*Moser*, 2003) represents one of the major property of the CMK which consists in recruitment and localization of circulating leucocytes. This process involves three sequential stages: vascular adhesion, extravasation and chemotaxis. Before extravasation, leukocyte interacts with adhesion molecules into the blood vessels. The functions of integrins are amplified by the participation of receptors for cytokines that ensures a firm adhesion of leukocytes to the vascular endothelium. During the next step, leukocytes migrate through the vascular endothelium. In the perivascular area, migration of leucocytes is directed into certain tissues according to the gradient of CMK action.

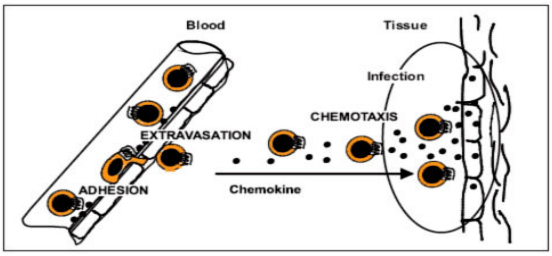

Fig. 5 The role of chemokines in the recruitment and localization of circulating leucocytes

Molecules belonging to the chemokine system and to the whole CMK system in general have some important characteristics who contribute to the achievement of their functions. Among those properties (*Mantovani*, 1999): the great number of members belonging to each family; redundant production and actions; high and steady efficiency, a main characteristic of biochemical mechanisms in general, as a result of a certain robustness of the chemokine system.

Chemokines are active upon cells belonging to many populations, such as:

- Alpha-chemokines: especially neuthrophilic granulocytes, T and B lymphocytes;
- Beta-chemokines have a wider action area, including monocytes, basophilic and eozinophilic granulocytes, T cells, dendritic cells and NK cells.

Taking into consideration chemokines as individual molecules, it has been noticed that their functions are realized in different ways regarding different cell types (*Taub*, 1998).

Beside chemokines, there are other molecules with chemoattractant properties such as (*Wilkinson*, 1998): cytokines (IL-2, IL-5, IL-16, TGF-beta, and TNF- alpha) and other molecules: C5a, B4 leukotriene, thrombin, etc.

In the context of activities related with the regulation of migration and other mechanisms, the main actions of chemokines are, mostly, similar, including: modifying the distribution, number and affinity of chemokines receptors; stimulating the migration (chemotaxis, chemokinasis); the production of certain granular azurophilyc and other granular products; modifying cellular adhesion to endothelial cells; influencing the angiogenesis and collagen production processes; stimulating the precursor of hematopoietic cells; amplifying the release of lysosomal enzymes; enhancing the destruction of tumoral cells by NK; increasing the destruction of pathogenic organisms by neuthrophils.

Chemokines network has an important contribution to the increasing of the polarized responses in which T_H1/T_C1 and TH_2/T_C2 lymphocytes are involved. IFN-gamma (produced by activated NK cells as well as T_H1 and T_C1 cells), induces the secretion of antagonistic molecules against CXCR3 receptor by the monocytes and endothelial cells: the IFN-induced protein (IP-10), the alpha-chemoattractant of T cells induced by IFN (ITAC), the monokyne induced by IFN (MIG). These CMK attract and locate a great number of cells. IL-4 and IL-13 induce the synthesis of eotoxines by cell belonging to many types and the production of chemokines derived from macrophages (MDC) by monocytes and dendritic cells. Production of MDC cells is blocked by IFN-gamma. These CMK attract T_H2/T_C2 lymphocytes.

An example of CCL18 (a CC chemokine) functions, during physiological and pathological processes, is represented in fig. 6 (*Schutyser et al*, 2005). It has been noticed that this CMK is secreted by monocytes and macrophages (Mo/Mf) as a result of some classic activation processes (induced by LPS or CD40L), of alternative activation (induced by IL-4, IL-10 and TFN-alpha) or, probably, maturation (determined by IL-4, IL-10 and GC). Immature dendritic cells (immDC) secrete CCL18 constitutively (through GC or prostaglandins' actions) or inductively,

following maturation (a controversial process induced by LPS and TNF- alpha) or alternative activation (initiated by LPS and TFN-alpha) or alternative activation (initiated by the action of IL-4 and IL-10). After being released from cells, CCL18 attracts immature T and B lymphocytes as well the immature dendritic cells; it also induces the deposit of collagen by fibroblasts. CCL18 also contributes to some mechanisms such as lymphocytes and dendritic cells oriented migration, setting off primary immune and tolerogenic responses. This chemokine is involved in some pathological processes such as neoplasia and inflammations.

Chemokines are involved in various immune mechanisms such as rejecting of grafts as well as in non-specific processes within some microbial infections and inflammations (*Fox-Marsh and Harrison*, 2002; *Moser* and *Williman*, 2007).

Fig. 7 (*Fox-Marsh and Harrison*, 2002) presents the role of natural immunity in the infiltration of xenografts and infectious processes sites with T cells. The involvement of chemokines and of some cellular and molecular effectors is shown. (acronyms used within this figure: DC: dendritic cell; T: T cell; PAMPS: pathogen-associated molecular pattern).

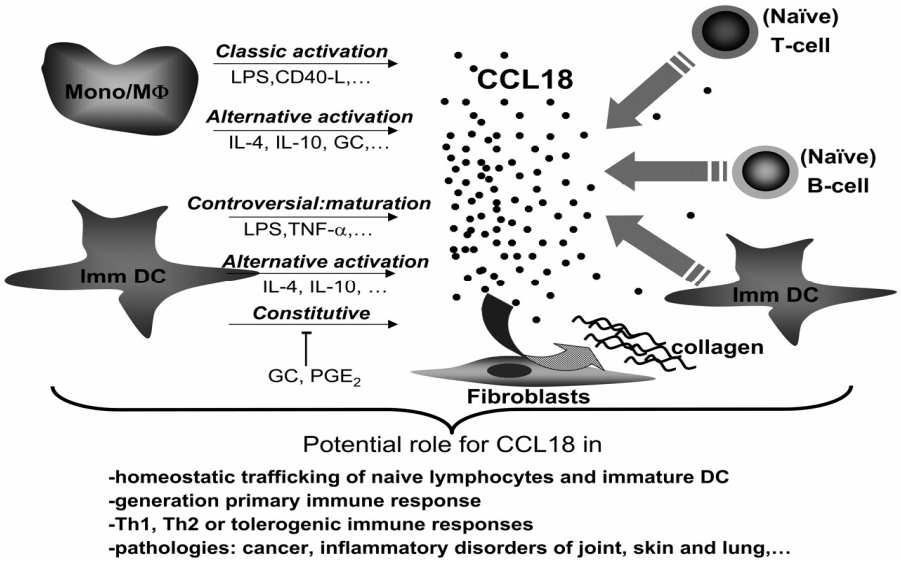

Fig. 6 Potential functions of CCL18 chemokine

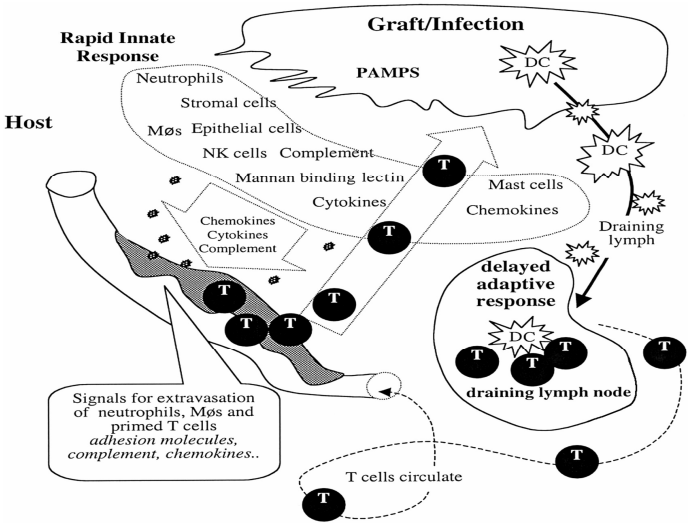

Fig. 7. Some aspects regarding the role of natural immunity in the infiltration with T cells of xenografts and sites with infectious processes

The involvement of some chemokines within pathogenic mechanisms in human asthma has been described by *Lun et al* (2006). The authors have described the role of IP-10, MIG and RANTES chemokines and of their receptors (CCR3, CCR5 and CXCR3), establishing that RANTES can be considered as a marker for this pathological status.

Chemokine functions in mycobacterial infections

An important chemokines' action has been described by *Orme and Cooper* (1999). The authors consider that, in case of tuberculosis, the two major mechanisms of the immune cellular-mediated response, protection and delayed type hypersensitivity (DTH), are independent phenomena, initiated and regulated by different mediators – interleukins (IL-1, IL-2, IL-12) and IFN-gamma, chemokines respectively. Macrophages and monocytes influx and the formation of granulomas at the site of infection as well as in the expression of a tuberculin reaction, are induced as a result of chemokines' actions. Cumulating these cells in the lungs is associated with the most intense synthesis of chemokines and keeps on going even after the activation of protective immunity.

Chemokine functions in viral infections

Some studies (*Price and col*. 1999, *Lalani and col*., 2000 and others) present chemokines functions regarding the antiviral immunity. CMK activities have a major involvement among interactions between leukocyte populations who participate in

generating and regulating antiviral immune responses, as well as among other adaptive cellular reactions.

Especially, chemokines released by citotoxic T lymphocytes (CTL), following their activation by the specific viral antigens, are essential for the antiviral mechanisms through the signals involved in the oriented migration of leucocytes. Secretion of CMK within the sites where the pathological processes induced by the virus develop, contributes in the realization of essential antiviral defense: the recognition of foreign antigens, signaling their presence, recruitment and attraction of effectors cells.

$CD8^+$ lymphocytes release alpha-chemokines as well as beta-chemokines. This CTL function depends on the recognition of a viral antigen through TCR which interacts with the complex formed by CMH cl. I molecules and antigenic peptides, at the surface of an antigen presenting cells. The property to induce the release of CMK has been proved in case of an infection with several viruses, that leads to the conclusion that this is a general property of cytotoxic cells. Intercellular regulation of this mechanism is differentiated depending on the chemokine involved.

Among strategies that help viruses avoid the immune response, an important place goes to the viral proteins production which oppose to the interactions between cytokines and their receptors, interferring with the induction of the differentiation process of specific effectors cells (*Lalani et al*., 2000). Viral coded proteins and expressed proteins are called virokynes; they mimic the structure or modulate functions of the same molecules with major immune activities, such as proteins that control the complement system, development factors and cytokines. Some of these proteins coded by viruses mimic cytokine receptors for TNF-alpha and IFN-gamma. Also, viruses release molecules that reproduce the structure of chemokines, of CMK receptors and of the antagonist of the receptors for CMK. There has been noticed the existence of an increased number of genes belonging to the pox viruses and herpes viruses who code the homologous molecules belonging to chemokines or CMK receptors. For the Marek disease virus there have been identified genes for three CXC chemokines and for the avian difterovariolic virus genes for 13 CC chemokines. All these molecules block or limit the recruitment of effectors cells with antiviral activity.

Chemokines described in animals

In the past few years there have been a lot of contributions in detecting genes for chemokines and their receptors in animals as well as the proteins coded by these. There have been identified genetic sequences involved in coding for many amphybian species (8 CC chemokines, 12 CXC chemokines) and reptiles (a CXC chemokine). In fish there have been described a great number of genes for chemokines as well as the elaborated proteins. For example, in *Cyprinus carpio* are known more than 40 CC chemokines, over 30 CXC chemokines and a C chemokyne. In birds (the reference species is *Gallus gallus*) there have been

known 13 CC chemokines, 8 CXC chemokines, a C chemokine and a CXC3 chemokine.

Recent studies regarding the presence of chemokines in farm and agreement animals have offered important data regarding the existence of an increased number of chemokines and their receptors.

Hughes et al. (2001) have identified, localized and phylogenetically analyzed the genes for three new chemokines in chickens. *Wong et al* (2005) have detected at the same species genes that code 23 chemokines (belonging to all four families) and 14 receptors for chemokines. Also, *Zhu et al* (2005) have analyzed the genes of *Gallus gallus*, identifying genetic sequences that code 14 new chemokines included in the same four families.

Heaton et al (1999) have analyzed genes for two bovine chemokines, expressed in the epithelial cells as part of an inflammatory response. *Werling et al* (2002) have described the role of bovine chemokines elaborated by dendritic cells in the proliferation of T cells induced by the respiratory sincityal virus.

Dunphy et al (2001) have isolated and characterized mRNAs involved in coding some CC chemokines (chemoattractant monocytary proteins) in sheep. *Nagaoka et al* (2003) have described, at the same species, the IP-10 chemokine induced by the action of IFN-gamma, who has the quality of recruiting and distributing the immune cells in the uterus during early stages of pregnancy.

More contributions have been reported regarding the chemokines identified in swine. For example, *Hu et al* (1997) have localized and described the coding genes for many cytokines as well as for the chemoattractant monocytary protein (MCP-2). *Shinkai et al*. (2003) have developed an original research by cloning the gene coding CCR7 receptor, whose ligand is a CC chemokine. *Kazuhico et al* (2004) have described the effects of some CXC chemokines and of CX3CR receptors on the development of integrins expression in swine fetuses. *Skjolaas et al* (2006) have established that bacteria belonging to *Salmonella enterica* family (*S. typhimurium* and *S. cholerae suis*) determine the expression of some chemokines in the ileal and jejunal epithelial cells.

The role of chemotactic cytokines in the mobilization of neutrophilic granulocytes has been investigated by *Franchini* and col. (1998), related with the pathogenesis of obstructive pulmonary disease. There have been identified chemokines reactive to those cells - IL-8 and MIP-2 .They are produced by activated alveolar macrophages.

Based on the results of some *in vitro* and *in vivo* investigations, there have been identified in dogs several chemotactic factors involved in the mechanisms of leukocyte recruitment: IL-8, RANTES, MCP-1 (*Felsburg*, 1998). *Endo* et al (1998) have cloned cDNAs for chemokines in cats, identifying the genes for MIP-1 alpha and MIP-1 beta, as well as these molecules.

Studies regarding chemokines and their receptors, identified in various animal species, reveal that these molecules have similar structures and functions like those previously identified in humans and some laboratory animals.

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