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Review article ■

Selective Immunotherapy by Engineered Chimeric Molecules

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SUMMARY

In many physiological processes, peptides play a critical role as neurotransmitters, hormones, antibiotics, etc. They have research importance in fields such as immunology, pharmacology, neuroscience and cell biology. There are many approaches for immunotherapies: some of them use the peptides as important components of chimeric molecules for immunosuppression, the others - as peptide-based vaccines for immunostimulation. These immunotherapeutic strategies offer the advantages of being safe, easy to produce, devoid of oncogenic potential, and can be chemically or genetically engineered into defined conformational active form. The peptides contain very important functional part called epitope, which is recognized by the immune system, specifically by antibodies, B or T cell receptors. Epitopes play a prominent role in the peptide-based vaccines and disease diagnosis. Protein-engineered or genetically engineered peptides conjugated to antibody-carrier could be used as a targeting device delivering the epitopes to the cells of interest.

Key words: **immuno-peptides, SLE, Inhibitory B cell receptors, chimeric molecules, complement receptors, DNA vaccines**

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PEPTIDES AND AUTOIMMUNITY

The antigens associated with autoimmunity may often include multiple epitopes that can be temporally, quantitatively and qualitatively involved in different ways and/or in diverse phases of the disease pathogenesis. Several artificial peptides have been tested as tolerogenic modulators of pathogenic autoimmunity (1). At the functional level, the binding of an artificial peptide to the MHC has to mimic the natural self antigen, and it has to lack the inflammatory response elicited by the native peptide. Although the primary structure of the artificial peptide must structurally resemble that of the self peptide that drives and/or sustains the autoimmune response, the conformational change of the artificial peptide makes the ligand/receptor binding that would lead to activation of APC or T cells either unproductive or partly ineffective. As a consequence, the artificial peptide will inhibit immune cell activation (2). Strategies based on development of therapeutic approaches targeting autoreactive B and T cells to prevent or suppress autoimmunity have been successfully used in experimental models. However, these approaches fail to discriminate between B and T cells specific for self- and foreign antigens. Peptide-based immunotherapies offer an approach to selectively target autoreactive cells, leaving the remainder of the immune system intact (3). Approaches of peptide immunotherapy that induce IL-4- or IL-10-secreting adaptive immunoregulatory CD4+T (aTreg) cells have proven to be effective for autoimmune diseases in which multiple autoantigens are regulated by T cells (3-6).

Various tissue - specific autoimmune diseases such as type 1 diabetes (T1D) are mediated by pathogenic T cells (7-9). Studies in NOD mouse, a spontaneous model of T1D, demonstrate that administration of β cell peptides induces aTreg cells, and suppresses differentiation of type 1 T effector cells that mediate destruction of the insulin-producing β cells (5, 10, 11). There are engineered peptide-soluble MHC class II-Ig (peptide-sMHCII-Ig) fusion proteins which consist of the extracellular domains of the MHCII α- and β-chains supported by an Ig scaffold (12-16). The peptide is tethered to the sMHCII β-chain ensuring that each bivalent fusion molecule presents a peptide, which binds T cells directly independent of APC. Administration of peptide-sMHCII recombinants has been shown to tolerize pathogenic T cells in mono-specific models of autoimmunity (17), including collagen induced arthritis (15), experimental autoimmune uveitis (18, 19), and experimental autoimmune encephalomyelitis (20).

More than thirty years ago, a synthetic analogue of myelin basic protein was designed (MBP), the glatiramer acetate (GA). It is a standardized mixture of polypeptides (range: 4.7-11 kDa) that are randomly polymerized from the L-amino acids glutamate, lysine, alanine and tyrosine in a molar ratio of 0.14:0.34:0.43:0.09 - which is the same ratio found in the amino acid sequence of MBP (21). The original idea was to create a synthetic molecular mimic of MBP that could induce experimental autoimmune encephalomyelitis (EAE) - a mouse model of human MS, because at that time MBP was not available and its primary structure was not known. GA, which was designed as a MBP analogue on the basis of the MBP amino acid composition, surprisingly prevented EAE, instead of inducing it (22). The confirmation of these studies eventually defined the beneficial effects of GA not only in EAE but also in human MS (23), where the clinical efficacy of GA was tested in a randomized clinical trial (24). GA also induces a shift in the phenotype of Th1 to Th2 cells (25) through the inhibition of the production of proinflammatory cytokines such as IL-12p70 from dendritic cells (26). Finally, GA has neuroprotective effects that involve the stimulation of neurotrophin secretion, which promotes axonal protection and myelin repair in the central nervous system (27).

To inhibit multiple T cell autoreactivities in EAE, artificial multiantigen/multiepitope molecules have been constructed to encode simultaneously several disease-relevant epitopes of MBP, proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (28, 29). When systemically administered in a tolerogenic fashion, a synthetic human multitarget autoantigen protein (hmTAP) efficiently inhibited EAE induced by immunization to PLP or by the transfer of encephalitogenic T cells reactive to MBP, PLP and MOG (27).

CHIMERIC ANTIBODY-PEPTIDE MOLECULES IN AUTOIMMUNITY

Systemic Lupus Erythematosus is a complex autoimmune disease characterized by the generation of IgG auto-antibodies specific to nuclear antigens - double-stranded (ds) DNA (30), histones (31), nucleosomes (32), etc. The primary auto-antigen that drives the autoimmune response in SLE is not known.

Several autoantibodies to DNA - the hallmark antibodies in SLE, contain amino acid sequences in their VH regions that induce *in vitro* T cell proliferation and cytokine secretion (33). The identification of T cell epitopes in many murine anti-DNA Ab indicated that immunogenic sequences had similar location and amino acid content (34-36). The recognition of peptide-mimics for dsDNA that are capable of inhibiting the binding of dsDNA with antibodies to dsDNA is a potential drug discovery route for SLE.

Autoreactive B lymphocytes play a major role in many autoimmune diseases as precursors of pathological autoantibody-producing plasma cells and as antigen-presenting cells that help break T cell tolerance to self molecules (reviewed in (37). The triggering of their immunoglobulin B cell (BCR) receptors by the specific antigen ultimately induces gene expression patterns that can promote cell activation, anergy or apoptosis. Their fate depends upon the availability of T cell help and

upon the balance of additional signals from their stimulatory and inhibitory surface co-receptors (38-40). Individual cell surface receptors may possess both activating and inhibitory signaling motifs and depending on ligand specificity and affinity, they may trigger inhibitory and/or activating signaling pathways to calibrate cell responses (41). Inhibitory co - receptors such as the low affinity receptor for IgG - FcγRIIb, CD22, CD72, etc., negatively regulate BCR signaling and thus prevent unphysiological overactivation of the B lymphocytes (42). These surface receptors on disease-associated B-lymphocytes are potential target molecules for therapeutic intervention. Deficiency of the inhibitory FcγR (FcγRIIb) on B cells results in imbalanced immune responses and in the development of autoimmune pathology (43, 44). The loss of CD22 function has been shown to be associated with autoimmunity (45) and it is regarded as a promising target for therapeutic antibodies (46).

The appearance of pathological DNA-specific B cells in lupus is regarded as a major event in the development of the disease because of the specificity of the antibodies they produce. Other investigators have recently claimed, however, that their main role in the lupus pathogenesis is as antigen-presenting cells that help breaking T cell tolerance to self DNA/protein complexes (37, 47). Regardless of the precise role that anti-DNA specific B lymphocytes play in the induction of the disease, their selective elimination or suppression is a legitimate goal in the efforts to control SLE. Immunoglobulin genes encoding dsDNA-specific IgG antibodies in lupus accumulate mutations in the same way as do VH and VL genes coding for antibodies against foreign antigens (48, 49). Both types of B lymphocytes are antigen driven, and we reason that if this is the case, autoreactive B cells should be susceptible to the same mechanisms that control the magnitude and duration of the IgG antibody response to foreign antigens (50). One of these mechanisms is the cross-linking of the surface immunoglobulins with the inhibitory FcγIIb receptors by IgG-containing immune complexes (for review see (42, 51-53). FcγIIb is the only Fcγ receptor expressed by B lymphocytes and mice deficient for it reacts with enhanced antibody production to T-dependent and independent antigens (54) and develop fatal glomerulonephritis (55). Memory B cells from SLE patients have decreased expression of FcγIIb receptors (56).

We constructed several chimeric molecules by coupling different self - mimicking peptides to a rat anti-mouse FcγRIIb-binding monoclonal antibody or Irrelevant mouse IgG as a peptide - carrier. The synthesis of peptides was carried out using a Fmoc-based manual solid phase peptide synthesis protocols on 2-Cl-Trt resin. The peptides were purified (≥95 % purity) by reversed - phase sample displacement chromatography (57) on a series of 3 x 50 mm Nucleodur 100-5 C_{18} columns (Macherey-Nagel, Germany).

The coupling of the antibodies to the peptides was performed using the classical EDC (1-ethyl-3(3'-dimethylaminopropyl) carbodiimide.HCl), (Fluka AG, Buchs, Switzerland) cross-linking technique (58). During the synthesis of the peptides an Ahx linker with lysine or a spacer ($H_2N(CH_2)_6H_2N$) were added to their C-end. The antibody was mixed with a 20-fold molar excess of the peptide and 60-fold molar excess of carbodiimide. The reaction mixture was stirred overnight at 4 ^{0}C, dialized against PBS and concentrated by ultrafiltration.

DNA-LIKE PEPTIDE CHIMERA

Engineered chimeric molecules for targeting of antigens to inhibitory human B cell receptors represent a tool for directed modulation of immune responses. Diamond et al., have previously screened a phage peptide display library with the murine IgG2b Ab R4A to identify a peptide mimotope for autoantigen. R4A binds to dsDNA and fibronectin and deposits in glomeruli of nonautoimmune mice. The 5-mer peptide DWEYS inhibited binding of R4A to dsDNA as well as binding of R4A to renal tissue (59). They have recently demonstrated that nonautoimmune BALB/c mice develop lupus-like autoimmunity when immunized with the peptide DWEYSVWLSN (60) (containing the DWEYS sequence) attached to a polylysine backbone. Peptide-immunized mice develop anti-dsDNA Abs as well as other autoantibodies characteristic of lupus, including anti-histone, anticardiolipin, and anti-Sm/ribonucleoprotein (RNP) Abs. At the 3rd month of age, immunohistochemical studies demonstrate the presence of IgM and IgG deposits in renal glomeruli of immunized mice (61).

We have constructed a chimeric antibody by coupling the DWEYSVWLSN dsDNA-mimicking peptide (62, 63) to a rat anti-mouse FcγRIIb - binding monoclonal antibody (Figure 1).

In this study, we have proven that by administration of the artificial antibody to MRL/lpr mice with lupus it is possible to suppress selectively the activity of disease-associated B lymphocytes and to change the natural course of a spontaneous autoimmune disease. We suppressed the differentiation of autoreactive B lymphocytes by engineered antibody chimera that binds to the inhibitory FcγRIIb, as well as to the immunoglobulin receptors on targeted dsDNA-specific B cells. The intravenous administration of the chimera prevents the development of the symptoms of lupus, the appearance of IgG anti-dsDNA antibodies and proteinuria in young lupus-prone MRL/lpr mice. When the treatment was started after its onset it prevents the progression of the disease and prolonged the overall survival (64).

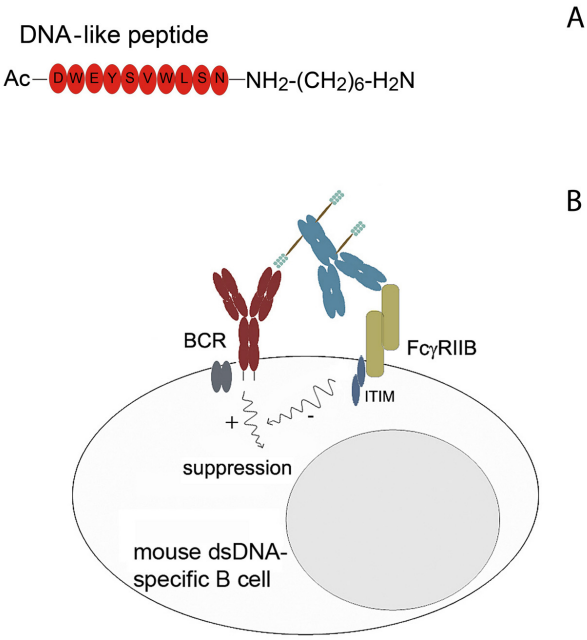

Figure 1.A. *Scheme of the DNA mimicking peptide*
B. *Receptor cross-linking by DNA-chimeric molecule*

HISTON 1-PEPTIDE CHIMERA

Nucleosomes from apoptotic cells have been shown to contain the self-antigens that induce disease-associated antibodies in (SWR x NZB)F1 (SNF1) lupus-prone mice (65). Auto-antibodies to nucleosome particles are considered to be the earliest nephritogenic antibody population in the SNF1 mouse lupus model, appearing even before the IgG anti-dsDNA auto-antibodies (66). Pure DNA is not immunogenic, but may be rendered so when coupled to proteins such as histones (66-68). Kaliyaperumal et al. have eluted a number of histone peptides from MHC class II molecules of an antigen-presenting cell line fed with crude chromatin from lupus-prone SNF1 mice (66). One of these peptides (H1`22-42) is a part of the Histone 1 molecule (STDHPKYSDMI VAAIQAEKNR). H1`22-42 has been shown to be a potent stimulator of autoimmune Th and accelerated lupus nephritis when administrated to SNF1 mice. Antibodies, specific to the H1`22-42 peptide, at later stages of the disease bound also to other nuclear antigens. Considering these results, we have constructed another type of chimeric molecule by coupling copies of the natural H1`22-42 peptide to a rat anti-mouse FcγRIIb-binding mAb (Figure 2).

The administration of these chimeric molecules to MRL/lpr mice with initial and with full-blown disease resulted in the reduction of the levels of IgG anti- Histone 1 antibodies, of the albuminuria levels, of the size of lymphoid organs and in prevention of the development of skin lesions. The observed effect was limited to lupus-associated B cells only, as the treatment did not decrease the IgG antibody response to an administered foreign antigen (69).

The beneficial effects seen after treatment with the described above chimeric molecules lasted until the treated mice started producing antibodies to the rat immunoglobulin that was a part of the chimeras. If antibody chimeras targeting the inhibitory FcγIIb receptor on selected autoreactive B cells in autoimmune patients are to be used in the future, they should be constructed using humanized antibodies or Fv antibody fragments binding only to the human FcγIIb receptor isoform (56, 70, 71).

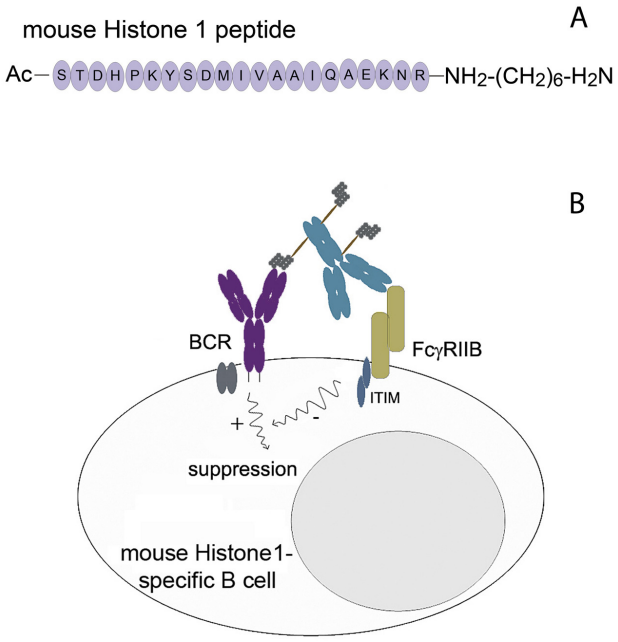

Figure 2. A. *Scheme of the H1`22–42 peptide*
B. *Receptor cross-linking by Histone 1-chimeric molecule*

DNA-LIKE - STN PEPTIDE CHIMERA

The exposure of disease-associated B-lymphocytes from lupus mice to bi-specific chimeric antibodies, cross - linking their dsDNA or Histone - 1 specific BCRs with the FcγRIIB resulted in the selective silencing of these pathological cells and in improved survival of the treated lupus-prone animals (64, 69). Next, we hypothesized that this beneficial effect of the antibody chimera would be even stronger if it targets simultaneously both inhibitory B-cell receptors - CD22 plus FcγRIIB. A chimeric antibody was constructed by coupling copies of a DNA mimotope peptide and of the CD 22-binding STN-peptide (STN-GGPGG) to a mouse monoclonal IgG antibody backbone (Figure 3).

The STN epitope has a free terminal sialic acid that is bound by CD22 (72, 73). We have demonstrated that the artificial tri-specific antibody induces signalling through both FcγRIIB and CD22, suppresses selectively the activity of targeted DNA- specific B-lymphocytes *in vitro* as well as *in vivo* and delays the natural aggravation of a spontaneous autoimmune disease.

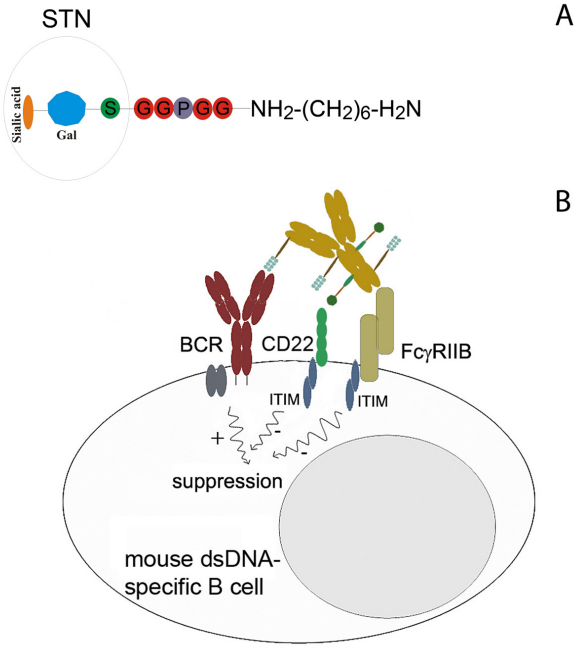

Figure 3. A. *Scheme of the STN epitope.* ***B.*** *Receptor cross-linking by tri-specific chimeric molecule*

ALLERGEN – PEPTIDE CHIMERIC MOLECULE

Many studies have been devoted to understanding the mechanisms of human allergic reaction, which is mainly characterized by an enhanced IgE production and a local inflammatory reaction. IgE responses depend on several factors including the nature of the antigen, the exposure conditions (dose of allergens, timing and route of allergen introduction), as well as the genetic background and the level of Th2/Th1 cytokine production. IgE antibody production is triggered as a result of the specific recognition of allergens by B-cells receptors (BCR), followed by internalization, processing and presentation (74, 75). Several attempts for immune response modulation and inhibition of IgE production have been made using modified allergen epito-

pes (76, 77). A chimeric molecule composed of the constant region of a human IgG1 antibody coupled to the cat Fel d1 allergen induced a dose-dependent inhibition of Fel d1-driven IgE - mediated histamine release from cat-allergic donors' basophiles. The cross-linking of the FceR1 and the inhibitory FcγRIIb receptor was shown to be responsible for the observed result. This chimera would obviously also aggregate Fel d1-binding BCRs with the inhibitory receptors on allergen-specific B-lymphocytes. Its effect on the activity of the latter, however, was not studied (78).

House dust mites Dermatophagoides pteronyssinus (Dpt) are the most important indoor allergens (79-81). Der p 1, a 222 - amino acid glycoprotein, is one of the major allergenic molecules of Dpt. Different approaches have been used for mapping B- and T- cell epitopes of Der p 1. Using conventional predictive methods and a tridimensional model of Der p 1, four peptides were selected as potential B cell epitopes and were shown to exhibit IgE-dependent biological activities (82, 83). One of them, peptide p52-71 (NQSLDL AEQELVDCASQHGC), is used as a model epitope in the engineered chimeric molecule.

The pathological Der p 1 - specific B cells in allergic patients play a key role in the development of the disease as producers of allergen specific sensitizing antibodies. Therefore, the selective elimination or suppression anti- Der p 1 B-lymphocytes is a reasonable therapeutic approach. It has been shown that the complement receptor type 1 (CR1, CD35) mediates inhibitory signals to human B lymphocytes (84). We have constructed a chimeric protein molecule, containing a monoclonal antibody specific to human inhibitory receptor CR1, coupled to p52-71 peptide from Dermatophagoides pteronyssinus (Figure 4).

After the coupling of the Allergen - mimicking peptide to the anti-CD35 antibody, the former retains its ability to be bound by anti - allergen antibodies. The effect of the Allergy chimera was limited to disease-associated B cells only. The incubation of PBMCs from allergy patients with the chimeric molecule induced the apoptosis of CD19+ PBMCs.

INFLUENZA - PEPTIDE DNA CHIMERA

The use of live and attenuated strains of microbes and viruses for vaccination comprises the risk of evoking B and (or) T cell epitopes possessing undesirable characteristics. This necessitates the application of epitope-specific vaccines.

As we have previously shown targeting of an epitope to the available epitope-specific B cells may have a dramatic positive or negative effect on their response. This targeting can be achieved by a hybrid molecule containing both B and/or T-cell epitopes, and a whole molecule or scFv fragment from an antibody specific to

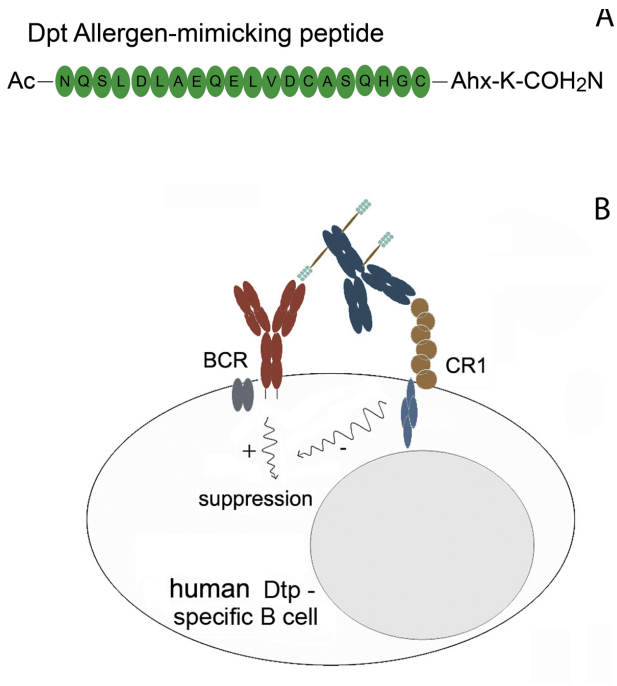

Figure 4. A. *Scheme of the allergen-mimicking peptide*. ***B.*** *Receptor cross-linking by allergen-chimeric molecule*

a an activating or inhibitory cell surface receptor. Such chimeras can be produced either by genetic or by protein engineering.

Killed viral vaccines are known to induce primarily antibody responses. By contrast DNA vaccination using naked DNA encoding viral antigens induces both humoral and cellular immune responses. Various approaches have been used to construct DNA vaccines with build-in adjuvanticity. Using gene-engineered chimeric molecules for targeting of antigens to the activating receptors gives tools for modulation of the immune response to the desired course.

Single-chain variable fragment (scFv) antibodies are genetically engineered molecules containing the variable regions of the antibody's heavy and light chains. These monovalent antigen-binding molecules retain the antigen recognition properties of the intact immunoglobulin. When a chimeric molecule is created by genetic coupling of a scFv and a defined peptide epitope, the latter could be directed to the targeted cells. Such a gene-engineered molecule could be used as a naked DNA vaccine.

We hypothesized that sequences encoding a common epitope of influenza A virus hemagglutinin jointed to sequences encoding a scFv antibody fragment to a costimulatory B cell surface receptor would result in the *in vivo* expression of a chimeric viral peptide with increased immunogenicity.

Mouse Complement receptors type I and II (CR1 and CR2) are expressed on B-lymphocytes and on follicular dendritic cells. The crosslinking of the antigen- and complement receptors reduces the threshold for activa-

tion of antigen-specific B cells up to 1000 times (85). Targeting influenza virus antigens to murine CR2-expressing cells by using single chain antibody fragments resulted in an enhanced antigen presentation by B-cells (86).

We hypothesized that it is possible to generate protective levels of anti-influenza antibodies in mice by administering to them a chimeric DNA molecule, encoding a single-chain variable fragment (scFv) from a monoclonal antibody against the activating receptor CR1/2, coupled to immunogenic peptide (MVTGLRNIP SIQSRGLFGAIAGFIEGGGSGGGV) (Figure 5) from Influenza virus hemagglutinin (86, 87).

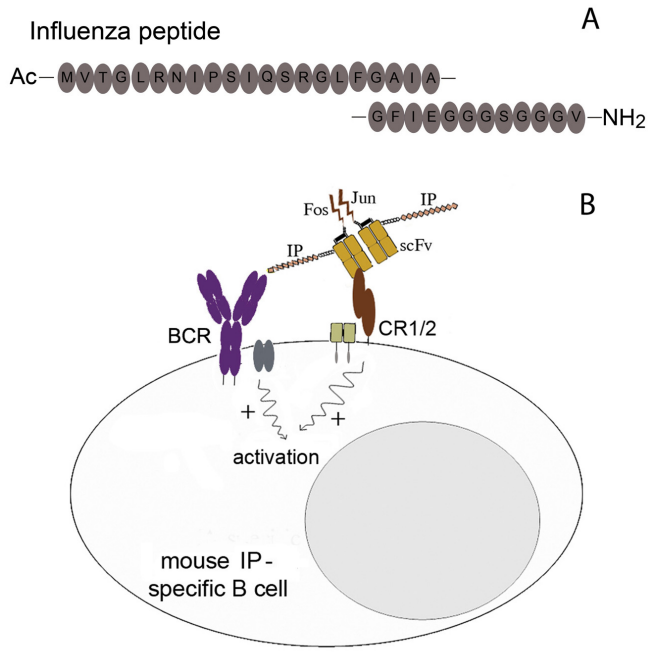

Figure 5. A. *Scheme of the Influenza peptide*. ***B.*** *Receptor cross-linking by expressed chimeric molecule*

The peptide contains an I-E^d restricted T- cell epitope (88) and a conformation-dependent B cell epitope (89). Such a hybrid DNA molecule was constructed by us, and a single or double vaccination with a plasmid containing the described construct was performed. The immunized mice produced high levels of IgM and IgG anti-IP and anti-whole virus antibodies. We examined the cytotoxic (CTL) activity of freshly isolated spleen cells from all animals against influenza virus-infected 3T3 cells six months after the first or second immunizations. Treatment of mice with a chimeric DNA molecule had a very strong cytotoxic effect. Gene vaccination appears to be very effective at inducing long-lived memory responses.

Artificial peptides can effectively downregulate or stimulate immune responses in mice and in humans. Peptide analogues of disease-related T or B cell epitopes hold highest promise for possible therapeutic applications. Protein- and gene - engineered chimeric molecules for targeting of antigens to the inhibitory and activating receptors are a tool for directed immune response modulation. In both cases antibodies (or their fragments) were used to transport antigens or epitopes to the cells of interest when targeted to their natural ligands.

The above findings demonstrate that immune response may be effectively influenced through the use of peptides complementary to the major antigenic regions of disease-specific target proteins.

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SELEKTIVNA IMUNOTERAPIJA POMOĆU HIMERA DOBIJENIH MOLEKULARNIM INŽENJERINGOM

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Sažetak

U mnogim fiziološkim procesima peptidi imaju ključnu ulogu kao neurotransmiteri, hormoni, antibiotici itd. Imaju istraživački značaj na poljima poput imunologije, farmakologije, neronauke i biologije ćelije. Postoje mnogi pristupi imunoterapijama: neki od njih koriste peptide kao važne komponente himeričnih molekula za imunosupresiju, drugi za vakcine bazirane na peptidnoj imunostimulaciji. Ove imunoterapijske strategije su u prednosti jer su bezbedne, jednostavne za primenu, lišene onkogenog potencijala i mogu biti formirane u konformaciono aktivne forme pomoću hemijskog ili genetskog inženjeringa. Ovi peptidi sadrže veoma važan funkcionalni deo, epitop, koga imuni sistem prepoznaje, naročito antitela, B i T ćelijski receptori. Epitopi igraju važnu ulogu kod vakcina baziranim na peptidnoj imunostimulaciji i mogu se koristiti u dijagnostičke svrhe. Peptidi dobijeni proteinskim ili genetskim inženjeringom vezani za antitela-nosače mogu poslužiti za predavanje epitopa ciljanim ćelijama.

Ključne reči: **imunopeptidi, inhibitorni receptori B ćelija, himerični molekuli, komplementni receptori, DNK vakcine**