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New strategies for antigen delivery and modulation of specific immune response

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Abbreviations:

APL	acute promyelocytic leukemia
APC	antigen presenting cells
BMDC	bone marrow-derived dendritic cells
BSA	bovine serum albumin
CFA	complete Freund´s adjuvant
CFSE	carboxyfluorescein diacetate succinimidyl ester
CTB	cholera toxin B subunite
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EGFP	enhanced green fluorescent protein
EGFP-VLP	pseudocapsids composed of VP1 major capsid protein
EGFP-t-VP3	fusion protein
ELISA	enzyme-linked immunosorbent assay
GM-CSF	granulocyte-macrophage colony-stimulating factor
HPV	human papiloma virus
IEL	intraepithelial lymphocytes
IFN-γ	interferon gama
IL	interleukin
mAb	monoclonal antibody
MLC	mixed lymphocyte culture
MPyV	mouse polyomavirus
PI	proliferation index
PML-RAR	the human promyelocytic leukemia–retinoic acid receptor
t-VP3	truncated C-terminus of VP3 minor capsid protein
Treg	regulatory T cells
VLP	virus-like particles
TAA	tumor-associated antigen
TSA	tumor-specific antigen
TLR	Toll-like receptor
Th	helper T cell
VP1-VLP	pseudocapsids composed of VP1 major capsid protein of MPyV
X-SCID	X linked severe combined immunodeficiency

1. Introduction:

Regulation of immune response still remains serious challenge for the present immunology. The immune system is composed of many independent cell types that collectively protect a body against bacterial, parasitic, fungal and viral infections and against the growth of tumor cells and also keep nonresponsiveness to "self". The cells of the immune system can engulf bacteria, kill parasites or tumor cells, or kill virus-infected cells. The reactivity of these cells often depends on the activation signals from helper T cells (Th). Number of costimulatory molecules and cytokines is involved in the process of an immune response induction. Regulation of the immune response is a key factor keeping homeostasis of the organism. Understanding the mechanisms of regulation of the immune system is crucial for comprehension how to modulate immune response. To realize the root of immune deficiencies and to recognize potential directions of possible modulation of immune response, in the case of specific diseases, are first steps to find out or improove possible therapy. Attenuating or increasing the strength of immune response is the main goal of regulation of the immune system.

Different approaches can be employed to regulate the immune system. Various vaccination protocols were improved during last years not only to induce antibody responses which save organism mainly from infections but protocols were also developed to induce cytotoxic T cell response, which is important especially in defence against virus-infected cells or against tumor. DNA vaccination or use of viral pseudocapsids seems as a promising approach in this field. On the other hand it is necessary to develop effective strategies, saving the organism from uncontrolled self-destructive immune response. Such strategies are fundamental for protecting the body from autoimmunity or allergy. Mucosal delivery of antigens was intensively studied in this context.

Recently rediscovered regulatory T cells (Treg) appear as potentially important target cells influencing the regulation of immune response. Evidence cumulates for importance of Treg in regulation of immune response and also indicates Treg as potential therapeutic target in many disorders, as it has been shown especially in chronic infections and in tumor immunology. Treg are also studied in the context of various immunization protocols, because it has been discovered that induction of Treg after immunization can be an obstacle for acquiring immune response after vaccination. Intensive research of Treg is focused on preparation or induction

of Treg, which can suppress immune response in treatment of autoimmunity or rejection of graft. On the other hand, the role of Treg in pathology has been noticed. Such Treg for example infiltrate solid tumors and block essential immune response against tumor antigens, so the possible targeted elimination of Treg could be an important tool.

1.1. Antigen delivery, ticket to get response

This introduction intends to summarize several new data concerning the immune system manipulation and its regulation, comparing several different tools and strategies .

Presence of an antigen has the main influence on the immune system and its regulation. The form of antigen together with immunological environment on the site of antigen entrance determines immune response.

The way of entrance of antigen and its other immunological characteristics play a crucial role for the outcome of immune reaction. Detailed knowledge of all these factors could rise new more safe and more effective strategies to manipulate or control immune response. Other imporatnt factors affecting the outcome of immune reaction are for example: presence of antigen presenting cells (APC), cytokine environment, etc.

Induction of antibody response by vaccination is already well introduced, but usually sufficient „only“ for prophylactic vaccination. Situation in therapeutic vaccine development is more complicated. Antibody response is usually easy induced by vaccination with soluble proteins. Despite these successes, to induce immune response against intracellular pathogens or tumor transformed cells, which requires induction of cell-mediated immunity is either not available or not uniformly effective. This lack of immunization protocols involves particularly malaria, leishmaniasis, human immunodeficiency virus infection, or already established mycobacterium tuberculosis infection. But in the cases where neutralizing antibodies are not the main arm, standard immunization protocols often fail. The mechanisms involved in generating long-lasting cellular immune responses have tremendous practical importance also in cancer therapy, where cellular response is essential. For the reasons given above, a new forms of vaccines which could induce also cellular response are under an intensive investigation. For example: vaccination using plasmid DNA that contains a gene for the antigen of interest and start its expression in targeted tissue, or antigen delivered by virus-like particles as effective way of delivery, where viral coat protein ensures cell entry and also

adjuvat effect, then immunization with dendritic cells loaded with antigens of interest have been shown as effective.

1.2. DNA vaccination

In 1990 Wolff and Malone showed new approach based on the delivery of antigen in a form of plasmid [1]. These authors demonstrated long-lasting (2 months) expression of chloramphenicol acetyltransferase, luciferase and β-galactosidase after intramuscular injection of DNA and RNA expression vectors to mice. This observation lead to development of new immunization protocols, recently known as DNA vaccination. General construction of DNA vaccine consists of bacterial vector with a strong viral promotor, the gene of interest and terminal sequence. Plasmide is grown in the bacteria, purified and injected into the host. DNA plasmide is taken up by the host cells and encoded protein is produced.

During last years efficacy of DNA vaccination has been reported in promising experiments, where DNA vaccines were used to induce anti-tumor and anti-viral immunity. New protocols for immunosupresive treatments of autoimmunity have also appeared.

DNA vaccination provides sufficient protection against some viral infections. Positive results were reported for a bird flu DNA vaccine [2]. Ulmer et al. used DNA vaccine to protect mice from influenza [3]. Study of induced immune response has shown, that application of a DNA vaccine strategy can provide highly cross-reactive cellular immunity against lethal influenza infection [4]. Also therapeutic vaccination in combination with antiviral medication seems to reach an effective control of HBV infection [5].

Surprisingly, suppressive DNA vaccination preventing multiple sclerosis was reported as being effective in preliminary studies. [6;7]. Authors demonstrated that DNA vaccination with DNA encoding peptide derived from myelin basic protein or with part of T cell receptor from autoreactive T cells can protect against experimental autoimmune encephalomyelitis induced by myelin basic protein.

The main interest in DNA vaccination is focused on induction of anti-tumor immunity. Approaches that specifically activate the immune system to control tumor growth in vivo have been a long-standing goal in cancer immunology. Historically, the first therapeutic cancer vaccines were cell-based, irradiated tumor cells themselves were used as a source of antigen to induce anti-tumor immune responses [8;9]. This approach was not completely successful because of the complexity of many different antigens. Later, the efficacy of cancer

vaccines was improved by identifying tumor specific-antigens (TSA) and tumor-associated antigens (TAA). The vaccines were changed from cell-based to vaccines directed against TSA of TAA. When antigeneic target is determined and has a known sequence, then DNA vaccination appears promising approach.

DNA vaccination was shown partially effective in several clinical trials, reviewed by Liu and Ulmer in [10]. The mechanisms mediating the protective effect are still under intensive investigation. Better understanding of mechanisms together with use of apropriate adjuvants could bring final success of DNA vaccinations. For example, presence of CpG DNA in the DNA vaccine, which binds to Toll-like receptor-9 (TLR-9) helps to activate APC function and finally leads to preferential induction of Th1 immunity [11]. Binding of CpG DNA to TLR-9 leads to cellular activation with production IL-12 and IL-18 cytokines, expression of costimulatory molecules, and increased antigen presentation.

Plasmid DNA represents a novel immunization strategy (Fig. 1) that is capable to elicit both humoral and cellular arms of immune response, in addition to being safely administered and easily engineered and manufactured.

DNA vaccination was shown as a very effective and long-lasting immunization. However, so far only a few applications in clinics are known. Unfortunately, while DNA vaccines have performed well in preventing and treating malignancies in animal models, their overall application in human clinical trials have not impacted cancer regression to date. Since the establishment of these early trials, progress has been made in terms of increasing immunogenicity of DNA vaccine and subverting the suppressive properties of tumor cells. Therefore, the success of future plasmid DNA use in cancer patients will depend on combinatorial strategies that enhance and direct the DNA vaccine immune response while also targeting tumor evasion mechanisms.

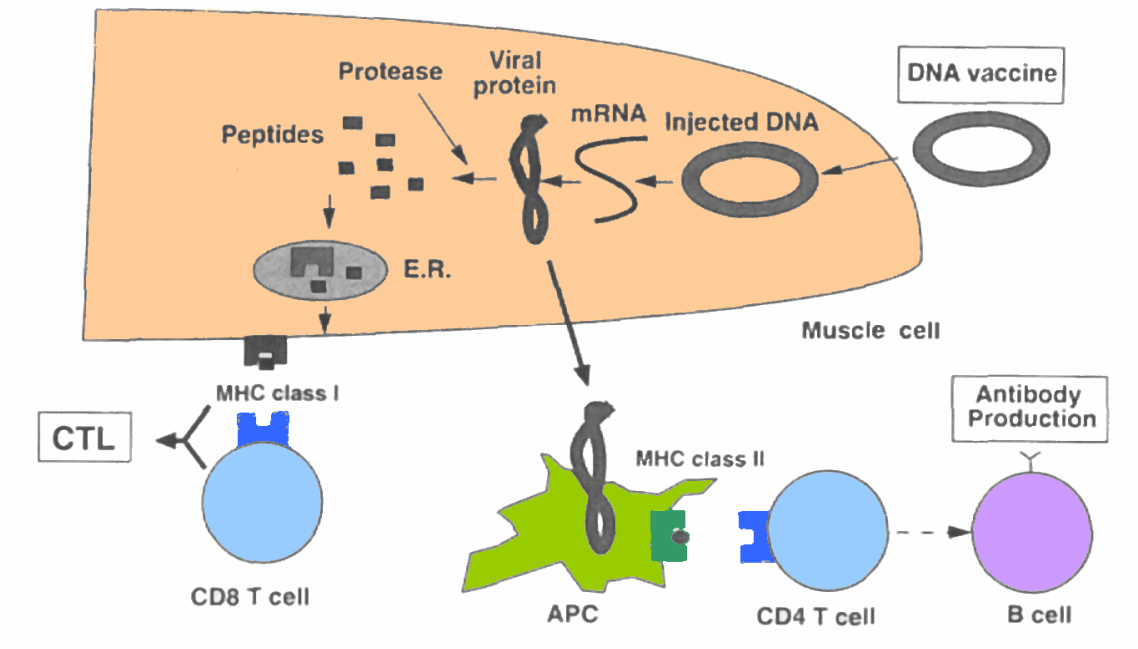

Figure 1: DNA-based immunization: The plasmid vector expressing the protein of interest (viral protein, tumor antigen) under control of apropriete promotor is injected intradermaly or intramusculary to the host. After uptake of plasmide the protein is expressed and intracellulary processed. Small antigenic peptides are presented in the context of MHC I and subsequently $CD8^+$ T cells are stimulated and induce cell mediated immunity. Protein peptides can be also presented in MHC II pathway by professional APCs, which can induce $CD4^+$ T cell response. Humoral immune response is also augmented by this mechanism.

Generally, plasmid DNA immunization is a promising strategy for generating cell-mediated immunity. However, while plasmid DNA immunogens can elicit potent cellular immune responses in small laboratory animals, they have proven less potent in human clinical trials [12]. DNA vaccination protocols are now developed or extensively studied in wide range of applications.

1.2.1 DNA vaccination in mouse model of leukemia

A large number of recent studies have investigated strategies for induction of immune response which would be able to recognize and eliminate malignant cells in the host [13;14].

Identification of novel specific TAA and TSA provides new possibilities in induction of cellular immune response and represents an attractive method for developing tumor-specific therapies. Identification of T lymphocytes invading and/or surrounding solid tumors in vivo suggested that T cells recognize tumor cells, although little evidence exists that these T cells are capable of killing cells within the tumor. In some leukemia patients, cytotoxic T lymphocytes (CTL) specific for the leukemic clones are able to kill the leukemic cells in vitro [15]. Despite the potential of T cells to detect and eliminate leukemic cells, the majority of acute myelocytic leukemia (AML) patients have failed to develop effective immune responses against their leukemic cells. The reasons for this failure remain unclear, but may include difficult accessibility of the tumor cells to the immune system, escape of malignant cells from immune response and/or inefficient stimulation of the immune system by the neoantigens of the tumor cell [16].

As an example of experimental use of DNA vaccination anti-TAA can serve acute promyelocytic leukemia (APL). APL is primarily associated with the t(15;17) (q22;q11.2) translocation, which leads to the expression of novel fusion proteins. Part of the promyelocytic leukemia (PML) gene is fused in frame with exons 3-9 of the retinoic acid receptor alpha (RARα) gene [17]. In myeloid leukemia, fusion proteins resulting from reciprocal translocations may provide a source of specific tumor-associated antigens. PML-RARα represents majority of the fusion proteins in acute promyelocytic leukemia (APL) and therefore it is a suitable potential target for DNA vaccination.

Padua et al. [18] used the APL-transplantable mouse model, in which leukemic cells from the spleen of an APL transgenic mouse (bearing the human *PML-RARA* oncogene) are transplanted into syngeneic recipients [19]. This model mimics human APL, both in its biological characteristics and its response to conventional therapeutic drugs such as ATRA or AS203 [19-21]. Vaccination with DNA vaccine fused from sequences of oncogene PML-RARA and tetanus fragment C (FrC) showed that DNA vaccine specifically targeted to an oncoprotein can have a pronounced effect on survival. The survival advantage is concomitant with time-dependent antibody production and an increase in IFN-γ production

In general, DNA vaccination against tumor antigens seems to be a promising approach because of many advantages, such as an easy and cheap preparation for every discovered TAA and an easy administration. Remaining problems of efficacy can be overcome with use of adjuvants as for example CpG.

1.3. Vaccines using recombinant viral vectors

DNA vaccination is a promising approach, but also other methods have been developed for DNA delivery to the organism. DNA vaccination provides DNA coding antigens intramusculary or intradermaly without further influence on their expression. More effective way of DNA delivery represents use of live recombinant virus vectors (Fig. 2). A number of studies have investigated the effects of immunization with recombinant virus constructs encoding tumor antigens. In some cases this approach can by uneffective because of binding of preexisting neutralising antibodies against virus proteins [22], which can eliminate the virus even before its entry to the cell. Viral vectors are an attractive choice of antigen delivery system for cancer immunotherapy since they mimic a natural infection and provide potent danger signals. Numerous viral vector systems have been developed since the first recombinant vaccinia viruses were constructed more than 20 years ago [23]. In the field of cancer immunotherapy, a panel of recombinant viruses has been used to deliver immune modulators (e.g., cytokine or co-stimulatory molecules) or TAA.

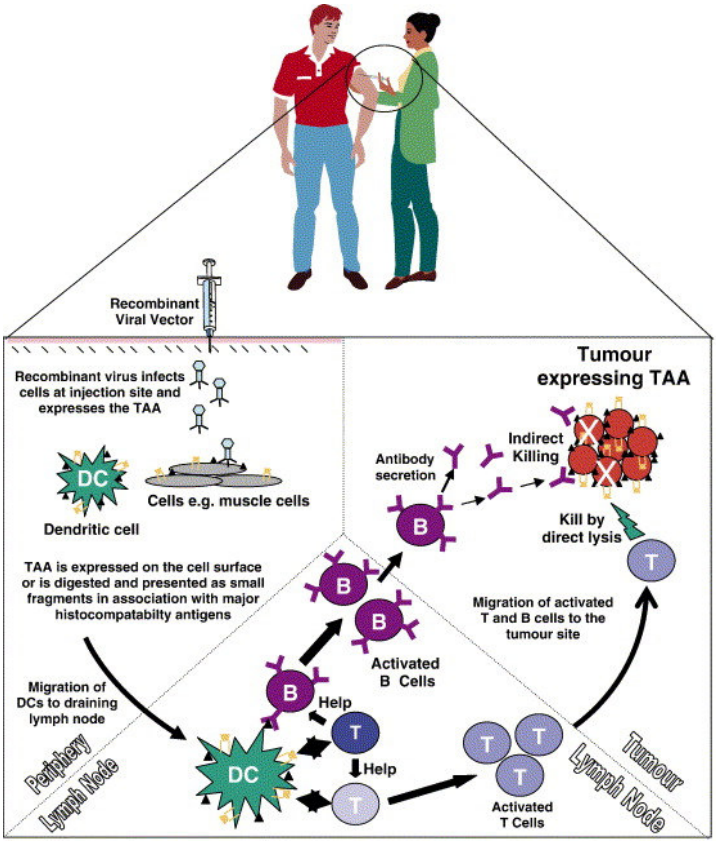

Figure 2: Viral vector-based immunization. Schema of the induction of a tumor specific immune response following vaccination with a recombinant viral vector expressing a tumor antigen.

Recombinant viral vectors derived mainly from vaccinia, avipox, adenoviruses or herpesvirus, have been already used as a cancer vaccine in many trials [24-27]. All live recombinant viral vectors are designed as nonproliferative antigen DNA carriers. Its role is to deliver DNA of potential antigens to the cells.

Safety issues had risen especially after some complications in clinical trials using retroviral vectors to treat X-linked severe combined immunodeficiency (X-SCID) [28-30]. The occurrence of leukemia in patients in a gene-therapy trial for X-SCID has highlighted an adverse effect of viral vector delivery technique for the therapeutic gene. Development of experimental model for vaccine safety testing still remains an important issue [31]. Part of safety problems with retroviral vectors can be overcome with use of virus derived pseudocapsids. Potential use of recombinant virus like particles is discussed in the next chapter.

1.4. Virus-derived pseudocapsids

Virus pseudocapsids or so called virus like particles (VLP) consist of virus proteins derived from the structural proteins of a virus. Virus coat proteins have a remarcable intrinsic activity to spontaneous self assembly to the virus coat. When these proteins are produced in recombinant expressing vector they give rise to VLP. VLP are usually prepared in insect cell lines infected with Baculovirus vector or in E. coli. Produced VLP are isolated by gradient centrifugation. Particles prepared in baculovirus mimic the structure of native virus and keep the same ability to entry the host cells. Pseudocapsids do not contain any virus nucleid acids. Such VLP fall in the general size range of viruses (22–150 nm). Size of particles appears to be optimal for uptake by dendritic cells [32].

1.4.1 Virus-derived pseudocapsids: an effective vaccine

Virus derived pseudocapsids, prepared in recombinant vectors, represent a potent and safe biologic material for development of new anti-virus vaccines. In last years evidence has been raising that property of VLP could by promising for their use as vaccines. Intensive research in this field led to introduction of new vaccines against human papiloma virus (HPV) which is an important factor in inducing cervical cancer (Fig. 3), reviewed in [33]. HPV pseudocapsids were prepared in yeast cells [34] and first trials showed safety and immunogenicity of pseudocapsids based HPV vaccine [35].

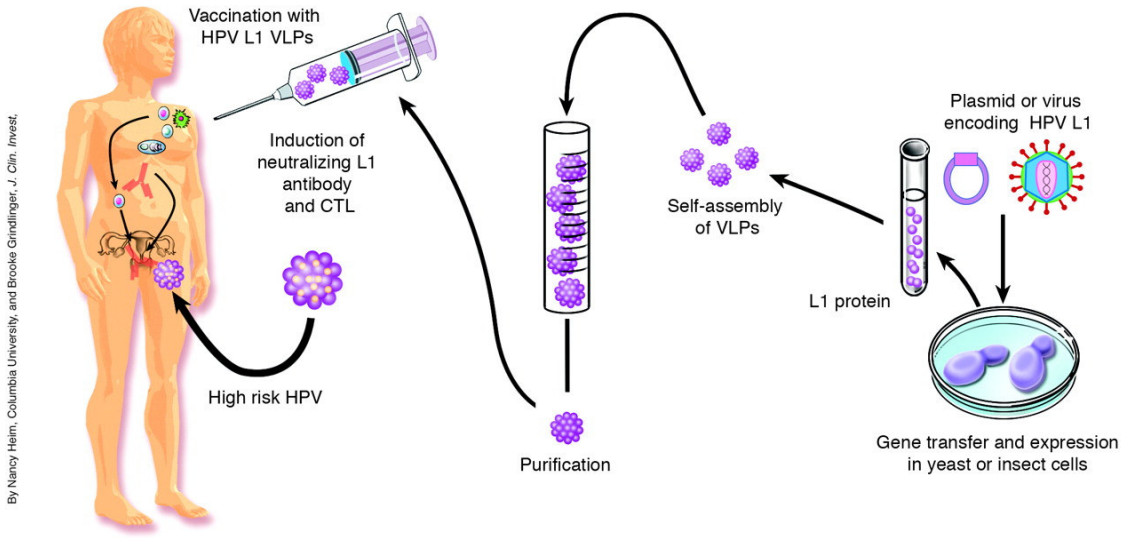

Figure 3: Virus-like particles based vaccine: Vaccination against human papilomavirus (HPV) infection using HPV-VLP. Recombinant HPV-16 or HPV-18 L1 capsid protein made in yeast or baculovirus-infected insect cells self-assembles to form VLP that are very potent at inducing neutralizing antibodies but are not infectious because they lack any virus nucleic acid. Such VLP vaccines show promise for prevention of HPV infection and HPV-associated cervical cancer. DC present antigen to Th (blue) and B (pink) cells and immune response leads to B cell induction and maturation into the plasma cells (shown as ellipses). Plasma cells then generate antibodies (red) capable of neutralizing the virus [36].

1.4.2 Virus-derived pseudocapsids: an effective antigen delivery

Antigen delivery manner has often an important influence on quality of immune response. Natural pathogens as viruses or bacteria have selected, during millions years of evolution, very efficient way of entrance. Antigen structures providing cell entrance are naturally first antigens recognized by the immune system.

First it has been shown that VLP can be use for effective delivery of DNA plasmides. Artificial polyomavirus VP1 pseudopcapsids were able to deliver DNA plasmides of different genes of interest, as for example beta-galactosidase gene or the chloramphenicol acetyl transferase (CAT) into cultured mammalian (including human) cells or directly into animals (mouse or rat) for relatively long term expression (from several weeks up to 2 months) [37-

39]. Intraperithoneal and intranasal administration of VLP with plasmid DNA lead to longer transfection and gene expression than the gene expression obtained after administration of DNA alone. Transgene products were also found in brain. The observation indicates that immunization with VLP is also able to overcome barrier of immunologicaly priviledged sites [38;40]. Results of these authors indicate that mouse polyomavirus derived pseudocapsids are another promissing target of research in this field.

The production of the recombinant VP1 protein offers a safe way to obtain a highly purified, nonproliferative and nonpathogenic vehiculum, which can be in addition genetically ingeneered to carry therapeutic peptides or protein. VP1 protein has also a DNA binding activity (N-terminus of VP-1 protein contains a DNA- binding domain and a nuclear localization sequence). VLP carrying the antigen provide effective delivery of antigen to the cells and also ensure activation of immune response. Presence of virus capsid protein can also provide an adjuvant effect.

Several studies demonstrated the ability of MPyV-based VLP to deliver genes for their expression to many tissues of mouse [41;42]. Recently, VLP have become broadly studied for vaccination strategies, primarily against the viral antigens forming the capsid surface [43-45] but also with the aim to induce immune response against heterologous antigens introduced into the pseudocapsid [46]. Immunization protocols have included VP1 antigen/DNA complexes [40;47;48] or chimeric VP1 VLP with therapeutical peptides exposed on the surface of particles, inserted into VP1 surface loops [49;50] or inside pseudocapsids [51]. Intranasal immunization with VLP has been shown to generate strong antibody production and to activate T cell response without any other adjuvants (Frič et al., submitted).

1.5. Mucosal surfaces and antigen delivery

Mucosal surface is the widest natural surface in the contact with outside environment. Its main immunological function is to prevent effectively the entrance of pathogens but also to keep hyporesponsiveness to the non-pathogeneic agens. It has been shown that mucosal surfaces are target tissue to antigen delivery and manipulation with the immune system.

Administration of antigen through mucosal surfaces has been shown to be effective for modulating immune response and has been demonstrated as an approach for vaccination, but, in contrast, oral administration of antigen has also been suggested to be useful for inducing tolerance. Final immune response after oral immunization is still difficult to predict because

many factors (for example: Fig. 4), some of them so far not well recognized, can influence the outcome of oral immunization [52-54].

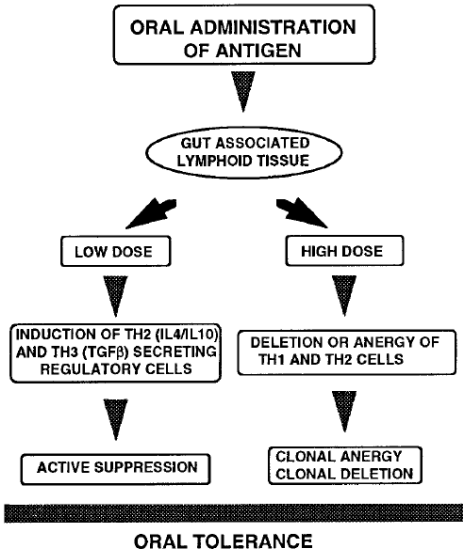

Figure 4: Importance of dose in oral administration of antigen. Dose of administrated antigen leads to different mechanisms of tolerance acquisition. Low doses of antigens often induce active T cell dependent supression. while high doses of antigen apparently lead to clonal anergy [55].

All mucosal surfaces were experimentally tested for hypothesis of tolerogeneic antigen delivery. Above all, experimentally successful were intratracheal delivery and than delivery through oral and intranasal route. Especially oral and intranasal route were tested later in clinical trials.

Multiple mechanisms of tolerance are induced by oral or nasal administration of antigen. Oral tolerance has also been tested in human autoimmune diseases. Trials have been conducted in multiple sclerosis with orally administrated myelin antigens [56;57], in rheumatoid arthritis with orally administrated colagen type II antigen [58-60], in uveitis with oral administration of retinal S antigen [61], and in type I diabetes with orally administrated insulin [62]. However, results of these clinical trials often have remained ambiguous.

The mechanisms of development of the nonresponsiveness after delivery of antigens through mucosal surfaces remains still unclear but the main factors are already known.

Low doses of antigen favour the active suppression, whereas higher doses support clonal anergy or deletion (Fig. 5). Orally administrated antigen induces Th2 cells producing IL-4 and IL-10, Th3 cells producing TGF-β ls and $CD4^+CD25^+$ regulatory T cells (Treg) expressing FoxP3. Induction of oral tolerance can be enhanced by IL-4, IL-10, anti-IL-12, TGF-β, cholera toxin B subunit (CTB), Flt-3 ligand, anti-CD40 ligand and continuous feeding of antigen. In addition to oral tolerance, nasal tolerance has also been shown being effective in suppressing inflammatory conditions with the advantage of a lower dose requirement. Mucosal tolerance is an attractive approach for treatment of autoimmune diseases because of easily repeated administration of antigens and antigen-specific mechanism of action. The successful application of mucosal tolerance for the treatment of human diseases will depend on dose (Fig. 4), route (nasal versus oral), size and form (soluble or particular) of antigen, presence of mucosal adjuvants, combined therapy and the state of disease at the beginning of administration of antigen.

1.5.1 Oral delivery of antigen

One of the most important forms of acquired tolerance is oral tolerance. In some cases, inhibition of immune response after oral administration of antigen was observed and this phenomenon has been called oral tolerance [63]. Oral tolerance is the specific suppression of cellular and humoral immune reactivity to an antigen by prior administration of the antigen by the oral route. It is an important mechanism to prevent hypersensitivity reactions to food proteins and bacterial antigens present in the mucosal flora. (Fig. 5). Antigens that continuously contact the mucosa represent a borderline between foreign and self environment. Oral tolerance evolved to treat external agents that gain access to the body via a natural route as internal components without danger signals. Failure of oral tolerance is attributed to the development and pathogenesis of several immunologicaly based diseases, including inflammatory bowel disease (Crohn's disease and Ulcerative colitis).

Administration of antigen through mucosal surfaces is very effective way of antigen delivery. Oral tolerance was intensively studied as promising tool of tolerogeneic antigen delivery in various experimental models including animal models of human diseases, reviewed in [64] or transplantation models [65;66].

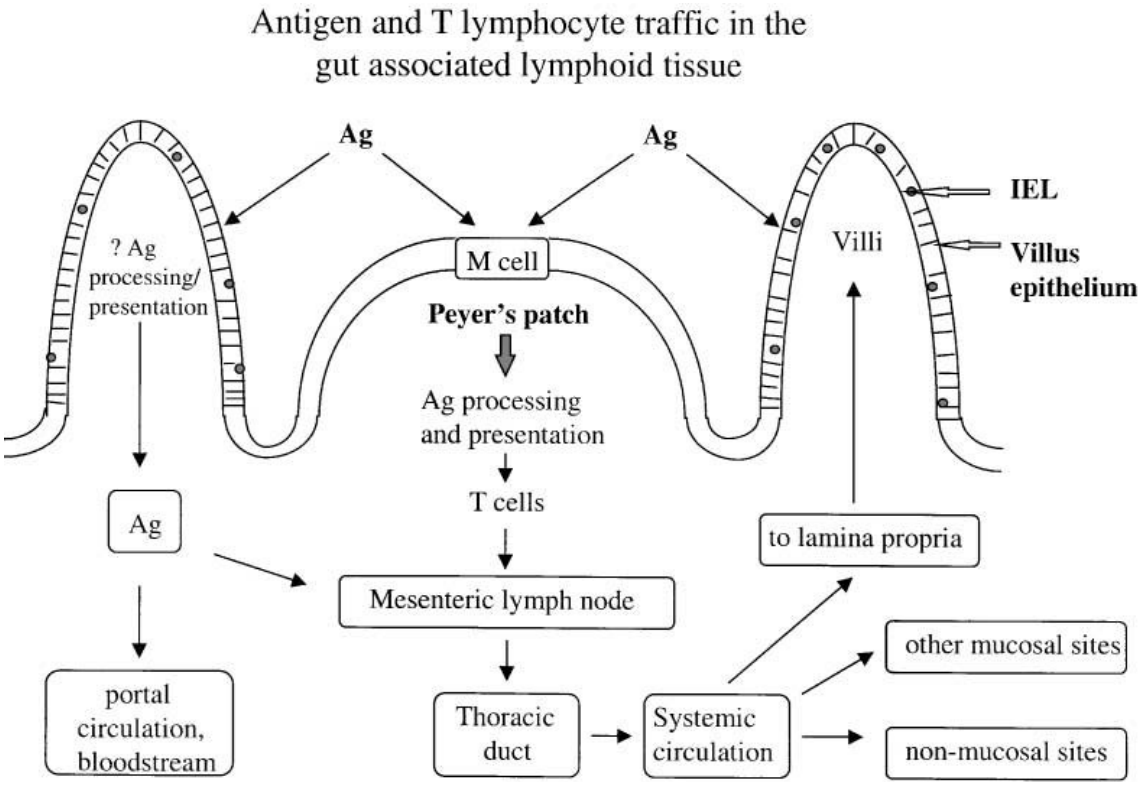

Figure 5. Antigen and T cell traffic in the gut-associated lymphoid tissue. Particulate antigens are preferentially taken up by M cells and soluble antigen by the villus epithelium. Primed T cells migrate to the systemic circulation via the mesenteric lymph node and thoracic duct and then migrate back to the lamina propria and to other mucosal and nonmucosal sites. The villi contain intraepithelial lymphocytes (IEL), which are $CD8^+$ T cells unique to the gut. T cells in the lamina propria are in a different state of activation to those in Peyer's patches. Also cytokine environment in the gut mucosa is exclusive and important for result of immune response. TGF-β is one of the main cytokines responsible for immunotolerant environment of the gut mucosa [55].

1.5.2 Intratracheal delivery of antigen

Intratracheal delivery has been shown as being effective in prolongation of heart transplant survival. Alloantigen administrated via the trachea could modulate immune response to allografts. Hearts from C57BL/10($H2^b$) mice were transplanted into CBA($H2^k$) recipients. Recipient mice were given donor splenocytes into the trachea 7 days before transplantation. This treatment significantly prolonged the survival [67]. Several modified immunization protocols were developed, but this approach did not achieve broad use.

1.5.3 Intranasal delivery of antigen

Together with oral delivery, several protocols for effective intranasal delivery of antigen were tested. Experiments were focused mainly on delivery of alergen epitopes or antigens identified as important in several autoimmune diseases. Intranasal delivery of alergen epitopes together with suitable adjuvants has been shown as immunosupressive.

A significant improvement has been achieved by co-administering with immunomodulating agents to enhance the tolerogenic activity of autoantigens as well as allergens given orally or nasally. The most promising among such agents is the B subunit of cholera toxin (CTB). Conjugation or co-administration of CTB with autoantigens or allergens have been shown being capable enhance markedly tolerance induction in already sensitized animals and thereby effectively suppress progression of various autoimmune diseases, especially in experimental autoimmune encephalomyelitis [68], spontaneous autoimmune diabetes [69] and experimental autoimmune arthritis [70].

Induction of tolerance can be facilitated when the antigen is linked to CTB, an efficient mucosal carrier. Kataoka et al. have recently shown that CTB fused on alergenic epitope Bet v 1 and administrated intranasaly, induced suppression of Th2 response to allergic sensitisation [71]. This results confirmed previous similar observations, that way of linkage is important for final result of immunization together with CTB [72;73]. Kataoka et al. have also shown that upregulation of FoxP3, IL-10 and TGF-β, mRNA expression can be detected in

splenocytes after pretreatment with unconjugated allergen but not with the fusion molecule, indicating that antigen conjugation to a mucosal carrier modifies the immunomodulating properties of an antigen/allergen. This important observation can explain some ambiguities in previous studies using CTB. On the other hand, cholera toxin CT is one of the most potent mucosal adjuvants and feeding CT abrogates oral tolerance when fed with an unrelated protein antigens.

1.5.4 Mucosal delivery in models of allotransplantation

Mucosal delivery of antigen is broadly used in experiments focused on suppression of various autoimmune diseases or alergies. Because of partial successes in induction of hyporesponsiveness in these models, the research of mucosal tolerance has been made attempt induction of nonresponsiveness in allograft models.

It has been shown, that administration of alloantigens through mucosal surfaces prolongs survival of skin [65] and renal [74] allografts and inhibits acute rejection of cardiac allografts [75;76]. Oral administration of donor cells enhanced corneal graft survival [66] and use of cholera toxin adjuvant markedly enhances the efficacy of oral tolerance. Even a single oral dose of donor cells significantly reduces the incidence of rejection [77].

The first experiments which have achieved specific alloantigen immunoresponsiveness, have used feeding with allogeneic cells. Improved immunization protocols were developed for use of allogeneic peptides. Allogeneic peptides could be eventually used also in clinic. Several studies have shown beneficial effect of mucosal immunization with allogeneic peptides in rats. Oral administration of mixture of different allogeneic peptides down-regulated the systemic cell-mediated immune response in an antigen specific fashion [78].

1.6. Regulatory T cell, a key to immune regulation?

The immune system is regulated by several mechanisms (antigen concentration, anti-idiotypic antibodies, cytokines, inhibitory molecules, etc.). Deficiency in regulation of immune reaction can lead to several problems such as chronic responses, autoimmunity or recently found complication in therapy of malignancies. Therefore, understanding of the immune regulation is indispensable. New era of research on Treg started after 1995 when Sakaguchi's group showed in a model adoptive transfer of autoimmunity that suppressor activity resided exclusively in the $CD4^+CD25^+$ T cell subset [79]. Later several authors have shown that activation and expansion of self-reactive T lymphocytes, which have escaped thymic clonal deletion, is actively suppressed in the periphery by naturally occurring $CD4^+$ regulatory T cells (Treg), the majority of which constitutively express CD25 (IL-2 receptor α-chain) [80;81]. FoxP3 transcription factor has been found as a key molecule responsible for induction of regulatory phenotype [82-84]. Since its re-discovery by Sakaguchi, the Treg occupy one of the the top positions in immunological research.

1.6.1 Regulatory T cell, induction of tolerance

Treg are intensively studied for potential therapeutical use in autoimmunity treatment or for prolongation of allogeneic graft survival. Main objective is to induce or prepare sufficient number of Treg which specificaly inhibits unwanted immune response.

Treg can be divided into two populations; naturally occurring $CD4^+CD25^+FoxP3^+$ Treg that are generated in the thymus and enter the periphery as functionally mature T cells [82;85] and adaptive or induced $CD4^+$ Treg that are either derived from $CD4^+CD25^-$ precursors or expanded from $CD4^+CD25^+$ cells in the periphery [86;87]. Until recently adaptive Treg were thought to develop exclusively from naïve cells, but recent analysis of human $CD4^+$ cells has revealed that $FoxP3^+$ Treg are also generated from rapidly dividing and highly differentiated memory T cells [86]. All these processes are strongly dependent on actual immunological environment, presence of cytokines and other stimulatory molecules (Fig. 6 and 7).

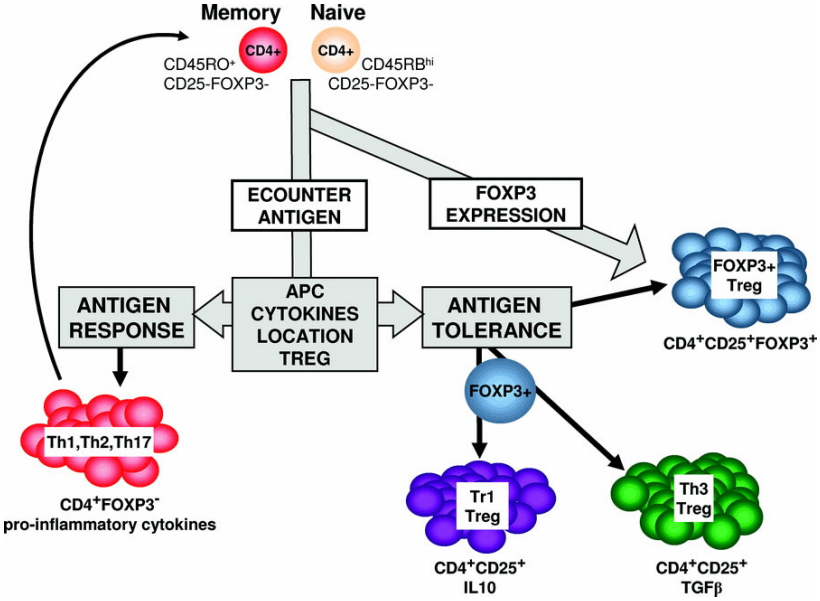

Figure 6. The balance between immune response and antigen tolerance [88].

It has been recently shown that once the FoxP3 expression is established in the cell, that FoxP3 amplifies and stabilizes Treg gene expression, which maintains Treg homeostasis and lineage stability [89].

Presence of Treg infiltrating allografts has been shown as beneficial. It has been shown by Muthukumar et al., that the presence of mRNA for FoxP3 can correlate with the state of renal rejection [90]. Higher levels of FoxP3 mRNA are associated with reversible acute rejection, and lower levels with graft failure. This indicates that during occurrence of acute rejection, $FoxP3^+$ Treg may limit donor alloantigen immune responses and that the lack of regulation by Treg may result in unrestrained effector-cell activity and graft failure. This indicates the potential of FoxP3 as a marker of ongoing graft tolerance and rejection episodes.

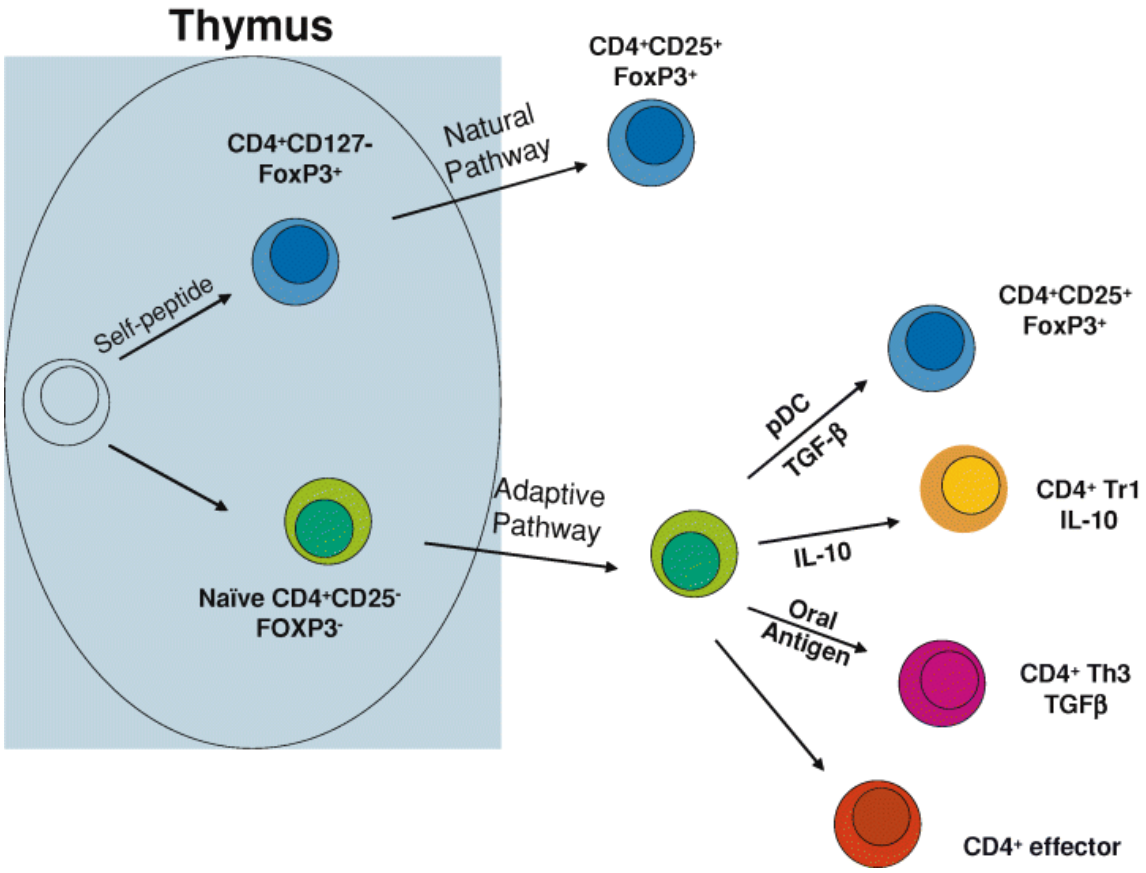

Fig. 7. Developmental pathways of natural and adaptive T regulatory cells. [91]. Other regulatory T cell subsets have been observed in various immune processes, and have demonstrated therapeutic efficacy in some transplant models. Although the $CD4^+$ T cells producing IL-10 (Tr1), Th3 producing TGF-β and other lymphocyte subsets do not generally express Foxp3, and lack characteristic surface markers that allow selective identification and isolation, they are all part of the pool of T cells with immunosupressive characteristics.

Only a few successes were achieved in experimental prolongation of graft survival with treatment based on Treg cells. Real therapeutic potential of Treg in the suppression of allogeneic response is still discussed [91]. Understanding the factors that affect FoxP3 expression both in mice and humans should allow us to optimize protocols in vitro or in vivo that will direct $CD4^+$ cells towards to a stable Treg commitment.

1.6.2 Regulatory T cell as an obstacle to therapy

Recent evidence cumulates that long-lasting immune reaction can lead finally to increase Treg number, sometimes already before elimination of antigen, which can lead to persistent and chronic infections. Therefore Treg seriously complicate the problems in many experimental and also therapeutic treatments. Thus, Treg are now intensively studied in majority of immunization protocols. Treg cells regulate peripheral self tolerance and possess the ability to suppress anti-tumor responses, that cause difficulties especially in anti-tumor immunotherapy in mouse [92] and may partially explain the poor clinical response of patients with cancer undergoing active immunization protocols. Clinical trials where $CD25^+$ cells were depleted were not completely successful yet [93]. Treg infiltrating the tumor site presumably obstruct immune reaction against tumor antigens together with immunosupressive environment caused by IL-10, TGF-β [94].

Depletion of tumor infiltrating Treg is one of proposed strategies. It has been shown that depletion of Treg increases survival of tumor-bearing mice [86;95-97] and improves anti-tumor immune response in patients [93]. On the contrary, diverse data appeared during study of follicular lymphoma, where Carreras and others showed that high numbers of FoxP3 positive T cells correlate with prolonged survival of follicular lymphoma patients [98].

Viral infections can change the distribution of FoxP3 positive cells in early state of infection. Evidence rises that viral infection finally accumulates Treg in organs or tissues which are infected and presence of Treg facilitates propagation of virus. This phenomenon was observed in infections with HCV [99] or HIV [42;100;101]. The results indicate that Treg can be important therapeutical target in chronic infections, but also suggest the necessity of Treg study in experimental models of vaccination with virus-like particles.

It was also shown that other immunoadjuvants affect distribution of FoxP3 positive cells [99;102;103]. The experimental data show the importance of Treg also in immune responses induced by vaccination. Protocols for Treg elimination seem to be generally accepted soon as an addition to clasical treatment.

2. Aims of thesis

Regulation of the immune response is important in therapy of many disorders. Numerous different approaches are used to manipulate immune response in positive or negative manner. This thesis compares different approaches in immune system manipulation and regulation and summarizes their advantages and disadvantages.

DNA vaccination is a promising approach to delivery antigen directly to the tissue and ensures its expression and presentation on a context of MHC class I or class II in the targeted cells. This expression of new antigen leads to specific stimulation of the immune response. Therefore DNA vaccination was used in several experimental models focused on development of cellular immune response. The mouse model of acute promyelocytic leukemia, presented in this thesis, is an example of promising tool for therapeutical DNA vaccination. Observation that treatment with plasmid DNA was beneficial for mice survival led to intensive study of detailed pathways of anti-leukemia immune response.

Later, a model using polyomavirus derived chimeric pseudocapsids have been available. Questions were adressed how efficient can be antigen delivery mediated by mouse polyomavirus-derived pseudocapsids. Changes in immune response, studied after intranasal administration of virus-like particles carrying the enhanced green fluorescein protein (EGFP), were only a preliminary study before introducing the antigen derived from chromozome translocation causing the chronic myelogenous leukemia. The main aim of these experiments was to induce immune response to the antigen carried inside of the particle and to study the antigen processing after delivery and a potential adjuvant effect of viral capsid protein which could lead to efficient immune response.

Mucosal surfaces were identified as a tissue with tolerogeneic environment, therefore continuous administration of antigens through mucosal surfaces is usually considered as tolerogeneic. We tested the tolerogeneic potential of mucosal surfaces in a model of allogeneic mouse transplantation, with the main goal to validate this approach as usable for inducing allogeneic hyporesponsiveness.

The importance of regulatory T cells (Treg) is now considered in many experimental models of immune response manipulation. Not only detailed study of Treg functions but also experiments targeting the quality and the number of Treg are necessary to understand immune response. Aim of this thesis was also to prove the involvement of changes of Treg after immunization with virus-like particles carrying EGFP, as potencionaly important vehiculum for antigen delivery and amplification of immune response. On the other hand it was tested whether the inhibition of immune response maintained by Treg is inconvenient in anti-tumor immune response development. This was confirmed in experiments showing the beneficial role of Treg depletion in mouse model of human papilomavirus inducing tumors.

4. Discussion

Regulation of the immune system still remains a key issue. Several main fields related to this thesis will be discussed.

Induction of sufficient antibody response by vaccination is already well established, but mainly sufficient just for prophylactic vaccination. Antibody response is usually easily induced by vaccination with soluble proteins. However, in the cases where neutralizing antibodies are not the main arm of defence, standard immunization protocols often fail. Situation in therapeutic vaccination is usually more complicated. Presence of specific antibodies has usually not sufficient effect to treat many disorders as for example tumor cells or virus infected cells. Cytotoxic $CD8^+$ T lymphocytes (CTL) have been shown to be important mediators of anti-tumor immunity in various animal models. Several observations also suggest the important role of CTL in the control of tumor growth in humans. Biopsies from human regressive melanoma and in situ amplification of CTL with anti-tumor activity suggest that CTL may contribute to the host dependent elimination of tumor cells [104;105]. Only an antigen specific cellular cytotoxic immune response has beneficial role in malignacies.

Therefore immunization inducing strong cellular, mainly $CD8^+$ mediated response is under intensive research and represents one major goal in the design of cancer vaccines development[106].

Recently, different vaccination approaches are intensively studied. DNA vaccination, delivery of antigen by virus-derived pseudocapsids and also immunization with antigen loaded dendritic cells [107] are promising. The studies in mouse experimental models were published, showing beneficial effect of DNA vaccination in leukemia [18], solid tumor [108] and also promising results with VLP inducing immunity against Her-2/neu expressing tumors [109;110].

Sensitive techniques such as tetramer staining essay or ELISPOT assays detecting IFN-γ production or recently even more sensitive granzyme B [111] have been used to demonstrate the generation *in vivo* of anti-tumor T cells in vaccinated patients. The shortage of clinical responses in these patients has made difficult to validate any of these assays as a useful surrogate of clinical response. These conclusions can indicate that also on the level of animal model experiments, the detection of CTL with described sensitive techniques should be

confirmed in *in vivo* effects. Majority of authors do not show a direct evidence of cytotoxic T cells in mice, they usually detect CTL after extensive restimulation with specific peptides of other forms of antigens, and than detect IFN-γ by ELISPOTs or tetramers staining [112]. These results can lead to overestimate expectation for the future use of these vaccines. This can explain weak or nondetectable activity of CTL without restimulation in our model of leukemia [18] and also after immunization with VLP which is in the contrast with observations of other autors [10;44;113-115], but could be explained by different techniques of CTL detection.

Very important observations were recently done in immunology of CTL. In addition to $CD8^+$ T cell responses, $CD4^+$ T cell responses have been found to be critical in the maintenance of effective $CD8^+$ T cell function and control of infection with HIV and HCV [116-118]. In addition, memory $CD8^+$ T cells have now been subdivided into effector memory T cells, which home to tissues, and central memory cells, which recirculate in the body [119-121]. Chronic antigen stimulation during a persistent infection may inhibit the change of memory $CD8^+$ T cells to central memory cells. However, central memory cells are more effective in protection because they are more able to proliferate when re-exposed to antigen [122]. Thus, chronic virus infection may block effective immune response by preventing the development of the most effective form of T cell memory. Therefore the challenge for an effective vaccine is to induce long-lived central memory $CD8^+$ T cells as well as $CD4^+$ helper T cells.

Evidence now rises about the involvement of Treg in immune responses. Experiments showed that even in very successful priming of specific T cells response are those finally suppressed by Treg. This happens on the level of system immune response or locally in site of wanted immune reaction (as in the tumor). This serious obstacle can be potentially overcome by depletion of Treg as shown by Imai and Nair [97;123]. The depletion of Treg cells inhibited growth of the recurrences after surgery of HPV16-associated MHC class I^+ as well as MHC class I deficient tumors transplanted in syngeneic mice [13]. These results demonstrate that depletion of $CD25^+CD4^+$ Treg cells can be used as an efficient adjuvant treatment improving the results of surgery in the experimental systems mimicking human MHC class I+ and MHC class I-deficient, HPV16-associated neoplasms.

For cancer vaccines to be effective, it may require the elimination of Treg. Although reagents to selectively eliminate these cells in vivo are being developed, their clinical efficacy has to be established yet. Chemotherapy- or radiation-induced lymphodepletion can eliminate

regulatory cells but cannot be used in conjunction with cancer vaccines because the needed effector cells are also eliminated.

Mucosal administration of antigens is well described for induction of hyporesponsiveness to soluble antigens (mainly from food and comensal bacteria). Experiments with induction of hyporesponsiveness to alloantigens remains complicated. Final immune response after oral immunization is still difficult to predict because many factors (some of them so far not well recognized) can influence the outcome of oral immunization [53;54]. It has been shown that oral immunization with allogeneic cells diminishes allotransplantation immunity in vivo [65;66] and induces hyporeactivity in vitro [76;124]. In our experimental schema presented in two articles [125;126] it has been shown that continuous peroral administration of allogeneic cells induces alloantigen specific local and also systemic immune response. However, our experimental schema has been suggested as tolerogeneic. Genetically determined differences and different immunological reactivities of individual strains of mice thus appear to be the main factors responsible for the different results obtained. Some changes in cytokine production were also described by others, especially for increased IFN-γ. [127;128].

Knowledge of complicate network of immune regulation seems to be essential to our understantig of the immune system. Recent findings enable novel approaches to achieve better and more sophisticated manipulation of the immune system and to treat the immune disorders.

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