

A review of immunomodulators with reference to Canova

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Immunomodulators are substances which modify the immunity of an individual to favour a particular immunological response. The immune response and the function of the immune response regulation process are described, with special reference to cancer and autoimmune disease. Homeopathy and its role in immune regulation are discussed with special reference to Canova. Canova is a homeopathic product produced, according to the Hahnemannian homeopathic method, in Brazil. Its role in cancer, bone marrow and haematopoiesis as well as macrophage and monocyte activation is reviewed. Canova seems to stabilize platelet morphology in human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS).

The data suggest that the future of immunomodulators and homeopathic products which appear to have an effect on the immune response requires a better understanding of the relative need for immune activation versus immune modulation. Homeopathic products specifically need more attention. Homeopathy (2009) 98, 169–176.

Introduction

Immunity is the reaction of cells and tissues to foreign substances or pathogens. The immune response requires the timely interplay of multiple cell types within specific microenvironments to maintain immune homeostasis.¹ Innate immunity is the rapid response to the first encounter to a pathogen. A consequence of an initial exposure is adaptive or acquired immunity.² The contributors to innate immunity are neutrophils, monocytes and macrophages. Lymphocytes (B and T) as well as cytokines are directly involved in generation of adaptive immunity.³ Advances in recent years in the understanding of the cellular and molecular basis of the immune response and the unfolding of the intricate system point to an important role for immunomodulators in maintaining a healthy state.⁴

Immunomodulators

As the understanding of basic immune mechanisms has increased, so has the ability to develop immunomodulators.⁵ Immunomodulators are agents directed to enhancing a particular immunological response.⁶ Immunomodulators

are currently available or under development for use in various immune disorders; strategies include Toll-like receptor (TLR) agonists, cytokines, cytokine receptors, transcription factor modulation, cell surface molecules, TH subset balance, signalling sequences and gene activation.^{5,7} Approaches to immunomodulation can be divided into those that are specific to pathogens (pathogen-specific) and those that are not (non-specific). Pathogen-specific immunomodulators include antibody reagents and vaccines. Non-specific immunomodulators include cytokines, antimicrobial peptides, certain antimicrobial drugs and microbes such as probiotics.⁸

The immune response

Microbes enter the body mainly through the skin (by contact), the gastrointestinal tract (by ingestion) and the respiratory tract (by inhalation). The epithelial tissue found in these areas contains antigen-presenting cells (APCs).⁹ Dendritic cells are specialized antigen-presenting cells that efficiently initiate immune responses. Other APCs include circulating monocytes, monocyte-derived cells and macrophages.¹⁰ Dendritic cells capture the antigens of microbes that enter the epithelium, by phagocytosis (for particulate antigens) and pinocytosis (for soluble antigens). Surrounding macrophages and endothelial cells produce cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), which are part of the innate immune system. TNF and IL-1 then act upon the dendritic cells, causing them to lose their adhesiveness and move (containing the

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antigen) with a chemo-attracting receptor via lymph nodes to T cell rich lymph nodes.⁹ Dendritic cells are particularly important in the presentation of antigens to, and activation of, T cells that have not previously encountered the antigens and have not previously been activated.¹¹ During migration dendritic cells mature and start to express major histocompatibility complex (MHC) molecules. Dendritic cells mature in response to microbes. MHCs display the antigen to T cells and other molecules called costimulators.

MHC consists of two sets of polymorphic genes: class I and class II molecules, these are membrane proteins, each contains a peptide-binding cleft at its amino-terminal end. Although the subunit composition of class I and class II molecules differs, the overall structure is similar. Both types contain peptide-binding clefts for display to T lymphocytes and invariant portions that bind CD8 (the $\alpha 3$ domain of class I) or CD4 (the $\beta 2$ domain of class II).⁹ Normally approximately 65% of T cells in the blood circulation are CD4+ and 35% are CD8+.¹² MHC molecules must assemble both their chains and bound peptide (derived from foreign e.g. microbial proteins) in order to achieve a stable structure.

Binding to the cell surface can occur by through receptors that recognize antibodies, microbes binding to surface receptors specific for microbial products, or products of complement activation attached to microbes (Figure 1). Internalization then occurs by uptake of the microbe by phagocytosis, endocytosis or pinocytosis. Following internalization, the antigen enters an endosome or phagosome which in turn fuses with a lysosome containing proteolytic enzymes. These enzymes digest the antigen into peptides. Even when the cell is not taking up antigens, APCs synthesize class II MHC molecules in the endoplasmic reticulum (ER). Each class II molecule includes a class II invariant chain peptide (CLIP) that binds in the peptide-binding cleft – making the class II molecule “inaccessible” to antigen peptides. As, the class II molecule moves towards the cell surface in a vesicle a class II-like protein called DM fuses with the vesicle containing the class II molecule and some of the peptides. DM is responsible for removing CLIP from the class II molecule. Once removed, the peptide can bind to the class II MHC molecule and move to the cell surface.⁹

Extracellular microbes are captured by APCs, including B lymphocytes, macrophages and dendritic cells and are presented by class II molecules on these APCs. CD4+ T lymphocytes have specificity for class II associated peptides and function as helper cells, stimulating B cells to produce antibodies (Th2 cells) and phagocytes (Th1 cells) to destroy microbes.¹²

Antigenic proteins found in the cytoplasm are from various sources: (i) proteins produced by intracellular viruses, (ii) phagocytosed microbes, (iii) products of mutated genes in tumors (Figure 2).^{9,10} These proteins are unfolded, tagged with a small peptide called ubiquitin and degraded by enzymes into small peptides. These peptides are now free in the cytoplasm; transporters associated with antigen processing (TAP) a specialized transport molecule found on the surface of the ER capture peptides and actively pump them into the ER. Here newly synthesized class I

MHC molecules bind the peptides and move them, in vesicles, to the cell surface.⁹

Cytosolic antigens are processed and displayed by class I MHC molecules, which are expressed on all nucleated cells. Class I associated peptides are recognized by CD8+ T lymphocytes which differentiate into cytotoxic T lymphocytes. These are able to kill infected cells with foreign antigenic determinants on their surface.¹² When microbes such as bacteria are captured by APCs, they produce a substance called lipopolysaccharide (LPS).¹³ In response to LPS, the APCs express surface proteins called costimulators, these are recognized by the receptors on T cells which then secrete cytokines. These cytokines bind to receptors on T cells the costimulators and cytokines have a cumulative effect with antigen recognition, stimulating proliferation and differentiation of T cells.⁹

Human MHC proteins are called human leukocyte antigens (HLA). There are three major class I molecules (HLA-A, HLA-B and HLA-C) and three major class II molecules (HLA-DP, HLA-DQ and HLA-DR) encoded by genes within the HLA complex on chromosome 6. HLA class I and II genes exist in a large number of allelic forms (i.e. they are polymorphic). Each of them preferentially binds different antigenic peptides and this is responsible for the variation in immune responsiveness to particular antigens between different individuals.¹²

TLRs are a family of pattern-recognition receptors (PRRs) that are activated by specific components of microbes and certain host molecules. They constitute the first line of defense against many pathogens and play a crucial role in the function of the innate immune system.¹⁴ TLRs play a key role in activating APCs and by blocking or stimulating TLRs a modification of the Th1/Th2 cytokine balance can affect various immunological states.⁷ Cells that express this type I transmembrane protein include dendritic cells, macrophages, natural killer (NK) cells, T and B lymphocytes and epithelial cells.¹⁵ Thirteen mammalian TLRs that can be activated by both exogenous pathogenic ligands and/or endogenous immune related ligands have been identified.¹⁶

Once an antigen is taken up by an APC TLRs become stimulated (Figure 3). TLRs form part of PRRs that are able to distinguish between self and non-self. Upon stimulation, an increase in MHC antigens as well as cytokine production will occur. Cytokines such as TNF- α , IL-1, IL-6, IL-8 and IL-12 may possibly increase. Due to this increase other APCs as well as T and B lymphocytes might become stimulated in order to activate a proper immune response.¹⁷

Drug development and autoimmunity

Immunomodulatory therapies involving single and/or multiple TLRs, their targets and signalling pathways may be of value in both infective and non-infective (autoimmune disorders) pathobiologies.¹⁶ Lipid A, the active component of LPS, is a TLR4 agonist that induces Th1 responses. Bacterial and viral genomes have immunostimulatory DNA sequences containing CpG (phosphorylated Cystein-Guanin) sequences that are suppressed and

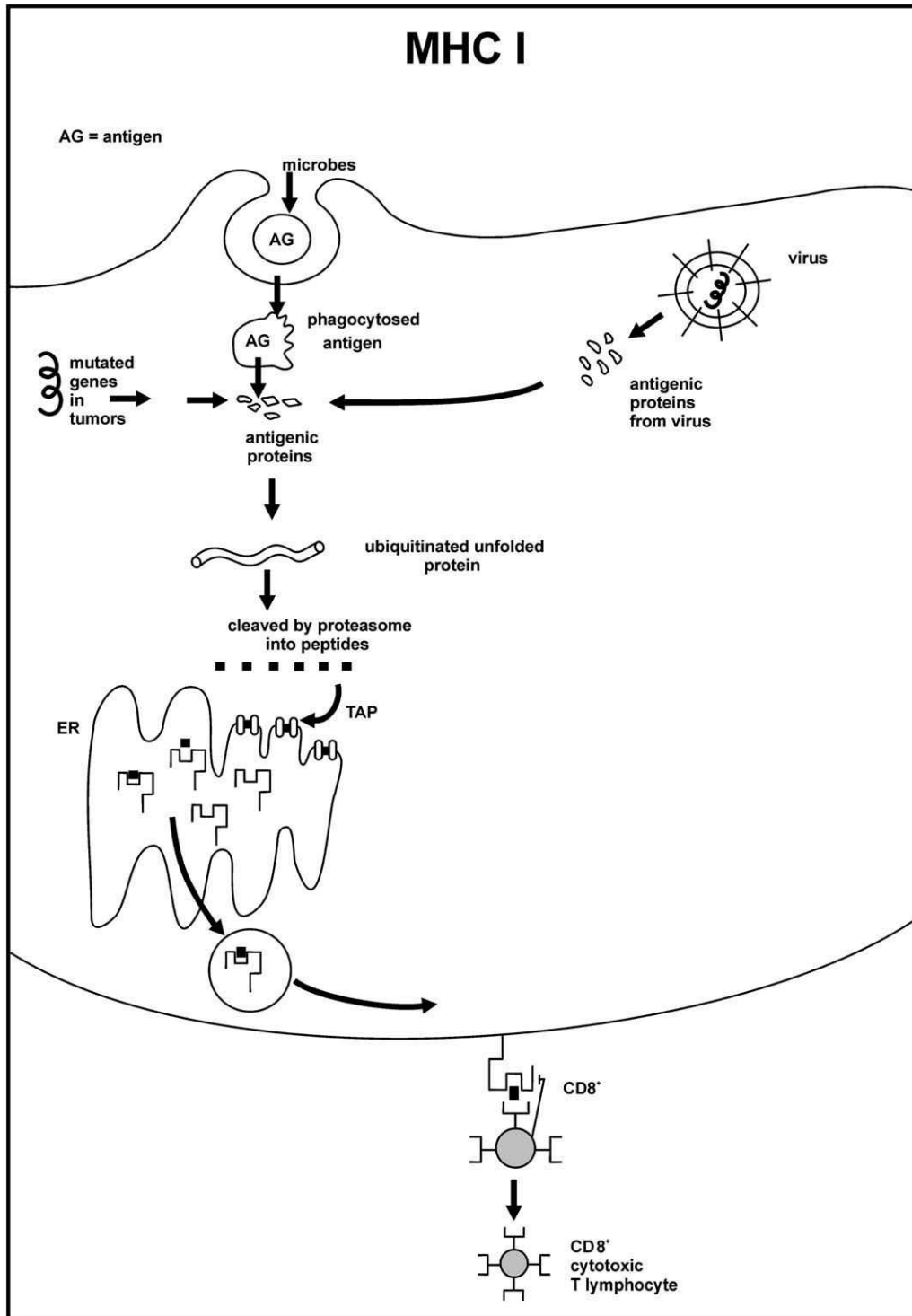


Figure 1 Schematic illustration of binding of microbe to cell surface by MHC class I surface receptors specific for microbial products or products of complement activation attached to the microbes.

methyated in vertebrate genomes. TLR9 is the receptor for CpG DNA, which in human subjects is expressed in highest concentration on B cells and plasmacytoid dendritic cells. TLR9 agonists activate both the innate and the adaptive immune system through these interactions. The application of TLR9 ligands is not limited to allergic disease. Human trials have demonstrated the effectiveness of CpG as either monotherapy or an adjunctive treatment in vaccines in a number of infectious diseases, such as hepatitis B,

hepatitis C, anthrax and influenza. CpG may also possibly be used for cancer treatment in combination with standard chemotherapy.⁷ Berinstein reported that CpG combined with a peptide vaccine containing the Melan-A 26-36 A2 analogue peptide with incomplete Freund's adjuvant resulted in rapid and strong T cell responses in melanoma patients.¹⁸ Imiquimod, a topical adjuvant, was the first TLR agonist approved for treatment of anogenital warts, actinic keratosis and superficial basal cell carcinoma in humans.¹⁹

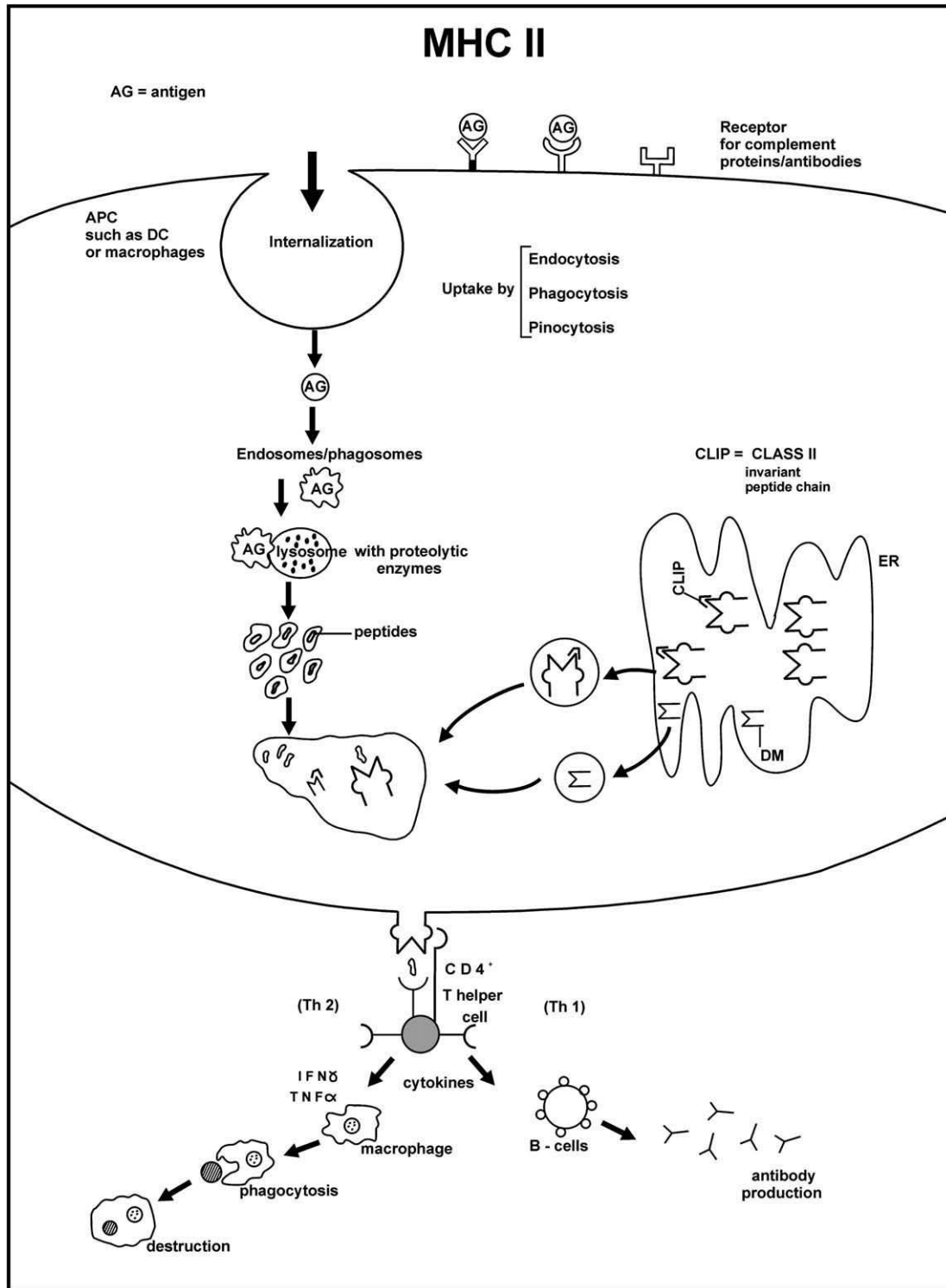


Figure 2 Schematic illustration of internalization process: uptake takes place via endocytosis, phagocytosis or pinocytosis following binding to MHC class II surface receptors.

Cytokines are a diverse family of polypeptide hormones and growth factors that regulate many cellular processes. Although they differ in detail, all cytokines are four-helix bundles. Cytokines are produced by a variety of immune cells in order to communicate and orchestrate an immune attack.²⁰ Any imbalance in the cytokine network may lead to initiation and perpetuation of auto-immune diseases, tumor growth and various other

diseases.²¹ Modulation of cytokine secretion may offer novel approaches in the treatment of a variety of diseases.²² Essentially, modulation or intervention can be implemented by administration of anti-inflammatory cytokines such as interferon (IFN)-b, growth factors such as transforming growth factor b, IL-4 and IL-10, or by neutralizing pro-inflammatory cytokines such as IL-2, IFN-g, IL-12, TNF-a and IL-1.²¹

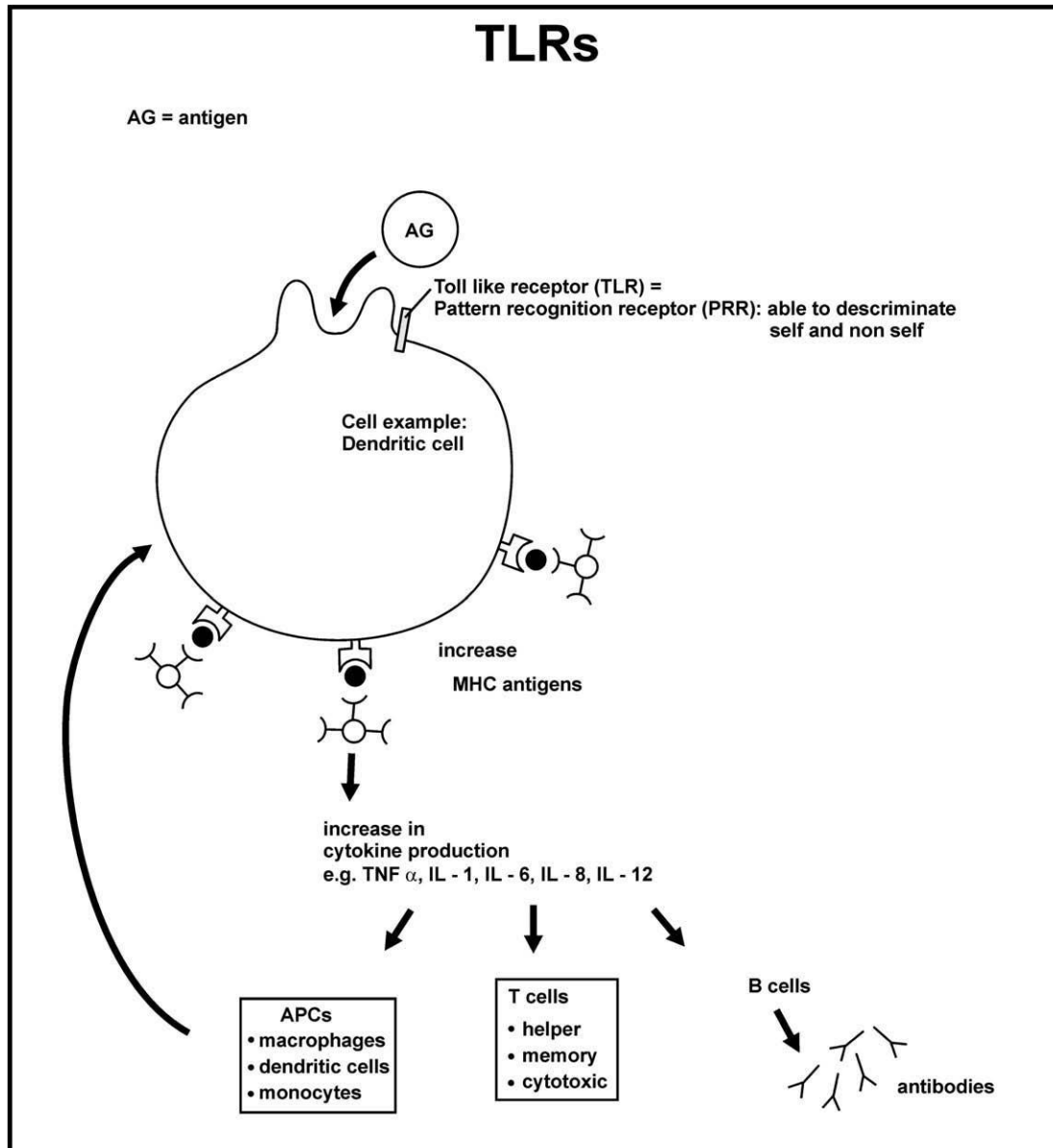


Figure 3 Schematic illustration of the process that takes place when an antigen is taken up by an APC such as a dendritic cell, where TLRs are stimulated.

Cytokines are critical mediators of the autoimmune process. They may be products of dendritic cells and/or induce the differentiation of immature dendritic cells into mature dendritic cells that in turn select autoreactive lymphocytes. Cytokines involved in the treatment of autoimmunity include:²³

IL-1: Inhibition of this highly pro-inflammatory cytokine is effective in rheumatoid arthritis in combination with methotrexate: the IL-1 receptor antagonist IL-1Ra (Anakinra) prevents the interaction of IL-1 to its cell surface receptors and thus acts as an IL-1 inhibitor.^{21,24,25}

IL-6: This displays pro- but also anti-inflammatory effects and plays a central role in hemopoiesis. IL-6 is secreted by B and T lymphocytes, monocytes, fibroblasts, keratinocytes, endothelial cells, mesenchymal cells and tumor cells. IL-6 induces the differentiation of B lymphocytes into plasma cells, proliferation of T lymphocytes,

differentiation of cytotoxic T cells and differentiation of macrophages and megakaryocytes. The humanized version is Tocilizimab (TCZ).^{23,24}

TNF-a: This cytokine was among the first whose dysregulation was proposed to contribute to the pathogenesis of various autoimmune disorders. TNF blockers are an effective treatment for rheumatoid arthritis, Crohn's disease and psoriasis. The blockers enhance the production of type I IFNs, by plasmatoid dendritic cells.^{21,23}

INF-a: Interferon alpha was the first exogenous cytokine to demonstrate antitumor activity in advanced melanoma. INF has immunoregulatory, antiproliferative, differentiation-inducing, apoptotic and anti-angiogenic properties in various malignancies. In 1995, INF-a2b became the first immunotherapy approved for adjuvant treatment of melanoma by the FDA.²⁶ IL-2 also has antitumor activity with or without lymphokine-activated killer cells.^{26,27}

The development of new agents that inhibit specific immunopathogenic mechanisms holds promise for benefit for patients at low risk. One such product is monoclonal antibody (mAb) therapy. Rituximab is a mAb directed against CD20, a surface marker on mature B cells. It was initially approved for B-cell lymphoma. It became apparent that depleting B cells with this antibody might be beneficial in autoantibody mediated diseases. A single intravenous administration of anti-CD4 leads to considerable decrease of CD4+ T cell counts, alleviation of asthma symptoms and increase in peak flow. Antibodies blocking the binding of CD28 to CD80/86 in murine models of allergic airways disease have been shown to diminish levels of Th2 cytokines and the degree of lung inflammation.²⁸

Homeopathy

One of the key concepts of Homeopathy is stimulation of the body's defence mechanisms and processes to prevent or treat illness. Most homeopathic medicines are from natural substances from plants, animals and minerals, these substances are diluted in a series of steps during preparation.²⁹ In homeopathic theory, patients are considered to have acquired a state that mimics the toxicity of a specific substance in nature. When they receive a dilution of a substance that matches their illness, healing occurs by the principle of Similars or 'like cures like'.³⁰ Most scientists and physicians reason that response to treatment with these dilutions (where there should be no single molecule or molecular remnant of the original substance present) would simply be due to a placebo effect. However it has been suggested that, in the process of succussion, there is an energetic 'imprinting' of the crystalline lattice of the water. It is interesting to reflect the 'potency' of single molecules of pheromones detected over vast distances by organisms the sensitivity of which defy ordinary measurement.³¹

Canova

Canova is a homeopathic product developed in Argentina and today is produced, according to the Hahnemannian homeopathic method, as a homeopathic remedy in Brazil.³² The final product is an aqueous, odorless and colorless solution that contains *Aconitum Napellus* (Ranunculaceae) + Associations, No toxicity or mutagenic effects have been reported. It is available as drops, and are used by researchers in concentrations range from 4% to 50%.³²⁻³⁵ 50 ml contains 49.965 ml water and 0.035 ml alcohol.

Canova is described as an immunomodulator and researchers⁶ have found that it stimulates host defenses in pathological states.³⁶⁻³⁹ It was investigated for its potential in cancer therapy by Sato et al. They studied the effect of Canova in normal and sarcoma 180-bearing mice. A comparison between the treated and the control groups revealed a delay in the development and reduction in size of the tumor. They also found an increase in the infiltration by

lymphoid cells, granulation tissue and fibrosis surrounding the tumor in the treated group. White blood cell count showed an increase in the leukocytes and lymphocytes count. These results reflect an enhanced immune response of the host.⁴⁰

Piemonte and Buchi analyzed the production of IL-2, IFN-g and TNF-a and ultrastructure of peritoneal macrophages treated with Canova. 86% of the macrophages treated with Canova were activated compared to 15% in the control group. Activation was determined by enlarged nucleus, more euchromatin than heterochromatin, spreading and projections. ELISA revealed no difference in IL-2 and IFN-g between the treated and control group, but TNF-a production was decreased compared to the control group.⁶ Lopes et al. investigated the cellular aspects of macrophage activation by Canova, studying the activation, morphological parameters and acid phosphatase activity of mice peritoneal macrophages exposed to Canova *in vitro* and *in vivo*. The results revealed a greater spreading ability in the treated macrophages, analysis of the acid phosphatase activity showed that Canova treatment stimulates an increase of the endosomal/lysosomal system. The authors concluded that it is an effective stimulator of macrophage activity.³³

These findings correlate with those of Pereira et al.³⁴ who investigated the immunomodulatory effect of Canova on experimental *Leishmania amazonensis* infection. The *in vitro* results showed that with 40% Canova the infection index was reduced and NO production was induced in the elicited macrophages. The *in vivo* tests also revealed reduced infection as well as decreased parasite load in the regional lymph nodes and spleen. The authors suggested that Canova modulates experimental infection, controls infection progression and limits dissemination.³⁴ In another study, the same authors investigated the proliferation of lymphocytes when stimulated by activated human macrophages treated with Canova and found that it indirectly induces lymphocyte proliferation.⁴¹

Abud and co-workers investigated the activation of bone marrow cells treated with Canova *in vitro*.⁴² As previous studies found that Canova induces up-regulation in a number of leukocytes and increase the number of CD4T lymphocytes as well as NK cells in peripheral blood,⁴⁰ the authors aimed to determine whether it can promote the differentiation, proliferation and survival of mouse bone marrow cells. This study showed an increase in the number of adherent, larger and activated cells in the Canova-treated group. Most of the adherent cells were macrophages (CD11b⁺). The authors concluded that it may have a role in diseases that affect bone marrow cells.⁴²

Canova, has been shown to activate macrophages *in vitro* and *in vivo*, with resultant enhanced spreading of the cells and formation of microvillus extensions. Since monocytes are the precursors of macrophages and dendritic cells, a study investigated its effects on the differentiation of human blood monocytes *in vitro*. Monocytes were isolated, grown in culture and exposed to 10% and 20% Canova without the addition of cytokines. After 48 h, monocytes were prepared for analysis, while cells kept in culture for 7 days and exposed to Canova on days 1, 3 and 4 were analyzed by flow

cytometry for alterations in the levels of expression of various markers. Scanning Electron Microscope (SEM) revealed monocytes exposed to 10% Canova had morphology similar to that of macrophages, cytoplasmic projections (pseudopodia) were observed. Flow cytometric analysis indicated high cell viability and up-regulation of CD80, compatible with differentiation into macrophages or dendritic cells. The authors concluded that exposure to Canova causes activation of monocytes with differentiation into large macrophage-like cells of indeterminate phenotype which have increased expression of CD80. Like cytokines, Canova induces differentiation of monocytes, this may underpin its immunomodulatory activity.⁴³

Pretorius et al. investigated the ultrastructural changes in platelet aggregates of human immunodeficiency virus (HIV) patients. The ultrastructure of platelet aggregates of patients with HIV shows membrane blebbing and ruptured platelets, indicative of apoptosis. HIV patients may develop thrombocytopenia as a result of peripheral platelet destruction. The differences in platelet morphology from control, Canova-treated HIV patients and non-treated HIV patients were investigated using SEM. Slight morphological changes compared to control in fibrin networks with Canova-treated HIV and untreated HIV patients were observed. Membrane blebbing was less pronounced in the Canova-treated patients compared to control. The authors suggest that Canova protects the membranes of platelets, concluding that these results support previous research where it was found that Canova protects the immune system, by protecting ultrastructure.⁴⁴

Aleixo et al.⁴⁵ evaluated the random amplification of polymorphic DNA (RAPD) profile of *Trypanosoma cruzi* II under the influence of Canova and Benzimidazole in mice. The authors found changes in the RAPD profile of the Y strain of *T. cruzi* and revealed that it may be

due to the Benzimidazole, the association between Benzimidazole and Canova and the course of the infection itself. The results showed no direct effect of Canova, but suggested that Canova interacts with Benzimidazole and concluded that the observations were compatible with immune selection or suppression of different sub-populations of *T. cruzi*.⁴⁵

Conclusion

Canova is a complex homeopathic medicine used as an immunomodulator. It appears to activate macrophages and to have effects on TNF- α which play an important role in inflammatory processes. Canova has also been shown to enhance the immune response in cancer-inoculated mice. It is difficult to place Canova in the traditional classification of immunomodulators, but it seems to activate macrophages, stimulate differentiation of monocytes and modulate other inflammatory processes. More research is needed to expand the possible uses and applications of this homeopathic immunomodulator.

The future development of immunomodulators in general requires a better understanding of the relative need for immune activation versus immune modulation in the context

of the immune response of one specific individual. Some diseases may cause an insufficient response, and others an overly exuberant response, different types of interventions may be required depending on the immune status of the individual.⁸ Immunomodulators should be investigated not only as a group of pharmaceuticals, but as individual response pharmacological agents. Homeopathic products merit particular attention.

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