Scuola Internazionale Superiore di Studi Avanzati - Trieste

ISAS-International School for Advanced Studies

Exploring new vaccination strategies: Genetic immunization models for enhancing the immune response in cancer immunotherapy

Thesis submitted for the Degree of

Doctor Philosophiae

Candidate
Mirza Suljagic

Supervisor
Dr. Oscar Burrone

Academic Year
2006/2007

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" Do you know what's best in life ?
Desire, my friend. "

(M. Selimovic, Fortress)

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1. Introduction

1.1 CANCER IMMUNOTHERAPY

The basic premise of immunotherapy for cancer, is to stimulate the immune system in some way to treat and even prevent cancer. Historical data show that the immune system clearly plays a role in cancer progression. Cancer is one of the three leading causes of death in the world today. As treatments for infectious diseases and the prevention of cardiovascular disease continue to improve, and the average life expectancy increases, cancer is likely to become the most common fatal disease.

Cancers are caused by the progressive growth of the progeny of a single transformed cell. This happens due to a variety of genetic defects that occur in genes that encode for proteins involved in cell growth. The components of the immune system, antibodies and T cells, do not recognize or respond to defective genes, but they do recognize and respond to the abnormal proteins the cancer-causing genes encode.

Immunotherapy for cancer consists of the stimulation of the immune system via a variety of reagents such as vaccines, infusion of T cells, or cytokines. These reagents act through one of several mechanisms:

1) by stimulating the antitumor response, either by increasing the number of effector cells or by producing one or more soluble mediators such as lymphokines;
2) by decreasing suppressor mechanisms;
3) by altering tumor cells to increase their immunogenicity and make them more susceptible to immunologic defenses; and
4) by improving tolerance to cytotoxic drugs or radiotherapy, such as stimulating bone marrow function with granulocyte colony-stimulating factor (G-CSF).

Over the last several years it has been shown that the host immune system interacts with the developing tumor and, in some cases, may be responsible for the arrest of tumor growth and tumor regression. This is based mainly on results of studies performed in various animal models of tumor growth and metastasis, which convincingly demonstrated the efficacy of the immune response as effective extrinsic tumour-suppressor system. Specifically, in animal models lymphocytes and INF- γ have been found to collaborate to protect against development of carcinogen-induced sarcomas and spontaneous epithelial carcinomas (Shankaran et al., 2001) . In human, the role of the immune system in the control of spontaneous tumors has been much less clear. The presence of antibodies (Abs) to tumor-associated antigens (TAAs) and of specific as well as nonspecific effector cells in the peripheral circulation of patients with cancer has been reported often. This implies that immune cells and Abs potentially capable of tumor rejection exist in these patients. *In vitro*, isolated effector cells—T cells, natural killer (NK) cells, Ab-armed NK cells, or macrophages—have been shown to be able to kill tumor targets in short-term assays by mechanisms such as osmotic lysis (cytotoxicity) or apoptosis and/or Ab-dependent cellular cytotoxicity (ADCC). *In vivo*, however, tumors seem to have evolved means to resist or hide from these immune effector cells, a phenomenon known as "immunologic escape". Two fundamentally different strategies aimed to overcome this resistance and induce cancer immunity have been developed, namely passive or active immunization. Recent vaccination-based clinical trials in patients with various malignancies have failed to demonstrate the relationship between clinical responses and the presence or frequency of antitumor effector cells in the patients' peripheral circulation.(Marchand et al., 1999; Yamshchikov et al., 2001).

In many cases, immune responses to vaccines have been weak or not detectable.(Cormier et al., 1997; Jaeger et al., 1996). In other cases,

peptide- or TAA-specific effector cells are demonstrable in the patients' circulation, and their frequency increases following vaccine administration without any obvious or measurable effects on the tumor (Lee et al., 1999)

1.1.1. Passive and Active immunotherapy

a) Passive immunotherapy

Antibodies or immunoglobulins are a crucial component of the immune system, circulating in the blood and lymphatic system, and binding to foreign antigens expressed on cells. Once bound, the foreign cells are marked for destruction by macrophages and complement. In the context of cancer immunotherapy, monoclonal antibodies have brought to light a wide array of human tumor antigens.(Old, 1996) In addition to targeting cancer cells, antibodies can be designed to act on other cell types and molecules necessary for tumor growth. For example, antibodies can neutralize growth factors and thereby inhibit tumor expansion.

Monoclonal antibodies are made by injecting human cancer cells, or proteins from cancer cells, into mice so that their immune systems create antibodies against foreign antigens. The murine cells producing the antibodies are then removed and fused with laboratory-grown cells to create hybrid cells called hybridomas. Hybridomas can indefinitely produce large quantities of these pure antibodies.

Thanks to the hybridoma technology (Kohler and Milstein, 1975) the reproducible and large-scale production of monoclonal antibodies allowed their wide employment in patients treatment. Now the passive transfer of immunoglobulins is mainly used to establish antigen-specific

immunity in host unable to mount a desired immune response, as in immunocompromised individuals.

Relative to treatment, monoclonal antibodies can react against specific antigens on cancer cells and may enhance the patient's immune response. Monoclonal antibodies can be programmed to act against cell growth factors, thus blocking cancer cell growth. We can conjugate or link monoclonal antibodies to anticancer drugs, radioisotopes, other biologic response modifiers, or other toxins. When the antibodies bind with antigen-bearing cells, they deliver their load of toxin directly to the tumor. Monoclonal antibodies may also be used to preferentially select normal stem cells from bone marrow or blood in preparation for a hematopoietic stem cell transplant in patients with cancer. A growing number of examples indicate the clinical potential of passive immunoglobulin therapy. These include treatment of autoimmune arthritis with anti-tumour-necrosis-factor (TNF) antibodies (Taylor et al., 2001) and the treatment of breast cancer and B-cell lymphomas with antibodies that are specific for HER2/NEU and CD20, respectively (Weiner, 1999). However, although the protection enhanced by passive transferred antibodies is immediate, it is temporary, lasting only so long as the transferred antibodies remain active in the recipient's body.

One of the concerns when using monoclonal antibodies for therapy is that a target antigen must be selected. It is important that this antigen is presented uniquely by all tumor cells and not on normal tissues.

(b) Active immunotherapy

The antibody-based therapies we have been discussing are a form of passive immunotherapy. That is, the effector molecules or substances are introduced into the body, rather than the body creating its own immune response. Vaccines, on the other hand, are considered active immunotherapy because they generate an intrinsic immune response. They are also considered a form of adoptive immunotherapy because they attempt to stimulate an immune response that can directly target the tumor antigens, in contrast to non-specific approaches such as cytokines that broadly stimulate the immune system.

1.1.2. Adoptive immunity and immunotherapy

1.1.2.1. Cytotoxic and helper T cells

Another focus of immunotherapy for cancer is the role of the T cells. T cells are either cytotoxic (CD8+) or helpers (CD4+). Unlike antibodies, which react to intact proteins only, the CD8+ T cells react to peptide antigens presented on the surface of a cell. Peptide antigens derive from proteins that have been digested by the cell, and presented as protein fragments or peptides, bound by MHC and displayed on the cell membrane. CD8+ T cells are specific for class I MHC molecules, while CD4+ T cells are specific for class II MHC molecules.

After attaching to the MHC-peptide complex expressed on a cell, the CD8+ T cell destroys the cell by perforating its membrane with enzymes or by triggering an apoptotic or self-destructive pathway. The CD8+ T cell will then move to another cell expressing the same MHC-peptide complex, and destroy it as well. In this manner cytotoxic T cells (CTL) can kill many invasive cells. Ideally, CD8+ T cells could engender a very

specific and robust response against tumor cells. One of the biggest breakthroughs in tumor immunotherapy in the past several years has been increased understanding of the role of helper T cells. The helper T, or CD4+ T cell, is the major regulator of all immune system activities (Guyton, 2000). These cells form a series of protein mediators called lymphokines that act on other cells of the immune system and on bone marrow. Some of the most important lymphokines secreted by the helper T cells include interleukin-2, interleukin-3, interleukin-4, interleukin-5, interleukin-6, granulocyte-monocyte colony stimulating factor (GM-CSF), and interferon-gamma. Without these lymphokines, the remainder of the immune system does not function as effectively as it would with the appropriate cytokine environment. Like the CD8+ T cells, CD4+ T cells also recognize MHC-peptide complexes in the context of class II MHC. CD4+T cells augment the immune response by secreting cytokines that stimulate either a cytotoxic T cell response (Th1 helper T cells) or an antibody response (Th2 helper T cells). These cytokines can initiate B-cells to produce antibodies, or enhance CD8+ T cell production. The function of the CD4+ T cell depends upon the type of antigen it recognizes, and the type of immune response required.

Lymphokines produced by helper T cells also regulate macrophage response. The lymphokines slow or stop the migration of macrophages after they have been engaged, allowing macrophages to accumulate at the site. These lymphokines also stimulate more efficient phagocytosis, so they can destroy rapidly increasing numbers of toxins. The helper T cell amplifies itself by secretion of lymphokines, particularly interleukin-2. This action enhances the helper cell response, as well as the entire immune system's response, to foreign antigens.

Adoptive immunotherapy involves removing lymphocytes from the patient, boosting their anti-cancer activity, growing them in large numbers, and then returning them to the patient:

LAK cells: Initial experiments in adoptive immunotherapy involved removing lymphocytes from the blood of a patient and growing them in the presence of the lymphokine interleukin-2 (IL-2), an immune stimulator. The cells were then returned to the patient. These lymphocytes were called lymphokine-activated killer (LAK) cells.

TILs: A stronger response against tumor cells is obtained using lymphocytes isolated from the tumor itself. These tumor-infiltrating lymphocytes (TILs) are grown in the presence of IL-2 and returned to the body to attack the tumor. Moreover, radiolabeled monoclonal antibodies for tumor antigens are used to even more closely identify lymphocytes specific for tumor cells.

Adoptive immunotherapy and dendritic cell immunotherapy are forms of cellular therapy, where ex vivo processed cells are introduced into the body. Adoptive immunotherapy uses immune effector cells (e.g., T cells), whereas dendritic cell immunotherapy uses antigen-presenting cells.

1.1.2.2. Cellular adoptive immunotherapy

Cellular adoptive immunotherapy is an treatment used to help the immune system fight cancer. A cancer patient's T cells are harvested and grown ex vivo in order to increase the number of T cells that are able to kill the cancer cells. These cancer-specific T cells are given back to the patient to help the immune system fight the cancer.
Exceptional progress has been made in understanding the molecular and cellular bases of T-cell-mediated anti-tumor responses. CD8+ T cells

have been identified as potent effectors of the adaptive anti-tumor immune response (Boon et al., 2006; Sakaguchi, 2005) (Rosenberg, 2001). The target antigens that are recognized by tumor-reactive CD8+ T cells have been shown to be mostly non-mutated self-antigens that are also expressed by tumor cells (and these antigens are denoted as self/tumor antigens). Tumor-specific CD4+ T cells have been also identified, but their functionality can be controversial because CD4+ T cells can help or hinder anti-tumor immune responses (Pardoll and Topalian, 1998; Sakaguchi, 2005). By contrast, the work of Sun *et* al. has demonstrated that CD4+ helper cells are necessary to both activate and sustain the survival of CD8 killer cells (Sun and Bevan, 2003) . The molecular signals that adjust and modulate T-cell activation, function and memory are currently being elucidated. Both positive and negative signals from co-stimulatory molecules have been shown to shape the anti-tumor response (Chambers and Allison, 1997; Lenschow et al., 1996). Cytokines, including those signaling through receptors that contain the common cytokine-receptor γ-chain , have been shown to alter the programming of effector CD8+ T cells (Chambers and Allison, 1997).
Active immunotherapy with therapeutic vaccines has been attempted despite the presence of many redundant negative influences of the host immune system(Klebanoff et al., 2005), and tumor microenvironment (Khong and Restifo, 2002; Schreiber et al., 2002).
By contrast, adoptive cell transfer (ACT) therapies achieve T-cell stimulation *ex vivo* by activating and expanding autologous tumor-reactive T-cell populations to large numbers of cells that are then transferred back to the patient (Bollard et al., 2004; Yee et al., 2002). Early attempts of ACT therapies using tumor-infiltrating lymphocytes (TILs) and immunoreplete patients met with some success (Rosenberg et al., 1994). However, previous preclinical studies indicated that immune

ablation is an effective preconditioning regimen that can increase T-cell responses after adoptive transfer (Cheever et al., 1980; North, 1982).

1.1.2.3. Cytokines

Cytokines are substances that appear to have application in the treatment of hematologic malignancies or immunogenic tumors. The major cytokines currently in use or under evaluation for cancer therapy include: interferon alfa, IL-2, GM-CSF, and interleukin-12 (IL-12). Recently, an adenovirus vector encoding human interferon gamma (IFN-γ) was successfully used to induce tumor regression in patients with advanced coutaneous T lymphomas and multilesional B cell lymphomas (Dummer et al., 2004)

Interferon alfa has many roles. It upregulates genes like MHC class I, tumor antigens, and adhesion molecules. It is also an anti-angiogenic agent. It is extremely active in the immune system, promoting B and T cell activity. Interferon alfa can stimulate macrophages, even dendritic cells, and upregulates Fc receptors. Interferon has been approved as treatment .in hairy cell leukemia (Quesada et al., 1986). A significant survival benefit of more than 89 months in a phase 2 trial in patients with chronic myelogenous leukemia suggests interferon has activity here as well (Allan et al., 1995; Ohnishi et al., 1995). This survival advantage was independent of cytogenetic improvement with interferon, which was also noted.

Interleukin-2 (IL-2) is a T cell growth factor that binds to a specific tripartite receptor on T cells. In dose escalation studies, patients treated with high doses of IL-2 showed clinical responses, although severe toxicity was seen. Giving IL-2 in high doses is comparable to inducing a controlled state of septic shock. Toxicity is nearly always reversible. A

study of 425 patients evaluated the activity of low-dose IL-2 in combination with interferon-alfa, as well as each agent alone (Negrier et al., 2000). IL-2 has shown activity in non-Hodgkin's lymphoma and leukemia and lymphoma post-stem cell transplant. Low-dose IL-2 was also evaluated in combination with histamine, but no differences in response were observed compared with IL-2 alone.

Granulocyte-monocyte colony stimulating factor (GM-CSF) is a well-known cytokine, approved for use in stem cell and bone marrow transplant to reconstitute the myeloid series. We have data suggesting it also might have application as monotherapy in melanoma (Spitler et al., 2000). In this trial, 48 patients with stage III and IV melanoma were treated with long-term, chronic, intermittent GM-CSF after surgical resection. The theory is that GM-CSF would reconstitute antigen-presenting cells, and thus the ability to mount an immune response.

Interleukin-12 (IL-12) is a heterodimeric protein that promotes NK and T cell activity and is a growth factor for B cells. It has demonstrated anti-tumor activity in mouse models. Alone, IL-12 shows minimal potential for therapeutic effect (Atkins et al., 1997). However, IL-12 may have value as a vaccine adjuvant. When IL-12 was paired with peptide vaccines in patients with stage 3 and 4 melanoma, IL-12 appeared to boost the response to the vaccine (Lee et al., 2001).

1.2. VACCINES

Vaccination, means of producing immunity against pathogens, such as viruses and bacteria, by the introduction of live, killed, or altered antigens that stimulate the body to produce antibodies against more dangerous forms. Vaccination was used in ancient times in China, India, and Persia, and was introduced in the West in 1796 by Edward Jenner. Jenner demonstrated that rubbing or scraping the cowpox virus (the term vaccine comes from the Latin *vacca,* cow) into the skin produced only a local lesion but was sufficient to stimulate the production of antibodies that would defend the body against the more virulent smallpox. Vaccination has eradicated smallpox worldwide and prevents such diseases as cholera, rabies, and typhoid fever. Vaccines work with the immune system's ability to recognize and destroy foreign proteins (antigens) that it determines are non-self. The same principle is used to help the body recognize antigens peculiar to cancer cells. It is also applied in an experimental birth control vaccine that tricks the immune system into believing that human chorionic gonadotropin (HCG), a hormone secreted by a developing fertilized egg, is foreign, thus inactivating it and inducing menstruation even if fertilization has occurred.

Therapeutic vaccination was tested first against infectious disease in 1885 by Louis Pasteur, who successfully vaccinated individuals infected with rabies. Fortunately, in this case the virus grown relatively slowly, giving time for induction of immunity, and it established the principle of vaccination as a treatment. For the bulk of infectious diseases with available vaccines, the preferred setting of prophylactic vaccination was used once the relative safety of vaccination was assured.

The previous treatment of infections by passive transfer of antibodies gradually declined but is still used occasionally for treatment of certain infections (Krause, 1999) Furthermore, antibody therapy has certainly been shown to be effective in the treatment of selected cancers. The advent of monoclonal antibodies allows infusion of measured doses targeted to selected antigens, and antibody is now an important "drug" for the treatment of B cell malignancies (Maloney et al., 1994) and solid tumors. Passive transfer of specific T cells can be used also to attack infections (Riddell et al., 2000) and cancer (Rooney et al., 2001).

For tumors, efficacy of transferred T cells is seen most clearly in allogeneic stem cell transfer, in which donor T cells recognize patient-specific polymorphic antigens expressed by leukemic cells (Goulmy, 1997). Balance between attack on tumor cells or normal host cells has to be clear to avoid damaging graft-versus-host disease.

A similar balance applies to active vaccination against cancer, because many candidate tumor antigens are self-proteins overexpressed by tumor cells (Nanda and Sercarz, 1995). The success of passive immunity has demonstrated the ability of immune effector pathways to suppress cancer, and the challenge for therapeutic vaccines is to prime and maintain those pathways. The idea of activating immunity against cancer is not new.

DNA vaccines provide testable vehicles for inducing immunity against candidate antigens from either infectious diseases or cancer and have now advanced to a crucial point (Stevenson et al., 2004).

1.2.1. DNA vaccines

Deoxyribonucleic acid vaccination has emerged as a simple yet potentially powerful strategy to prevent or to treat various diseases (Gurunathan et al., 2000a). DNA vaccination is a proposed experimental

technique for protecting an organism against disease by injecting it with naked DNA encoding antigenic proteins that are consequently expressed by vaccinated individual in order to produce an immunological response.

The ability of DNA vaccines to induce both humoral and cellular immune responses and to provide protection has been shown in a variety of animal species for a number of infectious disease models (Robinson and Pertmer, 2000). Efficacy of DNA vaccination against a range of cancer models has also been reported (Stevenson and Anderson, 2000). Clinical trials in infectious diseases are showing that DNA vaccines are well tolerated and are capable of inducing antigen-specific immune responses (Boyer et al., 2000; MacGregor et al., 1998; Wang et al., 1998). These encouraging results provide a platform for improvement in both design and in mode of delivery.

Optimal performance will be required for cancer therapy, and the flexibility of DNA vaccine design allows the incorporation of candidate genes that are able to direct and promote immunity. Several of these are now moving to clinical testing.

DNA vaccines are simple vehicles for *in vivo* transfection and antigen production, and the subsequent steps to the induction of immunity are now emerging.

The first is the activation of the innate immune response caused by the presence of hypomethylated CpG dinucleotide sequences with particular surrounding motifs in the bacterial plasmid backbone (Krieg, 2002), which may be a natural response to exposure to bacterial DNA and is a significant operational component of DNA vaccines. The outcome is an outpouring of cytokines including IL-6, IL-12, tumor necrosis factor-α, IFN-γ, and IFN-α (Wagner, 1999) and a polarization of the CD4+ T cell response toward T helper 1 (Th1) dominance. The most effective sequence motifs have been delineated by using synthetic oligonucleotides

and differ among species. Selected oligonucleotides are now being investigated as adjuvants for protein vaccines, with promising results emerging (Krieg, 2002). However, the data from oligonucleotides do not completely explain how plasmid DNA is perceived by the innate immune response. Oligonucleotides are known to require Toll-like receptor 9 (TLR-9) for activity, but DNA vaccines operate normally in TLR-9 -/- mice, pointing to the involvement of additional receptors (Heit et al., 2003).

Recognition of bacterial DNA by the innate immune response can be harnessed by DNA vaccines, with no apparent harmful side effects.

In terms of induction of immunity, there is an influence of the site and procedure used for injection, with muscle and skin cells clearly able to act as antigen depots but unable to prime the immune response. It seems that cross-presentation from these sites to antigen-presenting cells (APCs) is the major route to priming (Corr et al., 1996; Fu et al., 1997), but there is also evidence for direct transfection of APCs, especially when delivery is to skin sites through a gene gun (Porgador et al., 1998). The uncertainty on this point makes rational design more difficult, particularly because the process of cross-presentation is incompletely understood. However, a recent investigation of the route of access of exogenous phagosomes to the MHC class I pathway could have relevance. Phagosomes apparently carry elements of the endoplasmic reticulum (ER), creating organelles capable of antigen processing for induction of cytotoxic T cell responses (Guermonprez et al., 2003; Houde et al., 2003).

The process that conveys antigens to the APC seems highly efficient in that DNA vaccines that produce only very low levels of antigen can induce all arms of the immune response (Gurunathan et al., 2000a). However, there may be different requirements for priming or boosting

immunity, and to activate anti-tumor immunity, both processes need to be efficient. It is also essential that tumor cells alone can boost the vaccine-induced response so that continuing pressure is maintained against emergent cells.

1.2.1.1. Basic components of DNA plasmid vaccines

The DNA vaccines consist of bacterial plasmid DNA containing the gene encoding the antigen of interest, with expression generally driven by a promoter/enhancer derived from cytomegalovirus (Fig. 1). Following injection, the encoded protein is synthesized by the host in a form aimed to induce specific immunity. The bacterial DNA backbone can also contribute to activation of immunity, because of the possible presence of immunostimulatory sequences (ISS). The ISS are composed of non-methylated CpG dinucleotides with specific flanking motifs. CpG motifs are 20 times more common in bacterial DNA than in mammalian DNA and represent a form of pathogen-associated molecular patterns.
As the oligonucleotides incorporating these motifs have immunostimulatory activity, there is considerable interest in using them as immune adjuvants (Krieg, 2002). The intracellular receptor for CpG oligonucleotides is Toll-like receptor 9 (TLR9), which is expressed by B cells, DC and other cells of the innate immune system. Activation leads to an inflammatory response, with production of multiple cytokines including interferon (IFN)-α/β/γ, IL-6, IL-12, IL- 18 and tumor necrosis factor-α (Bauer and Wagner, 2002).

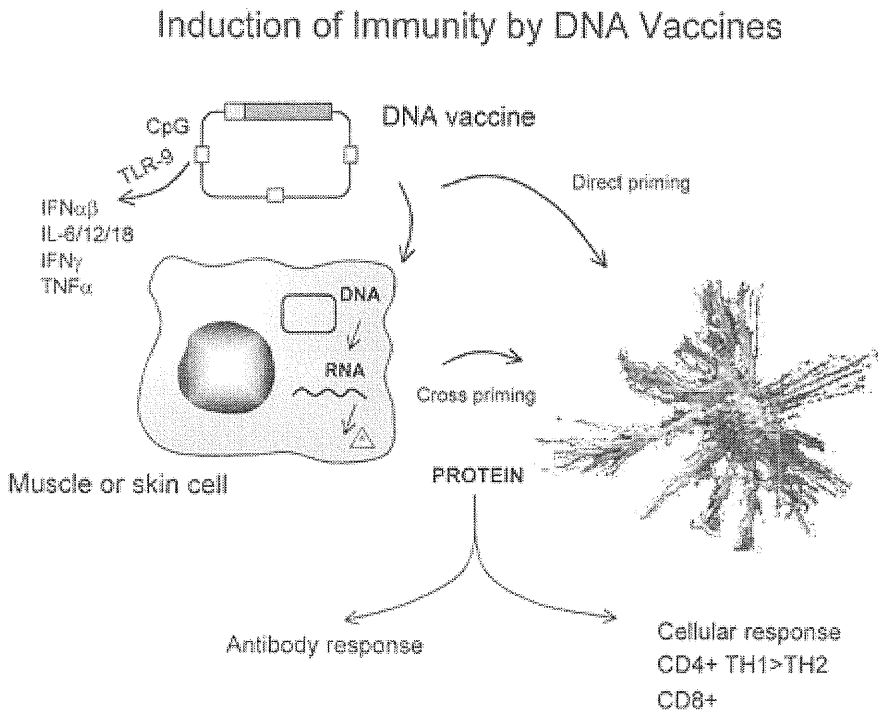


Figure 1. Induction of immunity by DNA vaccines. Plasmid DNA, with CpG motifs in the backbone, activates the innate immune response with production of a range of cytokines. Cross-priming from transfected muscle or skin cells leads to antigen presentation and induction of all arms of the immune response. Direct priming may also occur. Immune outcome depends on the nature of the encoded protein.

CpG oligonucleotides can also stimulate B cell proliferation and maturation of DC. Most studies of CpG effects are made using synthetic oligonucleotides, and comparison with performance of plasmid DNA is rare. Extrapolation from murine studies to human are also difficult, as not all receptors are expressed by the same cell population in each species. For example, the critical TLR9 molecule is expressed by monocyte-derived DC in mice, but only by lymphoid derived plasmacytoid DC in humans.

1.2.1.2. Mechanism of action of DNA vaccines

The DNA vaccines are usually administered by intramuscular (i.m.) or intradermal (i.d.) injection, or into skin cells with a gene gun, and antigens are produced endogenously by transfected cells. When DNA vaccines are delivered through the id route, the skin-derived DC (Langerhans cells)
are directly transfected and probably play a key role in antigen presentation to T cells (Akbari et al., 1999; Condon et al., 1996; Porgador et al., 1998).
Vaccine delivery by im injection predominantly results in the transfection of myocytes (Fig xx). However, muscle cells do not express MHC class II and co-stimulatory molecules that are critical for effective T-cell priming. Instead, there is compelling evidence that the bone marrow-derived antigen-presenting cells (APC), presumably DC, play a predominant role in the activation of T cells through a process termed 'crosspriming', whereby the antigen released by muscle cells is taken up, processed and presented on MHC class I by the bone marrow derived professional APC (Corr et al., 1996; Corr et al., 1999). The mechanism of this transfer is unclear but could involve ferrying of proteins or peptides via heat shock proteins (Srivastava, 2000). However, i.m. injection may also result in transfection of a small number of bone marrow-derived APC that could directly activate T cells (Casares et al., 1997). The outcome of DNA vaccination is to induce all arms of the immune response with a tendency for dominance of the Th1 subset of the CD4+ T-cell population (Gurunathan et al., 2000a).

1.2.1.3. Strategies for optimizing DNA vaccine performance

For maximizing the performance of DNA vaccination against infectious organisms there is the opportunity to modify sequence by codon

optimization, and to insert leader sequences to allow entry to the endoplasmic reticulum and secretion. The latter is particularly important in amplifying immune responses, but it has to be acknowledged that DNA vaccines are generally not as efficient as protein vaccines in inducing antibody responses. Part of this is because of the tendency to drive a Th1 dominant response, but the low levels of antigen produced may be another factor. To increase antigen levels, and therefore amplify antibody responses, there are physical methods available to increase transfection rates, such as electroporation (Tollefsen et al., 2002) or delivery as microparticles (Otten et al., 2003).

A popular strategy is to increase responses by 'prime / boost', which commonly uses a priming injection of naked-DNA followed by a second injection of DNA within a viral vector, such as modified vaccinia Ankara (MVA) (Schneider et al., 1998). This combined approach is showing encouraging results using a malaria antigen (McConkey et al., 2003). Clearly, various viral vectors can be used but there is a potential disadvantage for cancer, where it is likely that multiple injections will be required to control emergent tumour cells. The problem is that if viral proteins are made, they will be immunogenic and the immune response may then suppress the effect of further boosts. Vectors that make few proteins, such as alphaviruses, are being investigated and may be useful (Smerdou and Liljestrom, 1999).

The in vivo transfection efficiency of antigen presenting cells, such as dendritic cells, can certainly be enhanced by effective cytokine- or ligand-induced recruitment of these cells to the site(s) of immunization of the DNA vectors. Delivery of DNA to mucosal surfaces (e.g., intravaginally or intranasally) and directly to lymph nodes (Maloy et al., 2001) are examples of promising approaches that are likely to undergo expanded testing in coming years. If DNA can be effectively delivered transcutaneously, via bacterial carriers (Mollenkopf et al., 2001), or boosted orally through food ingestion (Webster et al., 2002), much of the

current research on regimens may need to be revisited. At this time, DNA vaccines are still in early development. The last 15 years of intensive DNA vaccine research in animal models have provided sufficient success to act as an incentive for further development of this powerful tool.

1.2.1.4. DNA vaccination against cancer

For the 10–20% of cancers, including cervical and gastric cancers, that are highly associated with an infective organism (*HPV* and *H.pylori* respectively), there is clear potential for prophylactic vaccination (Eckhart, 1998). However, in common with certain infections, such as human immunodeficiency virus and hepatitis C, most cancers will require therapeutic vaccination, which is a greater challenge for vaccine performance.

There are many candidate tumor antigens which can be delivered via DNA, and identification of aberrant cancer-associated molecules at the genetic level can be followed by incorporation of target sequences into a DNA vaccine. Data from gene expression profiling, which detects new candidate antigens, or which reveals overexpression of potential target molecules, can be linked directly to testing in models. However, there are important considerations to apply, in that candidate proteins must be expressed efficiently following transfection, and they must be delivered in an immunogenic form that will activate an appropriate effector pathway.

1.2.2. Viral vector vaccines

With the aim of improving Ag immunogenicity, the power of DNA vaccines can be combined to the advantages of a viral delivery system. One advantage of viral vectors is their intrinsic ability to initiate immune responses, with inflammatory reactions occurring as a result of the viral infection creating the danger signals necessary for immune activation. An ideal viral vector should be safe and should not introduce an anti-vector immune response to allow for boosting anti-tumour-specific responses. Recombinant viruses such as vaccinia viruses, herpes simplex viruses, adenoviruses, adeno-associated viruses (AAV), retrovirues and avipox viruses have been used in tumour animal models and, based on their encouraging results, human clinical trials have been carried out in solid tumours (Marshall et al., 1999; Marshall et al., 2000). These trials have demonstrated that recombinant viruses can break immune tolerance against self and/or weakly immunogenic tumour antigens. However, the employment of these vectors in immunocompetent animals is limited by the development of neutralizing antibodies, compromising repetitive administration.

Among these virus, AAV presents some features that make it particularly attractive. In fact, in contrast to other viral vectors, such as vaccinia virus or adenovirus, AAV does not express any viral genes. As in immunization with naked DNA, the only gene expressed is the antigen itself.

1.2.2.1. AAV vector vaccine

AAV are small (20-25nm), non-enveloped, linear single-stranded DNA (ssDNA) *Parvoviruses*. To date, seven molecular clones have been generated representing the serotypes of AAV (1, 2, 3a, 3b, 4, 5, 6). Serological studies on these clones suggest that AAV-2, -3, -5 and -6

frequently infect human population, while AAV-1 and -4 infects monkeys. Transduction of these viruses naturally results in latent infection, with complementation of the life cycle requiring helper functions not associated with AAV-viral gene products. In the absence of a helper virus or genotoxic stress, AAV integrates into the long arm of chromosome 19 (q19.3-qter) known as the AAVS1 site and enters a latent phase.

The genome of AAV is approximately 4.7 kb. The DNA is flanked by inverted terminal repeat (ITRs) of 145bp. The ITR serves as origin of DNA replication and are required for packaging. Also, the ITRs are the only CIS element essential to the AAV replication life cycle. Between the ITRs are two sets of ORFs, *Rep* and *Cap*. The *Rep* ORF encodes for nonstructural proteins that regulate AAV replication and site-specific integration, while ORF *Cap* codifies three structural proteins (VP1-3).

Trasducing vectors have been initially constructed from cloned proviral genomes of the AAV-2 serotypes by deleting the REP and Cap ORFs and replacing the deleted DNA with a transgene between the ITRs. rAAV were used to transfer genes into a wide variety of mammalian cells *in vitro* as well as *in vivo* and were able to transduce both dividing and non dividing cells. They were successfully used for short-term application, such as the delivery of suicide genes to tumours (Okada et al., 1996), as well as for application requiring long-term transgene expression in immunocompetent animals. These vectors in fact can transduce cells by a variety of mechanism, including episomal transgene expression (Duan et al., 1998) or random chromosomal integration of the vector genome (Rutledge and Russell, 1997) . If transduction is performed in the presence of *rep* gene, vectors can also integrate at the site-specific integration locus of wild-type AAV (Bertran et al., 1998).

Despite the capacity to transduce different type of cells, the rAAV-induced long-term expression is established mainly in tissues composed

of slow growing or post-mitotic cells (Xiao et al., 1996). These include the central nervous system, skeletal muscle, the lung and the liver. For example, therapeutically relevant serum levels of clotting factor IX are reliably established in mice following a single intramuscular injection of AAV vectors encoding factor IX (Herzog et al., 1997). Similar results have been observed with a diverse group of other transgene, including β-galactosidase (Xiao et al., 1996) and erithropoietin (Epo) (Kessler et al., 1996) expressed in muscle, tyrosine hydroxilase (Kaplitt et al., 1994) and the GABA receptor (Xiao et al., 1997) expressed in the brain and the cystic fibrosis transmembrane conductance regulator expressed in the lung (Conrad et al., 1996). In all cases examined above, the transgene expression remains at a stable level for more than 4 months and the level of expression is linearly dependent on the dose of vector. Due to this ability to induce long-term transgene expression, AAV vectors have been used also in the treatment of genetic disease, such as β-talassemia, hemophilia B, cystic fibrosis, Parkinson's disease and inherited retinal degeneration. However, the use of AAV in clinical trials is in partial limited by the humoral neutralizing immune response in treated individuals. In fact, infection by wild-type AAV results in the production of neutralizing and complement fixing antibodies and 50 to 80% of adults have neutralizing antibodies to AAV, with antibodies against AAV-2 being the predominant serotypes (Blacklow et al., 1968). The fact that additional transduction events are not observed after readministration of AAV-2 vectors in animals, suggest that the host immune response can completely prevent transduction (Halbert et al., 1997). This problem may be now overcome by using different vector serotypes, which in fact elicit distinct humoral responses (Rutledge et al., 1998).

Besides its employment in the treatment of genetic diseases, AAV has been successfully used as tool to genetic immunization. Manning *et al.* demonstrated that intramuscular injection of mice with an AAV vector expressing herpes simplex virus type 2 glycoprotein B led to the

generation of both CTLs and antibody-mediated anti-gB responses (Manning et al., 1997). Interestingly, this immunization strategy was more effective than plasmid DNA or protein in generating antibody responses. Intramuscular injection was also efficiently used to *in vivo* deliver a chimeric gene containing an epitope of HPV E7 protein fused with the heat shock protein gene. A single immunization could eliminate tumour cells in syngenic animals and induce CD4- and CD8-dependent cytotoxic activity *in vitro* (Liu et al., 2000).

It should be noticed, that AAV was found able to efficiently transduce human monocytes as well as *in vitro* generated DCs (Ponnazhagan et al., 2001), but in a different work Xin *et al.* reported the low efficiency of AAV infection in mouse bone marrow DC, highlighting real limits to the reliable employment of this viral vectors for ex-vivo immunotherapy (Xin et al., 2002).

However, proteins, plasmidic DNA as well as vectors based on different viruses, such as retrovirus or adenovirus, are currently widely and used to pulse DCs and have been found to successfully induce cancer immunity *in vivo (Akiyama et al., 2000; Chen et al., 2003; Paglia et al., 1996; Wan et al., 1997).* One limit to the employment of DCs in clinical trials depends on the intrinsic difficulty to obtain a large numbers of clinical grade human DCs (Caux et al., 1992; Sallusto and Lanzavecchia, 1994). In addition, the finding that two healthy volunteers receiving immature DCs pulsed with influenza matrix peptide (FMP) had a reduction in FMP-specific $CD8^+$ T cell activity raised further concerns about the use of immature DCs (Dhodapkar and Steinman, 2002). Therefore, despite the promising efficacy of this approach to circumvent some strategies that tumours use to evade immune system, there are still many technical issues to be resolved for a wide and safe employment of DCs in cancer immunotherapy.

1.2.3.Principles of "Prime-boosting"

Prime–boosting has emerged as a powerful approach for establishing both, humoral and cellular immunity and recent results have demonstrated the efficacy of prime–boost vaccines in generating protective immunity in both animal models and in the clinic. The idea of 'boosting' immune responses has been around as long as vaccines and repeated administrations with the same vaccine (homologous boosting) have proven very effective for boosting humoral responses. However, this approach is relatively inefficient at boosting cellular immunity because prior immunity to the vector tends to impair robust antigen presentation and the generation of appropriate inflammatory signals. One approach to circumvent this problem has been the sequential administration of vaccines (typically given weeks apart) that use different antigen-delivery systems (heterologous boosting). The initial priming events elicited by a first exposure to the expressed antigens appear to be imprinted on the immune system. This phenomenon is particularly strong in T cells and is exploited in prime–boost strategies to selectively increase the numbers of memory T cells specific for a shared antigen in the prime and boost vaccines. The basic prime–boost strategy involves priming the immune system to a target antigen delivered by one vector and then selectively boosting this immunity by readministration of the antigen in the context of a second and distinct vector. The key strength of this strategy is that greater levels of immunity are established by heterologous prime–boost than can be attained by a single vaccine administration or homologous boost strategies. With someof the earlyprime–boost strategies this effectwas merely additive, whereas with some of the newer strategies (usually involving poxvirus or adenovirus boosting) powerful synergistic effects can be achieved. This synergistic enhancement of immunity to the target antigen is reflected in an increased number of antigen-specific T cells, selective enrichment of high avidity T cells and increased efficacy

against pathogen challenge (Estcourt et al., 2002; McShane, 2002). In addition, although early studies focused predominantly on CD8+ T-cell responses, it has now become clear that both CD4+ and CD8+ T cells can be strongly induced using appropriate prime–boost strategies.

1.3. TUMOR IMMUNITY

Tumor regression in vivo is mediated by a complex interplay between two main mechanisms: innate and adaptive immune response (Tables 1,2), that is involved with the immune recognition of cancer cells. Innate mechanism [involving soluble and cellular components, (Table 1) (Diefenbach and Raulet, 2002; Dranoff, 2004) may trigger inflammatory events in the tumor microenvironment and in presence of a local adequate cytokine combination (IL-2, IL-12, IL-18, IL-23), stimulate dendritic cells (DCs) (Palucka and Banchereau, 1999), the most specialized antigen presenting cells (APCs), to react against tumor specific surface antigens (TAAs) (Davis et al., 2003; Schirrmacher et al., 2003). After engulfment by DCs, TAAs are presented to naive T cells associated to MHC (Lord and Frelinger, 1998). Naive T cells activation occurs when the antigenic peptide-MHC complex interacts with the T Cell Receptor (TCR).

• Dendritic cells	• Complement
• Neutrophils	• Fever
• Macrophages	• Defensins
• NK cells	• Interferon producing cells
• Cytokines	• B Cells
• Chemokines	• γ δ T cells

Table 1. Principal cellular and soluble anti-tumoral components of innate immune system

However TCR receptor binding is not sufficient for a full activation of T cells unless costimulatory molecules interact with the specific ligands on

the surface of APC. The presence or absence of costimulatory signals like B7-1(CD80), B7-2(CD86) and CD40/CD40L, determines whether immune response becomes anergic or tolerant. CD40/CD40L is expressed transiently following TCR activation on the surface of $CD4^+$ cells and it is a key molecule in mediating the activation of B cells and in controlling CD8 T cells. Antigens can be associated to MHC I or MHC II class complexes and are presented by DCs to TCR of $CD8^+$ and $CD4^+$ T cells, respectively associated to the proper costimulatory molecules (fig.1). Both $CD4^+$ and $CD8^+$ cells after activation and costimulation produce a series of cytokines that differentiate T- Helper ($CD4_+$) lymphocytes in two subpopulations (T_H 1, T_H 2 cells). TH 1 cells produce IL-2, IFN-γ, TNF-α and granulocyte macrophage colony stimulating factor (GM-CSF) that increase the activity of macrophages, and the expression of MHC class 1 molecules on the surfaces of $CD8^+$ cells. T_H 2 secretes another group of cytokines IL-4, IL-5 and IL-10, that induces naive B cells to produce antibodies. The shifting towards T_H 2 pattern has recently been associated with an increased tumor metastasisation and a decreased survival in many human and animal neoplasia (Davis et al., 2003; Nishimura et al., 1999).

• Dendritic cells	• B Cells
• Neutrophils	• CD4+
• Macrophages	• CD8+

Table 2. Principal anti-tumoral components of adaptive immune system

CD8+, cytotoxic T cells (CTLs) are the major effectors of tumor regression (Titu et al., 2002), however CD4+T cells collaborate to their activation. Once activated CTLs do not need costimulation, since MHC-1-bound antigen is sufficient. For eliminating target cells (neoplastic cells) CTLs

use three effector molecules: Perforins, Granzyme and Fas ligand. Associated to these killing mechanisms, CTLs secrete specific cytokines, such as: IFN-γ, TNF-α and TNF-β. This pattern of cytokines plays an important role in the activation of macrophages which can exert a direct tumor cytotoxic or, conversely, stimulate tumor progression, depending on the tumor microenvironment (Carlos, 2001). (Fig.2)

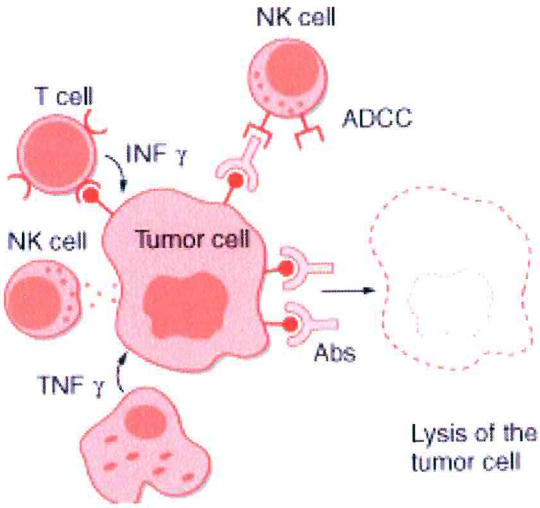


Figure. 2. Mechanisms responsible for death of tumor cells co-incubated with immune effector cells or antibodies. T cells, which recognize antigens expressed by the tumor, became activated and produce interferon IFNγ and lymphotoxin, which contribute to tumor cell death, as well as other cytokines capable of activating natural killer (NK) cells and macrophages. NK cells release perforin- and granzyme-containing granules. Macrophages release tumor necrosis factor TNFγ and other cytokines as well as reactive oxygen intermediates. Antibodies (Abs) to tumor-specific antigens (TSAs) induce arrest of tumor growth or mediate antibody-dependent cellular toxicity (ADCC) by arming effector cells endowed with FcR (NK cells or macrophages).

Other cells morphologically and functionally distinct, such as Natural Killer cells (NKs), macrophages and neutrophils, use pattern-recognition receptors and other cell-surface molecules to detect tumor cells directly (Carlos, 2001; Dranoff, 2004). Differently from T cells, NKs inhibit tumor growth in a MHC-non restricted manner. Frequently tumor cells (like stressed cells) express on their surfaces different glycoproteins (MICA and MICB) which function as ligands for NKG2D receptors on NK cells. Once activated, these receptors stimulate NK cell activity (Fauriat et al., 2003). By contrast, DCs use CD36 and $\alpha_v\beta_5$ integrin to recognize and phagocytize apoptotic tumor cells. Apoptotic tumor cells in turn release Heat Shock Proteins that, after specific interaction with CD91 receptors

on DCs, induce their maturation: thus providing an immune response (Hoos and Levey, 2003) (Fig.3)

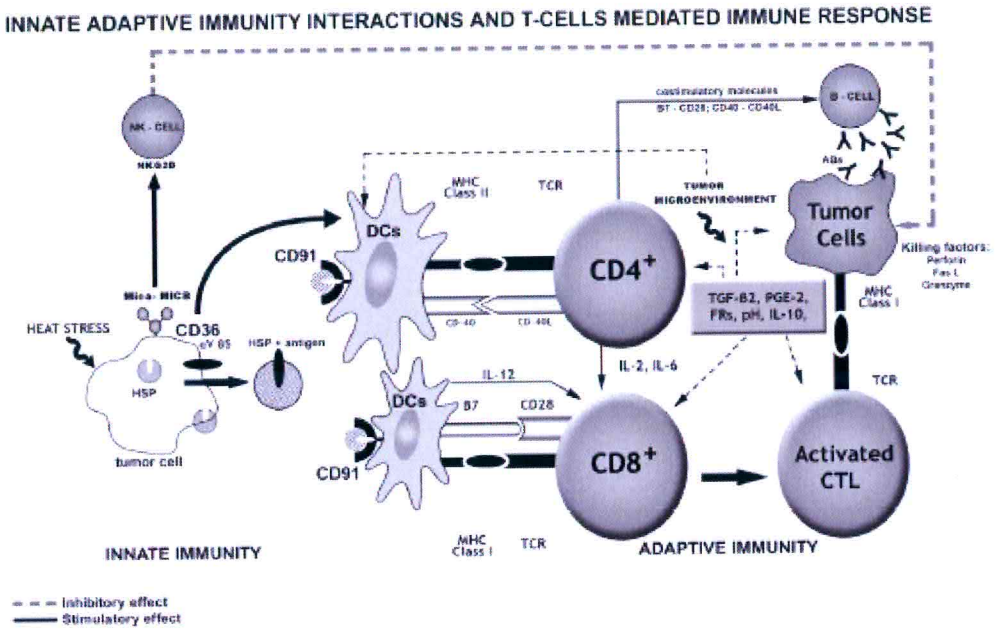


Figure 3. In this diagram the various mechanisms elicited by stress for stimulating innate and adaptive immunity against cancer are illustrated. DCs= dendritic cells; CTL= cytotoxic T lymphocytes; TCR= T-cell receptor; MHC= major histocompatibility complex; Abs= antibodies; FRs= free radicals; TGFB= transforming growth factor-β; PGE2= prostaglandins of E2 type.

1.3.1. Tumor markers

The largest number of immunologically defined tumour antigens has been discovered by the serological screening of phage-display libraries from tumours using the serum of cancer patients (Gure et al., 1997; Sahin et al., 1995). Although this approach identified hundreds of antigens recognised by antibodies circulating in cancer patients, it does not provide insights into which of these antigens could generate effective antitumour immunity. The screening of tumour libraries using tumour-reactive T cells from cancer patients is considered generally to be a

superior approach to identify tumour-rejection antigens, but this approach requires established T-cell lines and clones (Boon and Old, 1997; Robbins and Kawakami, 1996). Selective expression by the tumour relative to normal tissues is often considered to be an important feature of a tumour antigen; however, tissue-specific differentiation antigens are also potential targets in tumours of dispensable tissues, such as melanoma and prostate cancer. So, the quality of any candidate tumour antigen as an immunotherapeutic target depends on several interrelated factors, including tissue distribution, the T-cell repertoire and any pre-existing tolerance. Tumour antigens can be divided in categories:

- antigens unique to individual tumors, that arise as a result of somatic mutations in normal gene products (Mut1) (Mandelboim et al., 1995)
- tumour specific antigens, that are expressed in tumour cells but not in normal tissue and could be shared among cancer patients. This group is further subdivided into two subgroups. One subgroups consist of antigen that arise from mutations related to the oncogenic process. The second subgroup consist of viral antigens present in cancer of viral etiology, such as Epstain Barr virus (EBV)-induced lymphoma and human papilloma virus (HPV)-associated cervical cancer.
- tissue-differentiation antigens, that correspond to normal gene products with highly restricted tissue distribution.
- ubiquitous antigens expressed by normal and malignant cells.
- Cancer–testis antigens (CTAs), represented by proteins with restricted expression among tumor cells and germinal tissues including the family of MAGE genes (MAGE-A-1 to 12) (Boon and van der Bruggen, 1996) , BAGE, GAGE, LAGE, SSX-1 to 9, and NY-ESO-1 (Zendman et al., 2003), are also overexpressed in some hematologic malignancies.

- Differentiation antigens are shared among tumors that correspond to normal tissue specific gene products.

1.3.2. Target Antigens

Target antigens of hematological malignancies are expressed in different molecular forms, with distinct immune effector pathways appropriate for each. For example, glycoproteins at the cell surface, such as the surface Ig of B-cell malignancies (Stevenson and Stevenson, 1986), or the clonotypic T-cell receptor of T-cell tumors (Thirdborough et al., 2002), are susceptible to antibody attack. However, the vast majority of potential targets arise from intracellular proteins and are expressed only as peptides associated with MHC Class I or Class II molecules. The list includes novel or mutated peptides, the so-called cancer-testis antigens with expression limited to cancer or the testis, overexpressed autoantigens (Henderson and Finn, 1996) and the expanding category of proteins identified by microarray analysis. Attack on these must engage $CD8^+$ or $CD4^+$ T cells, and for autoantigens, careful assessment of possible consequences of autoimmunity must be made. The third category of tumor antigen includes secreted proteins, with the best studied example being the clonal Ig of multiple myeloma. It is becoming clear that immune $CD4^+$ T anti-idiotypic cells can attack MHC Class II-negative myeloma cells via an indirect process (Dembic et al., 2000), and this approach is now being tested in patients. Potential antigens for targeting on leukemia cells fall into several major categories (Fig. 4). First, many malignancies are associated with viruses, which will present unique epitopes.

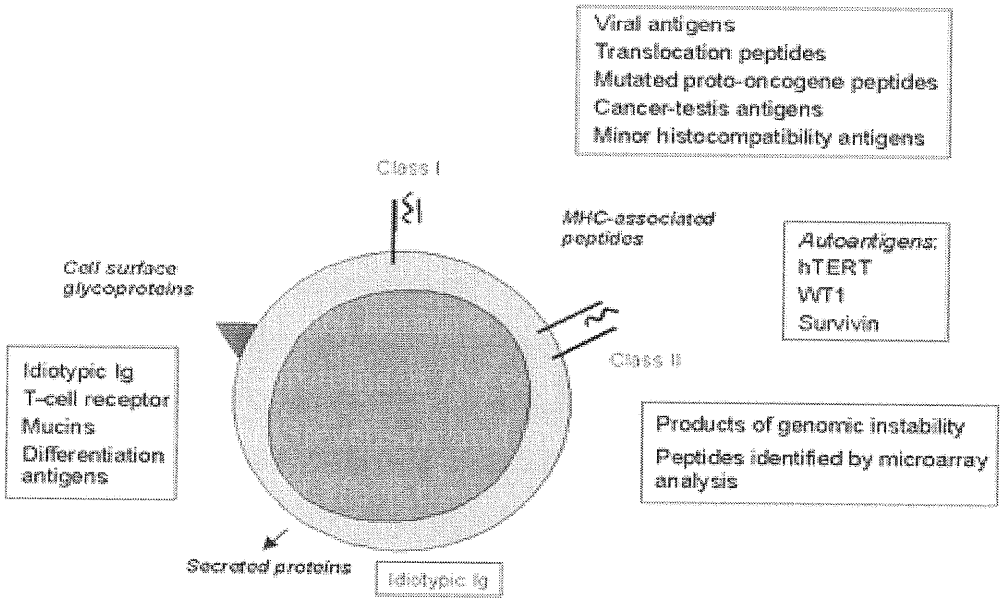


Figure 4 . Target tumor antigens of hematological malignancies. Target antigens can be expressed in 3 molecular forms: as cell surface glycoproteins, as peptides associated with the MHC Class I or Class II molecules, or as secreted proteins.

Epstein-Barr virus is associated with lymphoma in immunodeficient patients as well as with a subset of Hodgkin's disease and non-Hodgkin's lymphoma. Simian virus 40 has recently been reported to be significantly associated with some types of non-Hodgkin lymphoma (Vilchez et al., 2002). Another category of targets is differentiation antigens that are selectively expressed in tumor cells such as Proteinase 3 (PR-3), which is a serine protease overexpressed in CML and AML progenitors (Molldrem et al., 2000) and WT-1, which is expressed at higher level in leukemia than in normal hematopoietic cells (Gao et al., 2000).

1.4. B CELL LYMPHOMA

1.4.1. Non-Hodgkin B cell lymphoma

Non-Hodgkin lymphoma (NHL) is a heterogenous group of lymphoproliferative malignancies with differing patterns of behavior and responses to treatment (Armitage, 1993). Most (80-90%) NHLs are of B-cell origin. It usually originates in the lymphoid tissues and can spread to other organs. However, unlike Hodgkin disease, NHL is much less predictable and has a far greater predilection to disseminate to extranodal sites. The prognosis depends on the histologic type, stage, and treatment.

In general, with modern treatment of patients with NHL, the overall survival rate at 5 years is approximately 50-60%. Thirty percent of patients with aggressive NHL can be cured. Most relapses occur in the first 2 years after therapy. The risk of late relapse is higher in patients with a divergent histology of both indolent and aggressive disease (Cabanillas et al., 1992). A subset of aggressive lymphomas, Burkitt lymphoma and lymphoblastic lymphoma, are designated as high grade by the International Working Formulation (IWF) to reflect the rapidly progressive behavior of these subtypes.

In most cases, the causes of NHL are unknown. Immunodeficiency, including rare inherited diseases such as severe combined immunodeficiency and hypogammaglobulinaemia (Filipovich et al., 1992; Mueller and Pizzo, 1995), increases the risk of NHL. AIDS and posttransplant lymphoproliferative disorders can also lead to NHL. Patients with several autoimmune disorders (eg, Hashimoto's thyroiditis, coeliac disease) also have an increased risk of developing NHL (Mueller

and Pizzo, 1995). Some infectious agents are associated with NHL. Epstein-Barr virus has a role in the development of Burkitt's lymphoma, the nasal type of peripheral T-cell lymphoma, post-transplant lymphoproliferative disorders, some types of NHL associated with congenital immunodeficiency and AIDS, and in primary effusion lymphomas (DeVita and Bleickardt, 2001; zur Hausen, 1991). Human herpesvirus 8 is associated with primary effusion lymphomas and multicentric Castleman's disease (Cesarman et al., 1995; Soulier et al., 1995). The human T-cell lymphotrophic virus type I causes adult T-cell leukaemia or lymphoma and is most prevalent in southern Japan, South America, Africa, and the Caribbean (Blayney et al., 1983).Also, *Helicobacter pylori* has been implicated in the pathogenesis of gastric lymphoma (Wotherspoon, 1998).

1.4.2. Immunotherapy of NH B cell lymphoma

1.4.2.1. Idiotypic vaccination

The aim of vaccination is to target tumor cells not eradicated by current protocols, preferably in the setting of minimal disease load. The power of the immune system is clear from the effectiveness of passive immunity. Monoclonal antibodies, such as anti-CD20, now have a place in treatment of B-cell malignancies (Maloney et al., 1994). Similarly, passive transfer of cellular immunity from allogeneic transplant donors can suppress leukemia via recognition of minor histocompatibility antigens (Goulmy, 1997). The immune system is capable of attack and can maintain immune vigilance, features that could be usefully turned against cancer cells. A multiple immune attack on several target antigens should prevent the escape of tumor cells by the same principle as combination chemotherapy, but without the collateral damage.

Vaccination needs to activate the appropriate effector mechanism against chosen targets. Gene-based approaches facilitate rapid testing of vaccine designs, and insertion of genes encoding additional molecules can amplify and direct immune outcome.

Early studies in preclinical models have consistently demonstrated that vaccination with Id protein can suppress Ig expressing B-cell tumors (Stevenson et al., 1990). In lymphoma, vaccination with Id Ig, either alone or coupled to keyhole limpet haemocyanin (KLH), was able to induce protective immunity (Kaminski et al., 1987). The surprise at that time was that protection was largely mediated by anti-Id antibody. The additional advantage of active vaccination is that, in the presence of a polyclonal anti-Id antibody response, the tumor cell can not escape attack by modulating expression of a single Id determinant (Maloney et al., 1994). A further bonus is that CD4+ T cells can be induced, which may act as effector cells against the tumor (King et al., 1998). Success of Id protein vaccination in models (George et al., 1988; Kaminski et al., 1987), even in a therapeutic setting (Campbell et al., 1988), led to a clinical trial of Id protein coupled to KLH in patients with follicular lymphoma with encouraging immune responses (Hsu et al., 1997).However, lymphoma immunoglobulin itself appeared poorly immunogenic but induced high titers of anti-Id (anti-Idiotypic) antibodies and protection against tumor challenge when linked to a strong immunological carrier (keyhole limpet hemocyanin: KLH) (Campbell et al., 1990; George et al., 1988; Kaminski et al., 1987) even in animals with established lymphoma (Campbell et al., 1988). These pioneering studies demonstrated that, although nominally self-antigens, idiotypic determinants can become immunogenic when administered in a context that allows to overcome T-cell tolerance. These findings prompted for searching new simple methods to obtain idiotypic antigen.

1.4.2.2. DNA Vaccines Against Id antigens

The DNA vaccination against Id antigen was developed at an early stage of knowledge, as it was driven by the technical problem of preparing individual patient-specific Id vaccines (Hawkins et al., 1994). For B-cell tumors, the encoding VH and VL sequences can be readily identified, and can be assembled in a variety of molecular forms. One approach was to encode the two-chain Id Ig molecule within one cassette. In this case, the constant region sequences were derived from xenogeneic Ig, thereby providing some foreign protein to stimulate the immune response. This construct was effective in models, but a clinical trial in lymphoma patients produced only modest evidence of induction of immunity, even when GM-CSF was co-injected (Timmerman et al., 2002)

B cell tumors have several advantages for testing vaccine approaches, the main one being the expression of a defined tumor specific antigen, the idiotypic Ig (George and Stevenson, 1989). In B cell lymphoma, Id Ig is expressed at the cell surface, and the variable region sequences offer clone-specific targets for antibody or T cell recognition (Fig.5). Although Id Ig is a membrane-bound protein, Id-derived peptides are presented in association with MHC class II and can therefore be targeted by CD4+ T cells (Wilson et al., 1990). Id peptides also may be presented in association with MHC class I molecules (Cao et al., 1994), but the sequence restriction imposed for binding on peptides that differ between each B cell tumor hampers attempts to induce CD8+ T cells for therapeutic vaccines in human subjects.

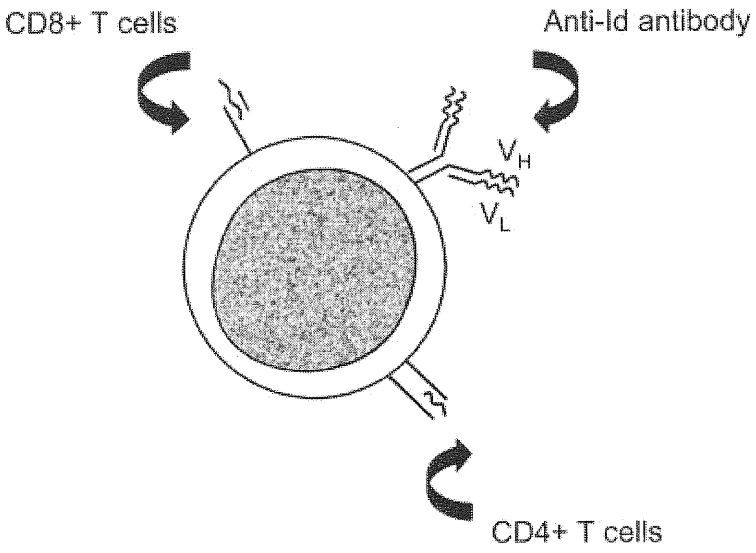


Figure 5. Target Id Ig antigen expressed at the surface of B cell malignancies. Id Ig is a clonotypic tumor-specific antigen expressed as a cell-surface glycoprotein available for antibody attack. Id peptides can also be expressed in the groove of the MHC class I or II molecules, in which they can be recognized by $CD8^+$ or $CD4^+$ T cells, respectively.

The first attempts to use a defined antibody to treat human B cell malignancy was with anti-Id antibody (Miller et al., 1982), which remains an effective strategy. The problem is that each Id Ig is different, and it is expensive and technically demanding to make individual anti-Id antibodies. A slightly easier approach is to use the Id Ig as a protein vaccine, which has succeeded in preclinical models (Stevenson et al., 1990) and in pilot clinical trials (Hsu et al., 1997). However, even making the Id Ig protein is difficult, and genetic technology appeared more attractive.

It is relatively simple to identify and isolate the variable (V) region genes, VH and VL, which encode the Id determinants, on an individual patient basis. Therefore, this antigen is a completely safe, tumor-specific target for testing in a DNA fusion vaccine format.

Assembled variable region genes as a single-chain Fv (scFv) sequence, are known to be capable of folding to display the Id determinants of the

parental Id Ig (Hawkins et al., 1994). Once in a DNA vaccine, it soon became clear that scFv alone was a typical weak antigen, and even injecting human scFv into mice induced only poor antibody responses. Addition of a gene to enhance T cell help was required. This has been achieved by genetically linking the idiotypic sequence of the desired Id to a cytokine sequence such as GM-CSF (Gurunathan et al., 2000b; Tao and Levy, 1993) or to different $CD4^+$ T-cell epitope carriers. King et al. tested plasmid-encoding idiotype (scFv) as a vaccine in the A31 lymphoma model (King et al., 1998). Initially, only low anti-idiotypic levels and poor tumor protection were achieved when the Id was used alone. However, when the scFv gene was genetically linked to a cDNA encoding the FrC of tetanus toxin, the majority of the animals tested were protected with significant enhancement of the anti-Id antibody response. On the same line, in the 38C13 lymphoma model, Syrengelas et al., showed that provision of a cDNA encoding a constant region of human Ig linked to the Id gene was required for specific anti-Id antibody induction (Syrengelas et al., 1996).

An ongoing phase I/II clinical trial in patients with NHL is evaluating a DNA vaccine incorporating the idiotypic scFv encoding fused to the gene of FrC of tetanus toxin within the same vector (Zhu et al., 2001). Another trial involved vaccinating lymphoma patients in remission after chemotherapy, with DNA encoding a chimeric Ig molecule (Timmerman et al., 2002). This contained the variable H and L chain Ig sequences derived from each patient's tumour, linked to the mouse IgG2a and kappa Ig constant regions chain, respectively. Althought the safety profile of the immunization procedure was demonstrated, only 1 of 12 patients developed a T-cell response specific to autologous Id upon treemonthly i.m. injections of the DNA in three dose escalation. In addition, two subsequent boosts with Id-DNA and the GM-CSF-DNA failed to improve anti-Id immune response.

The ease of manipulation and relatively low cost of production facilitates rational design and evaluation of DNA respect to protein vaccination strategies. These vaccine formulation could also be combined with other strategies, such as in *vivo* electroporation (Smith and Nordstrom, 2000) and DNA prime-recombinant virus boost (Ramshaw and Ramsay, 2000), to further enhance the vaccine potency.

1.5. DENDRITIC CELLS IN IMMUNOTHERAPY

1.5.1. Dendritic cells and anti-tumor immune response

The main role in immunological recognition, distinguishing 'self' from 'non-self' and activation of immune response, has been drown aside from the key role of lymphocyte to the main role of dendritic cells (DCs) – professional antigen-presenting cells (Matzinger, 2002; Medzhitov and Janeway, 2002). It was shown that DCs could activate both arms of the immune system: innate (Aderem and Ulevitch, 2000) and adaptive (Schnare et al., 2001), via recognition of 'non-self' in the presence of the so-called 'danger signals' during DC maturation. Moreover, the innate immune response plays an important role in the induction of subsequent acquired immunity and successful memory formation (Akira et al., 2001). Knowledge of the molecular mechanisms of immune response allowed development of a wide list of techniques based on the fine tuning of the immune system to treat some previously non- curable diseases.
One of the promising fields is the control of malignant growth. The idea came out in the early 1900 when for the first time Paul Ehrlich proposed that tumor cells can arise from the body normal cells, but usually neoplastic cells should be eliminated by the immune system. Later, Lewis Thomas has demonstrated that the main cells involved in tumor elimination are cytotoxic T lymphocytes (CTLs). The main

problem is that the result of interaction between the immune system and new antigen at the periphery depends on the microenvironment of the meeting (Ohashi et al., 1991).
Generally, when a pathogen injury (penetrate, invade) the body at the periphery, a lot of 'alarms' or 'danger signals' like reactive oxygen intermediates, extracellular matrix components, heat-shock proteins, nucleotides, soluble mediators, and cytokines are released in inflammation site.
These molecules induce activation and maturation of DCs, whereas antigens derived from pathogens are effectively presented to lymphocytes and the typical result is activation of the corresponding clones of effector cells (Gallucci and Matzinger, 2001). In case of tumor formation, endogenous tumor cell growth occurs without any inflammation and tissue damage, at least in the initial stages. Accordingly, in the absence of 'danger signals', tumor antigens cannot be effectively presented by immature DCs and the typical outcome of this immune system 'blindness' is tumor cell recognition as a 'self' and not dangerous (Khong and Restifo, 2002; Pardoll, 1998).

DCs have become known as professional antigen-presenting cells and inducers of immune response. Moreover, at present, they are recognized as the most important cells in induction of innate and primary adaptive immune responses (Aderem and Ulevitch, 2000; Schnare et al., 2001). There are exciting reports of the possibilities of influencing these cells to enhance tumor antigen presentation (Timmerman and Levy, 1999). There is a wide range of studies, which involved DCs targeting tumor antigens. Pioneered work in this field belongs to the E Gilboa lab. First, the phenomenon was shown for DCs loaded with RNA isolated from tumor cells. In stimulation of CTL response, DCs pulsed with unfractionated RNA (total or polyA+) from OVA-expressing tumor cells were as effective as DCs pulsed with OVA

peptide. In the poorly immunogenic, highly metastatic B16/F10.9 mouse melanoma tumor model, a dramatic reduction of lung metastases was observed in mice vaccinated with DCs pulsed with tumor-derived RNA. It is worth mentioning that only total or polyadenylated RNA induced protective vaccination, whereas polyA(-) RNA fraction did not(Boczkowski et al., 1996). Later, the potential opportunity to activate immune response with mRNA-transfected DCs was demonstrated for a number of characterized tumor antigens, for example prostate-specific antigen (PSA) (Heiser et al., 2000), carcinoembryonic antigen (CEA) (Morse et al., 2003; Thornburg et al., 2000), E6 and E7 proteins of human papillomavirus (Thornburg et al., 2000). A clinical trial utilizing such approach also has been reported.(Morse et al., 2003) Next modification of the approach was the use of DNA transfected DCs. For modification, genes encoding cytokines, tumor antigens, and molecules involved in antigen presentation were used (Nishioka et al., 1999).

Dendritic cells possess several mechanisms that make them highly efficient antigen-presenting cells. DC can endocytose a large variety of exogenous antigens for presentation via MHC class II molecules and for cross-presentation via MHC class I (Wilson and Villadangos, 2005). The cross-presenting capacity of DC is unusual, because most other cell types are only able to present endogenous antigens (i.e., antigens synthesized by the antigenpresenting cells themselves) on their MHC class I molecules. Thus, DC possess specialized machinery, as yet not fully defined, that allows delivery of exogenous antigens into the MHC class I presentation pathway for cross-presentation (Cresswell et al., 2005; Touret et al., 2005).

1.5.2. DCs and cross-presention.

M. Bevan showed in the mid-seventies that cytotoxic T lymphocyte (CTL) responses may be initiated by antigen-presenting cells that do not express the antigens themselves (Bevan, 1976). He called this process cross-priming. The antigen-presenting cells involved in cross-priming must therefore internalize and present antigens to $CD8^+$ T cells in the context of MHC class I molecules. This process is often referred to as "cross-presentation." Cross-presentation by antigen-presenting cells in vivo results in either cross-priming (initiation of $CD8^+$ T cell responses) or in cross-tolerance (induction of $CD8^+$ T cell unresponsiveness) (Heath and Carbone, 2001b). These results raised the question of the nature of the "cross-presenting cells." In vitro, dendritic cells cross-present antigens more efficiently than any other antigen-presenting cell (Thery and Amigorena, 2001). They are also the only antigen-presenting cells that activate naive T lymphocytes (Banchereau et al., 2000). Dendritic cells, indeed, are sufficient for cross-presentation *in vivo* (Kurts et al., 2001).

DCs are equipped with the biochemical machinery for processing and presenting peptide fragments of protein antigens on MHC molecules, rather than just digesting them (Mellman and Steinman, 2001; Villadangos, 2001). APCs usually present exogenous antigens on MHC class II (MHC II) molecules, whereas they usually present endogenous antigens, from self-components or a viral infection, on MHC class I (MHC I) molecules. However, it is clear that certain antigens outside the APC system somehow enter the MHC-I processing pathway, both to generate cytotoxic T cells in response to viruses that do not infect the APCs themselves, and to maintain self-tolerance to non-APC components (Heath and Carbone, 2001a; Heath and Carbone, 2001b). Many experiments now point to DCs being highly potent cross-presenting APCs

(Pooley et al., 2001; Shen et al., 1997; Svensson et al., 1997). The receptors and biochemical machinery that lead exogenous antigens into the MHC-I presentation pathway of DCs now need to be elucidated.

1.5.3. Subtypes of mouse mature DC.

Distinct subtypes were initially more evident among mouse DCs than among human DCs, because of the ready availability of different murine lymphoid tissues and the expression on mouse DCs of markers not present on human DCs. Mouse DCs that are classed as 'mature' express CD11c and the co-stimulator molecules CD80, CD86 and CD40, and have high surface levels of MHC II, although the levels of all of these can be further elevated on activation. These features always correlate with ability to induce the proliferation of allogeneic T cells. Such mature DCs are heterogeneous in normal laboratory mice (Anjuere et al., 1999; Henri et al., 2001; Iwasaki and Kelsall, 2000). Surprisingly, the T-cell markers CD4 and CD8 are expressed on mouse DCs and are useful for segregating subtypes. CD8 on DCs is in the form of an αα-homodimer (rather than the αβ-heterodimer that is typical of T cells). Although CD4 is also present on human DCs, CD8α is not. So far, there is no evidence that either marker is functionally significant on mouse DCs. Other markers that are useful for segregating mouse DC subtypes include CD11b (the integrin αM chain ofMac-1) and the interdigitating DC marker CD205 (the multilectin domain molecule DEC205, originally known as NLDC-145).

Using these surface markers, five DC subtypes are consistently found in the lymphoid tissues of uninfected laboratory mice. Spleen contains three of these: the CD4–CD8+, the CD4+CD8– and the CD4– CD8– DCs (Vremec et al., 2000). The CD8+ DCs are concentrated in the T-cell areas and the CD8– DCs in the marginal zones of most laboratory mice, but

the CD8– DCs migrate into the T-cell zones on stimulation with microbial products (Iwasaki and Kelsall, 2000; Reis e Sousa et al., 1997). The CD4–CD8+ DC subtype, which is CD205+CD11b–, is also found at moderate levels in LNs, but is the dominant subtype among thymic DCs. LNs contain two extra DC subtypes that are not normally found in spleen, which have apparently arrived in the LNs through the lymphatic system (Anjuere et al., 1999; Iwasaki and Kelsall, 2000). Found in all LNs,CD4–CD8–CD11b+ DCs express moderate levels of CD205, in contrast to spleen CD8– DCs. This LN DC subtype is believed to be the mature form of tissue interstitial DCs. Another distinctive DC subtype, found only in skin-draining LNs, expresses high levels of langerin, a characteristic marker of epidermal Langerhans cells, and is believed to be the mature form of this Langerhans cell (Henri et al., 2001). It also expresses a range of myeloid markers, including CD11b, stains at low levels for CD8α and has high surface levels of CD205, as high as on the CD8hi DC subtype. These Langerhans DCs in LNs are also distinguished by their high surface levels of MHC II and high expression of CD40, CD80 and CD86 (showing many characteristics of fully activated DCs).

1.5.4. DCs and T-cell response.

The experimental outcome of a DC–T-cell interaction is often simply T-cell proliferation. Although triggering of T cells into cell cycle progression is a central function of DCs, it is now clear that DCs can also influence, and perhaps dictate, the subsequent development of these dividing T cells. T-cell activation and proliferation might lead to immunity or to tolerance, to the generation/activation of effector T cells or regulatory T cells, and to T cells that secrete different patterns of cytokines, including the extreme cytokine-polarized T helper 1 (Th1) and Th2 responses.

Although the signals from DCs that govern T-cell cytokine polarization have not all been determined, the production of the TH1-inducing cytokine interleukin-12 (IL-12) is an important factor (Moser and Murphy, 2000). The production of the bioactive p70 form of IL-12 by DCs is tightly regulated and involves separate control of the production of the p40 and p35 components. Several factors are needed to induce IL-12 production by DCs, including microbial products, the CD40 ligand CD154, stimulation from activated T cells and the appropriate cytokine milieu (Schulz et al., 2000). Notable examples of controls on IL-12 production include the negative-feedback effect (in which the Th2 cytokine IL-4 acts as a strong promoter of IL-12 production by DCs (Hochrein et al., 2000)), temporal regulation (whereby DCs that have produced a pulse of IL-12 become 'exhausted' and do not respond to subsequent stimuli (Langenkamp et al., 2000)), and the subtle discrimination by DCs of microbial stimuli - such that *Candida albicans* stimulates IL-12 production in its yeast stage but IL-4 production in its hyphal stage (d'Ostiani et al., 2000). It is therefore not surprising that DCs show considerable plasticity in their ability to induce Th1 or Th2 responses.

1.6. ANTIGEN TARGETING TO DCs

1.6.1. Fc Receptors (FcRs) and Cross-Presentation in Dendritic Cells.

If cross-presentation is how dendritic cells initiate CTL responses, antigen targeting to and internalization by dendritic cells must represent a critical step in cross-priming. In vitro, targeting antigens to receptors for the Fc region (FcγR) of IgG, dramatically increases the efficiency of cross-presentation (Thery and Amigorena, 2001).

1.6.1.1. FcγRs

FcγRs are a family of membrane glycoproteins expressed on hematopoietic cells (Ravetch and Bolland, 2001). Most FcγRs do not bind IgG, unless IgGs are themselves bound to multivalent-specific antigens (i.e., immune complexes). Thus, FcγRII (CD32) and FcγRIII (CD16) bind monomeric IgG quite inefficiently, but bind immune complexes with very high affinity. FcγRI (CD64), in contrast, binds monomeric IgG with high affinity, but, like high affinity receptors for IgE, it does not signal unless IgGs are cross-linked by their specific polymeric ligands. Thus, FcγRs may be functionally considered as antigen receptors.

Targeting antigens to FcγR promotes cross-presentation by several orders of magnitude in mouse bone marrow–derived dendritic cells (Machy et al., 2000; Regnault et al., 1999). The intracellular mechanisms leading to cross-presentation after FcγR-mediated uptake have been analyzed. In dendritic cells, but not in other cell types, FcγR-mediated internalization very efficiently targets antigen for a unique dendritic cell–specific antigen

transport pathway resulting in delivery to the cytosol. Once in the cytosol, internalized antigens are degraded by the proteasome. The resulting peptides are translocated into the lumen of the ER and loaded on MHC class I molecules (Rodriguez et al., 1999). These results suggested that antigen-specific humoral immune responses may promote the generation of specific CTLs.

FcγR-mediated cell signaling results from a delicate balance between activation and inhibition signals triggered by different FcγRs (Ravetch and Bolland, 2001). The same immune complexes or opsonized particles may simultaneously engage activation and inhibitory receptors. Coaggregation of these two types of receptors results in inhibition of cell signaling. Therefore, the outcome of FcγR engagement depends on the relative expression of activation and inhibitory receptors.

In mouse, IL4 (a cytokine used for the differentiation of monocytes into dendritic cells) promotes the expression of FcγRIIB, an inhibitory FcγR isoform (Snapper et al., 1989). IFN-γ, by contrast, promotes the expression of activation FcγR isoforms, such as FcγRI. In addition, the extent and specificity of FcγR engagement depend on the size of the immune complexes, and on the isotype and species origin, of the Abs used to form the immune complexes. The pattern of FcγR expression in vivo, in dendritic cell subsets or during maturation, is unclear. Therefore, it is not very surprising that depending on the type and maturation status of the dendritic cells used, the effect of immune complexes on maturation may differ. This point is particularly important, because different subpopulations of dendritic cells and dendritic cells at different stages of maturation have different functions. For example, mature dendritic cells induce T cell priming, whereas immature dendritic cells are believed to induce tolerance.

1.6.1.2. Fc ε receptors

Mast cells and basophils are granulated cells that play a pivotal role in allergy and inflammation. Their granules contain inflammatory mediators such as histamine, proteases, lipid mediators, and cytokines. The activation of mast cells induces exocytosis and fusion of cytoplasmic granules with the plasma membrane, followed by the release of inflammatory mediators within minutes of stimulation. One potent stimulus is the aggregation of high affinity receptors (Fcε receptor I [FcεRI]) by the Ag–IgE complexes. The high affinity receptor for IgE - FcεRI expressed on these cells is a critical component in allergic responses, since this receptor allows the cells to bind IgE. Ligation of IgE-bound FcεRI by multivalent Ags results in the activation of multiple signaling pathways leading to diverse effector responses, including the release of mediators responsible for allergic inflammatory reactions. Thus, the binding of Ag-specific IgE confers specific reactivity to that Ag on mast cells and basophils (Turner and Kinet, 1999).
IgE binding to FcεRI induces the up-regulation of FcεRI expression on mast cells and basophils in both humans and mice (MacGlashan et al., 1998; Yamaguchi et al., 1997).
Immunoglobulin E-dependent mast cell activation is a major triggering mechanism for acute and chronic allergic reactions and host defense against certain parasites . Activated mast cells release preformed pro-inflammatory chemical mediators (such as histamine and serotonin), proteases, and nucleotides, and release or secrete de novo synthesized lipids (such as leukotrienes and PGs) and polypeptides (such as

cytokines and chemokines). These substances contribute to the development of allergy and other forms of inflammation.
The FcεRI on murine mast cells consists of four subunits: an IgE-binding α subunit; a signal-amplifying, receptor-stabilizing β subunit; and two disulfide-bonded γ subunits that are the main signal transducer, making an αβγ2 tetramer (Kinet, 1999).

In addition to FcεRI expression by mast cells and basophils, in human subjects antigen-presenting cells, such as dendritic cells and monocytes, express FcεRI (Novak et al., 2003a). Among DCs, only DC1 cells or DC1-like populations have been reported to express FcεRI, including the peripheral blood precursor DC1 (pDC1) subset, (Dzionek et al., 2000) skin-derived Langerhans cells,(Wollenberg et al., 1995) inflammatory dendritic epidermal cells, (Wollenberg et al., 1996) and in vitro differentiated DC1 cells (Novak et al., 2003b).
The observation that human FcεRI cell is expressed on a variety of cells other that mast cells and basophiles led to a surprising finding. In contrast to the easily detectable α and γ transcripts, those for the β chain were not detected in monocytes even using the polymerase chain reaction (PCR) (Maurer et al., 1994). The inability to detect FcεRβ transcripts in monocytes, Langerhans cells, and dendritic cells suggested that the structure of FcεRI on these cells is indeed different from that on mast cells and basophiles. Moreover, in these cells (monocytes, Langerhans cells, and dendritic cells), the FcεRI contains only αγ2 trimers, was shown to mediate small calcium fluxes in monocytes (Maurer et al., 1994), and to promote IgE mediated antigen presentation (Maurer et al., 1995). Also it has been reported that this Ag presentation function is more effective in human dendritic cells (Maurer et al., 1996). However, the αγ2 isoform of the FcεRI was not seen in rodents so far.

Apart from FcεRI in humans, the most recently discovered antigen presenting function was shown, mediated by IgE via the low-affinity receptor for IgE, FcεRII/CD23 on the B cells (Getahun et al., 2005). The biological role of CD23 is not well understood.

Unlike other Fc receptors, it does not belong to the super Ig family but is a type II integral membrane protein with a calcium-dependent lectin domain in the C-terminal end of the extracellular part (Bettler et al., 1989). CD23 is expressed on the cell surface as trimers, which are pre-associated in the absence of IgE (Kilmon et al., 2004). In its bound trimerized form, CD23 has an affinity for IgE of 1.45×10^8 M^{-1} (Bartlett et al., 1995). The most well known in vivo function of CD23 is probably its ability to act as a negative regulator of IgE and, to a lesser extent, IgG1 production. This function is evidenced from several experimental systems. Transgenic mice, overexpressing CD23, have reduced production of IgE (Payet et al., 1999) and conversely, CD23-deficient mice (CD23 $^{-/-}$) have enhanced IgE production (Yu et al., 1994). Negative regulation of IgE responses via CD23 takes place after primary immunization with Ag in alum (the ammonium double sulphate of aluminium), and in most systems affects polyclonal IgE production (Payet et al., 1999). Because only very low initial IgE concentrations are available to ligate CD23 in a primary immune response, it is an open question whether IgE or another CD23 ligand is involved in the pathway leading to negative regulation of IgE production.

The other in vivo immunoregulatory role of CD23 is to enhance Ab responses (Heyman, 2000). This effect is dependent on IgE, and was seen when soluble Ag, such as BSA-TNP or OVA-TNP, were administered to mice together with monoclonal IgE anti-TNP. The carrier-specific IgG responses in such animals were frequently >100-fold higher than in mice immunized with the Ag alone, and the effect was strictly dependent on CD23 (Heyman et al., 1993). Not only IgG, but also IgM and IgE levels

were shown to be enhanced (Westman et al., 1997). The remarkable immunostimulatory effect of IgE takes place without adjuvants (IgE and Ags are always given in PBS) and is Ag-specific, i.e., only responses to antigenic determinants within the IgE-Ag complex are enhanced (Gustavsson et al., 1994).

Possible mechanism underlying IgE-mediated enhancement of the Ab response in vivo is Ag presentation to specific Th cells by CD23+ B cells. Arguing against this hypothesis are reports that B cells are unable to present Ag to naive T cells (Eynon and Parker, 1992; Jenkins et al., 2001). Finally, it cannot be ruled out that co-cross-linking of the BCR and CD23 by IgE-Ag complexes increases B cell activation (Campbell et al., 1992).

2. Materials and Methods

Mice

C57BL/6 and Balb/C six to eight week old female mice were purchased from Harlan (Milan, Italy). OT1 mice, transgenic for a TCR that recognizes the SIINFEKL peptide of OVA presented on H-2K^b, have been previously described OT1 (Hogquist et al., 1994).
All mice were housed at the International Center for Genetic engineering and Biotechnology animal facility in accordance with institute regulations.

DNA constructs

Cloning of pBCL1 has been described previously (Benvenuti and Burrone, 2001) Cloning of constructs encoding membrane bound proteins: moε –BCL1, moα-BCL-1, was performed with primers (5'-GTGAATTCCTATGCCCTGGTCTGGAGGATGTT-3') and (5'-ATTCCGGAGGCTCTGGGAGCCAGCGCTCAGCC-3') for Cε4 and (5'-TATGAATTCTTCCGACAGACGGTCGATGGT-3') and (5'-GATGGATCCCGGGGACAGGGAGAGGCTCTTG-3') for Cα3, by amplification of the mouse ε_4 (mouse IgE)/α_3 (IgA) domains containing the extracellular-membrane-proximal domain (EMPD), the transmembrane and cytoplasmatic domains by RT-PCR from total RNA of purified mouse splenocytes. Primers for epsilon These amplified parts of Fc region of membrane immunoglobulins are then cloned as a BspEI/EcoRI into the corresponding sites of pBCL.
The cloning of membrane bound BCL1-GPI and BCL1-huε-GPI constructs were made by transferring the BCL1 idiotype from pBCL1 to pcDNA3 expression vector containing the GPI domain coding sequence (Carrasco et al., 2004), containing or not human ε4 domain, as Hind III/ BspE I fragment. The GPI-linked BCL1 constructs were transfected into MC38 cells and positive clones were selected in the presence of geneticin.

The engineering of biotin acceptor peptide (BAP) consisting the sequence GLNDIFEAQKIEWHEGS has been described elsewhere. (Barry et al., 2003). To construct BAP-E34, E34-BAP and E34 delta BAP , RT-PCR was performed on total RNA extracted from splenocytes derived from C57BL/6 mouse, using primers for amplification of epsilon 3 and epsilon 4 domains. After digestion with AgeI-BamHI , PCR products are than cloned to corresponding sites in pcDNA3 vector (Invitrogen, Leek, The Netherlands) containing BAP at amino or carboxy terminus.
For generating OVA-E34 and OVA-E4 , OVA peptide with flanking amino acids (LEQLESIINFELKLTEWTS) was made and cloned in pcDNA3 vector as Hind III – Age I fragment. ε3ε4 or ε4 domains were than cloned from E34-BAP , after digestion with AgeI-EcoRI.
The cloning of murine keratinocyte promoter (K14) promoter was described previously (Vassar et al., 1989) and the vector was kindly provided by Dr. Elaine Fuchs (Howard Hughes Medical Institute, Chevy Chase, MD). K14 promoter (2kb) was transferred as AvaI/AvaI fragment in pUC vector in preferred orientation to obtain pUC-K14. Than, pUC-K14 vector was digested with NdeI/EcoRI and ligated with annealed oligos containing Hind III site: k14Nde (5'-TACAACATCGTAAGCTTCGATC-3') and k14Eco (5'-AATTGATCGAAGCTTACGATGT-3'). This modified vector was then digested with HindIII/BamHI and fragment was cloned into pBCL digested with BglII/HindIII to obtain pK14-BCL1.
To produce pMHCII-BCL1 vector, the pDOI-5 vector (Kouskoff et al., 1993), kindly provided by Dr. Christhope Benoist (Joslin Diabetes Center, Boston, MA), containing 2.1kb murine MHCII promoter, was digested with BamHII to insert the oligos: BamHII(HindIII)5 (5'-GATCCAAGCTTGGTAGCG-3') and BamHII(HindIII)3 (5'-GATCCGTACCAAGCTTG-3'). This vector was then digested with XbaI to insert oligos Xba(BglII)5/Xba(BglII)3 (5'CTAGAGGTAGATCTAGTT-3') /

(5'-CTAGAACTAGATCTACCT-3'). The product is than digested with HindIII/BglII and fragment containing MHCII promoter was cloned into corresponding sites of pBCL1.
For construction of pBCL1-moCH2 , the murine CH2 domain of IgG2a was amplified from total RNA of purified mouse splenocytes by RT-PCR and PCR, with CH2(BspEI)5/CH2(BspEI)3 primers (5'-GTATCCGGAGAGCCCAGAGGGCCCACAATCAAG-3') / (5'-TCCTCCGGATTTGGGTTTTGAGATGGTTCTCTC-3'). After BspEI digestion, moCH2 domain was cloned as BspEI/BspEI fragment into pBCL1.
The correct construction of all vectors was confirmed by DNA sequencing.

Cell lines

MC38, a nonmetastatic colon adenocarcinoma cell line from C57BL/6 mice, was originally induced with oral dimethylhydrazine (McCart et al., 2001). BCL1 is spontaneous B cell lymphoma of Balb/C origin expressing high levels of membrane IgM (Slavin and Strober, 1978). It exists in two variants. The original BCL1 clone was maintained through serial passages in syngenic Balb/C mice. The BCL1-3B3 variant is *in vitro* cell line, that can be activated by LPS to secrete IgM.
The human keratinocytes (HaCaT) were passaged using 0.25% trypsin/1 mM EDTA (Invitrogen Life Technologies).
All cell lines were grown in DMEM or RPMI supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 1% penicillin/streptomycin (10,000 units/ml) Cell lines were maintained in an incubator at 37°C with 5% CO2 and serially passaged every 3–4 days.

Vaccination and tumor challenging

Mice abdominal area was shaved and shot with 1μm gold particles carrying 1-2 μg DNA at 400 psi using the BioRad gene delivery device (BioRad, Hercules, CA, USA). The sera was collected 2 weeks after the last shot. Also, at the same time mice were challenged intraperitoneally with $5x10^4$ of viable BCL1 tumor cells.
Intra muscular (i.m.) rAAV administration was performed by single injection in the leg – tibialis anterior muscle. Sera was collected and analyzed 15 /or 30/ days after immunization.

Ab response

Antibody response against human γ1-CH_3 was determined by ELISA using plate coated with 3 $\mu g/ml^{-1}$ of human IgG (Sigma Immunochemicals, St. Louis, MO, USA). Anti - Idiotypic mmune responses to mouse Id BCL1 was also detected by ELISA , with plates coated with 1 $\mu g/ml^{-1}$ of murine BCL1 IgM purified from 123BCL1 hybridoma supernatant, and by flow cytometry on BCL1.3B3 cells using FACScalibur (Becton Dickinson immunocytometry, San Jose, CA, USA) Sorting of cells was performed on the same instrument , as well. Immune sera was diluted and immunocomplexes detected with HRP or FITC conjugated goat anti-mouse IgG antibodies (Pierce, Rockford, IL, USA/ Kirkengaard & Perry, Gaithersburg, MD, USA)

Cell Transfections

Cells were stably and transiently transfected by electroporation or calcium-phosphate, respectively. For transient transfection cells were plated in a 30mm Petri dishes. Fresh medium was added 4h before transfection, and 5μg of plasmid DNA was resuspended in 50μl of o.1xTE (10mM Tris, 1mM EDTA). Mix A was prepared with 169μl of deionized water, 5μl of $CaCl_2$ 2M followed by drop to drop addition of 50

µl DNA mix, and another 26µl of $CaCl_2$ 2M. The mix A was added drop by drop to 250µl of 2xHBS (280mM NaCl, 10mM KCl, 1.5mM NaH_2PO_4, 12mM dextrose and 50mM Hepes), while bubbling. Total mix was added to cells after 5 min of RT incubation. Cells were kept in incubator at 37°C and 5%CO2 for 48h, when supernatants were collected and analyzed.

rAAV production

The rAAV vectors are based on the pTR-UF5 constructs that was kindly provided by N.Muzyczka (University of Florida, Gainesville, FL), which expresses the GFP gene under the control of human cytomegalovirus (CMV) immediate early promoter. Id-BCL cassette was obtained by digestion of pBCL1 vector with HindII/NotI and HindIII/KpnI, respectively, and cloned into the corresponding sites of pTR-UF5. Propagation of AAV plasmid was carried out in the XI10 Gold *E. Coli* strain. Infectious vector stocks were generated in HEK 293 cells, cultured in 150mm Petri dishes, by co-transfecting the plate with 15mg of vector plasmid, together with 45mg of the packaging/helper vector, pDG (kindly provided by JA Kleinschmidt, DKFZ, Heidelberg, Germany), expressing AAV and adenovirus helper functions (Deodato et al., 2002). Twelve hours after co-transfection, the medium was replaced with the fresh one, and 3 days later, collected. The cells were harvested by scraping. After three freeze-thaw cycles in dry ice/ethanol bath and 37°C water bath, cell lysates were fractionated using ammonium sulfate precipitation. rAAV particles were than purified by $CsCl_2$ gradient centrifugation in a SW41Ti rotor at 288000 *g* for 36h. 12-16 fractions of 10 drops were collected by inserting a G-21 needle below the rAAV bend, and their refractive index was determinated. The six fractions with index closest to 1.3715 (corresponding to a density of 1.40g/cm^3) were dialyzed against phosphate buffer saline (PBS) at 4°C overnight, and stored at -80°C. rAAV titers were determined by measuring the copy number of viral

genomes of pooled, dialyzed gradient factors. This was achieved by a competitive PCR procedure (Deodato et al., 2002), using primers and competitors mapping in the CMV promoter region common to all vectors. The purified viral preparations used, had approximately 10^{12} of viral genomes per ml.

Preparation of BMDCs

BMDCs were prepared as described previously (Kikuchi et al., 2004)). In brief, bone marrow cells were cultured for 8 days with interleukin- 4 (IL-4; 2 ng/ml) and granulocyte/macrophage colony-stimulating factor (10 ng/ml). Most differentiated cells (>70%) were determined to be CD11c positive when the cells were analyzed by flow cytometry.

OT1 activation

For preparation of activated OT1 CTLs, spleen and lymph nodes from OT1-transgenic mice were combined and crushed through a 100-μm filter to prepare a single-cell suspension. RBC were removed by a 2-min incubation in ACK buffer (sterile dH_2O containing 0.15 M NH_4Cl, 1.0 mM $KHCO_3$, and 0.1 mM EDTA adjusted to pH 7.2–7.4). OT1 cell were than purified using CD8α magnetic beads (Miltenyi Biotec). In vivo OT1 activation experiments were performed by injecting 1-2x10^6 of purified , CFSE labeled OT1 cells in tail vain of immunized animals. 24h later, mice were sacrificed and spleen and lymphnodes were harvested and CFSE+ cells were analyzed for the expression of T-cell early activation marker- CD69.

3. Results

3.1 B-CELL LYMPHOMA TUMOR PROTECTION

3.1.1 Engineering the vaccine

The focus of research in our laboratory in previous years was on anti-idiotypic vaccination against BCL1 lymphoma (Benvenuti et al., 2000). BCL1 lymphoma is a mouse B cell lymphoma, which also appears like human polymorphocytic leukemia (Tutt et al., 1985) , expressing IgM at the malignant B cell surface. The light *(VL)* and heavy *(VH)* variable regions of this immunoglobulin represents tumor specific idiotypic determinants as an actual tumor associated antigen.. These unique amino acid sequences are generated by *V(D)J* recombination (Haluska et al., 1986) and subsequent somatic hypermutation of variable (V) region genes and represent clonal signatures of the individual B cells. Although self antigens, these idiotypic determinants are potentially immunogenic to the host and may be used as target for active immunotherapy.
Since the first attempts of targeting BCL1 idiotype through genetic immunization resulted in induction of low-levels of anti Id antibodies, with no protection of animals after subsequent tumor challenging, our group have been focused on engineering an efficient DNA vaccine against BCL1 idiotype. For this purpose we constructed a BCL1 scFv fragment, in which V_L/V_H variable domains of BCL1 idiotype were separated by a linker of 18 amino acids (GSTSGSGKPGSGEGSTKG) for mimicking the natural conformation of BCL1 idiotype.

Moreover, the CH3 domain of human IgG1 heavy chain (γ1-CH3) was fused to our BCL1 scFv cassette to make pBCL1, a construct then used as a vaccine. This molecule, called Small Immune Protein (SIP), is folded and efficiently expressed and secreted by transfected mammalian cells (Li et al., 1997).

Vaccination with a scFv construct that contained only the VL and VH genes of the murine BCL1 lymphoma induced low levels of anti-Id antibody and no protection from subsequent tumor challenge (Stevenson et al., 1995). After genetic immunization of animals with pBCL1 SIP containing human heavy chain domain, we observed a high efficiency in breaking T cell tolerance and inducing specific anti-Id humoral response and protection of tumor challenged animals (Fig.6) (Benvenuti and Burrone, 2001; Benvenuti et al., 2000).

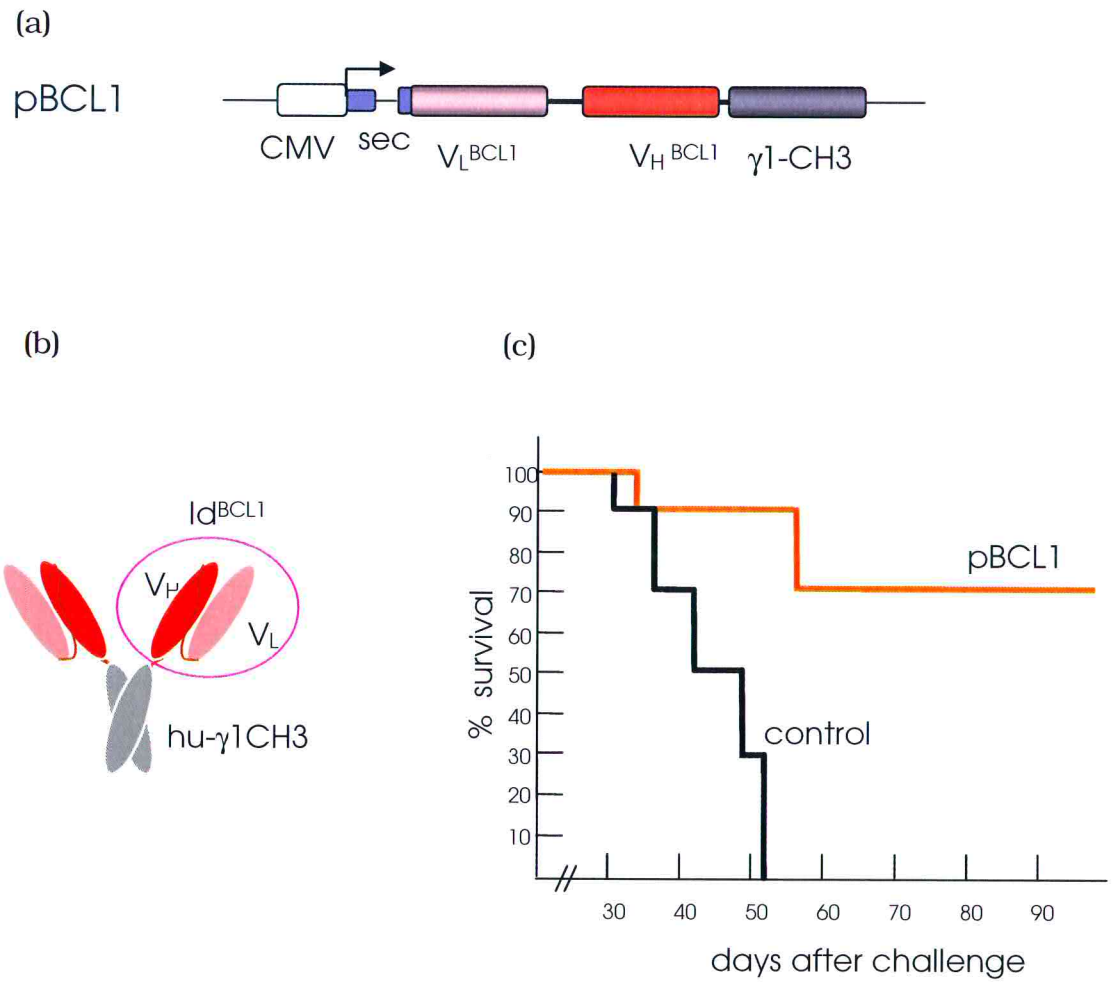


Figure 6. **(a)** and **(b)** Schematic representations of pBCL1 construct and dimerized Id^{BCL1} displayed protein. **(c)** Survival curve of mice immunized with pBCL1 or empty vector (pcDNA3, Invitrogen) as a control, and challenged with $5x10^4$ of BCL1 tumor cells per mouse.

3.1.2 Mechanism of tumor protection

More recently, it was found that this anti-idiotypic response is highly specific for BCL1 VL/VH association, after DNA genegun administration. We implemented a system based on this specificity to investigate the mechanism of BCL1 lymphoma protection induced by DNA immunization. Antibody response and survival of mice immunized with the tumour Id scFv were compared with those of mice immunized simultaneously with two chimeric scFvs, containing either the tumor-derived V_H or V_L paired to an irrelevant V_H or V_L domain, respectively (Cesco-Gaspere et al., 2005). Animals vaccinated with one or both chimeric constructs were not protected, despite the exposure to all putative tumor Id-derived MHC class I and class II T-cell epitopes. In addition, conformational antibodies induced by DNA vaccination caused tumor cells apoptosis in vitro and transferred protection in vivo (Cesco-Gaspere et al., 2005).
Therefore, we concluded that BCL1 lymphoma protection induced by idiotype DNA vaccination is entirely dependent on anti-idiotypic antibodies.

3.2 TRANSFERRING THE MODEL OF TUMOR PROTECTION

Since we could observe significant delay in tumor progression and a complete tumor rejection in 40-70% of challenged mice in our B cell lymphoma model, better understanding and exploiting the potency of our Id^{BCL1} immunization strategy, may allow us to develop an efficient strategy for cancer immuno-protection.
We put forward the question of weather the tumor protection of the B cell lymphoma could be transferred to different tumors.
Because of its properties, we wanted to investigate whether the BCL1 idiotype could serve as a potent tumor associated target in other tumors. We hypothesized that BCL1 idiotype immunization could be able to protect animals challenged with other tumor types displaying on their membranes, the BCL1 idiotype. We designed several experiments with the final aim to investigate the capability of our protection model , when animals are challenged with indifferent tumor cell, which stably expresses BCL1 idiotype (Fig7a).

3.2.1 **Strategy of DNA genetic immunization**

For eliciting the anti BCL1 immune response we vaccinated mice with our immunogenic pBCL1 construct. DNA genegun immunization shots were performed for 3 times, each 2 weeks. At week 6 the sera were collected and analyzed for the presence of anti-BCL1 antibodies.

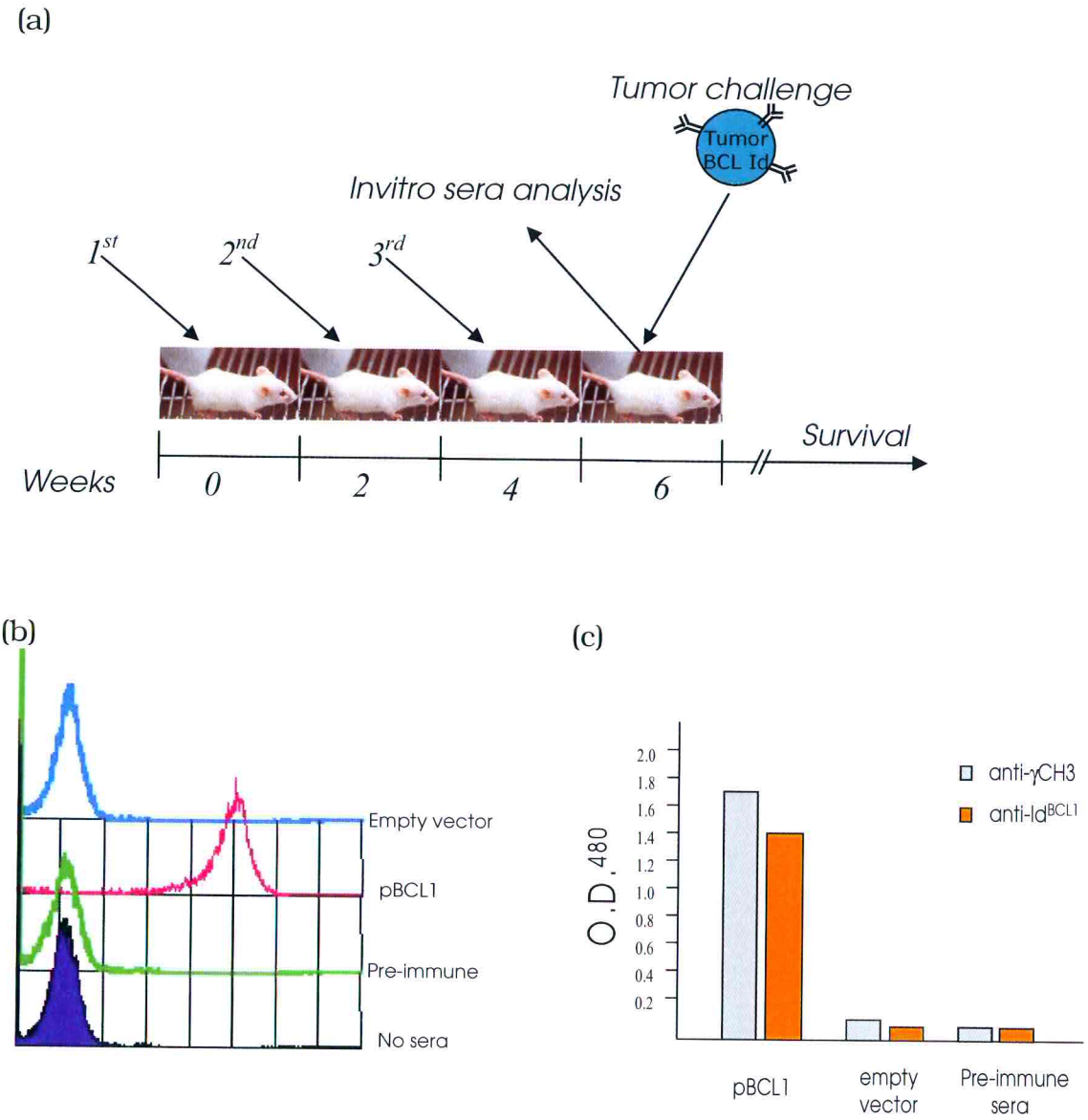


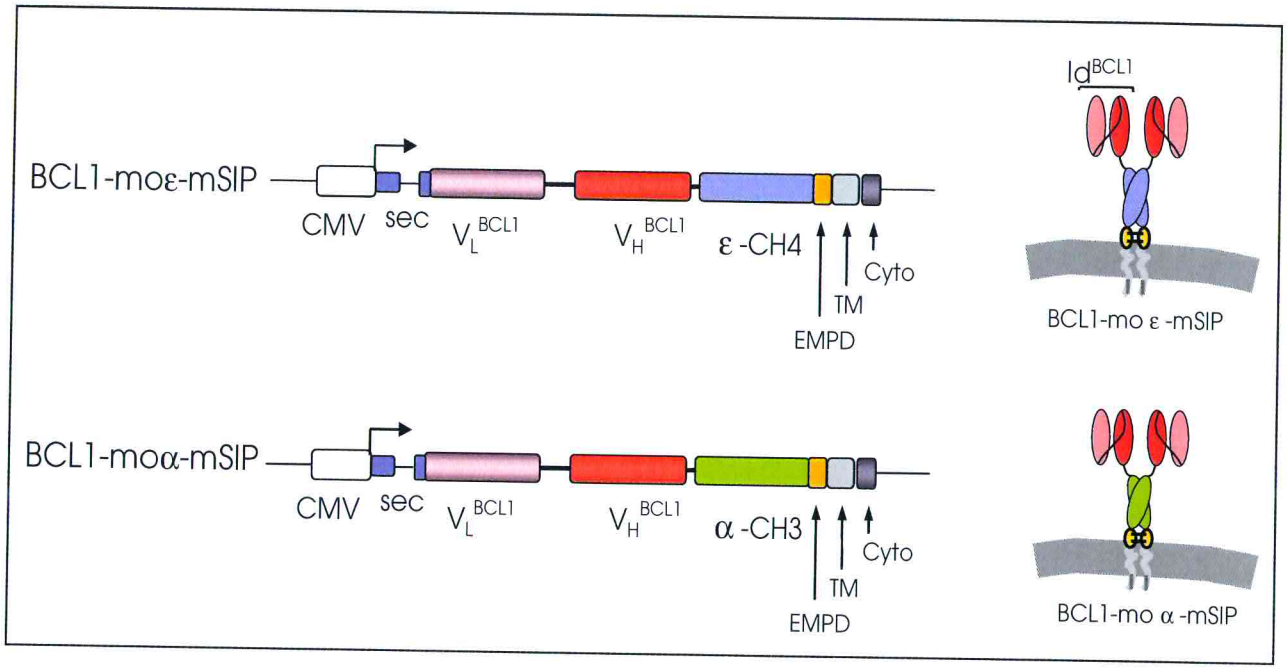
Figure 7. **(a)** Schematic representation of immunization strategy. Mice were immunized by genegun 3 times at day 0, day 14 and day 28 with pBCL1 and control constructs. **(b)** and **(c)** Analysis of sera collected at day 42. **(b)** Binding of sera to BCL1-3B3 cells. The pooled sera were diluted 1:200 and analyzed by flow cytometry . **(c)** Antibody levels measured by ELISA. Analysis of anti-CH3 and anti-BCL1 sera after 3 genegun immunizations with indicated constructs and sera from non-immunized mice.

Sera analysis showed the presence of anti –Id^{BCL1} and anti-human CH3 antibodies, and functional capability to bind BCL1 idiotype expressed by BCL1.3B3 cells (Fig7).
Genetic immunization with the DNA cassette encoding the BCL1 idiotype fused to xenogenic γ1-CH3 domain has proven to be an efficient way of inducing high levels of anti idiotypic BCL1 antibody response.

3.2.2 Generating the Id BCL1 expressing tumor cells

In order to challenge immunized animals with different tumor cells expressing BCL1 idiotype, we first had to generate stably transfected tumor cells. For obtaining stable membrane Id^{BCL1} positive clones, we designed several constructs containing the scFv Id^{BCL1}, connected to the C-terminal regions of mouse membrane bound immunoglobulins IgE or IgA H-chains, containing ε4 or α3 dimerizing domains, respectively, together with the extracellular membrane proximal domain, the trans-membrane domain and the cytoplasmic tail (Fig 8a). Such an expression format, which has been called the membrane SIP (mSIP), is efficiently expressed as a membrane protein in myeloma and B cells, has been described previously by our group (Bestagno et al., 2001).
These constructs were then used for transfection of the mouse neuroblastoma N2a, and mouse melanoma B16f10 tumor cell lines.
After a series of transfection assays performed on the above mentioned cells, we could observe very low expression of BCL1-α–mSIP on both cell lines. Moreover, multiple transfections of mo-ε-mSIP resulted in a complete failure of expression on both, N2a and B16f10 tumor cell lines. (Fig 8b). Successful mo-ε-mSIP transfectants could not be seen neither after infection with a recombinant BCL1-moε-mSIP retrovirus (data not shown).

(a)



(b)

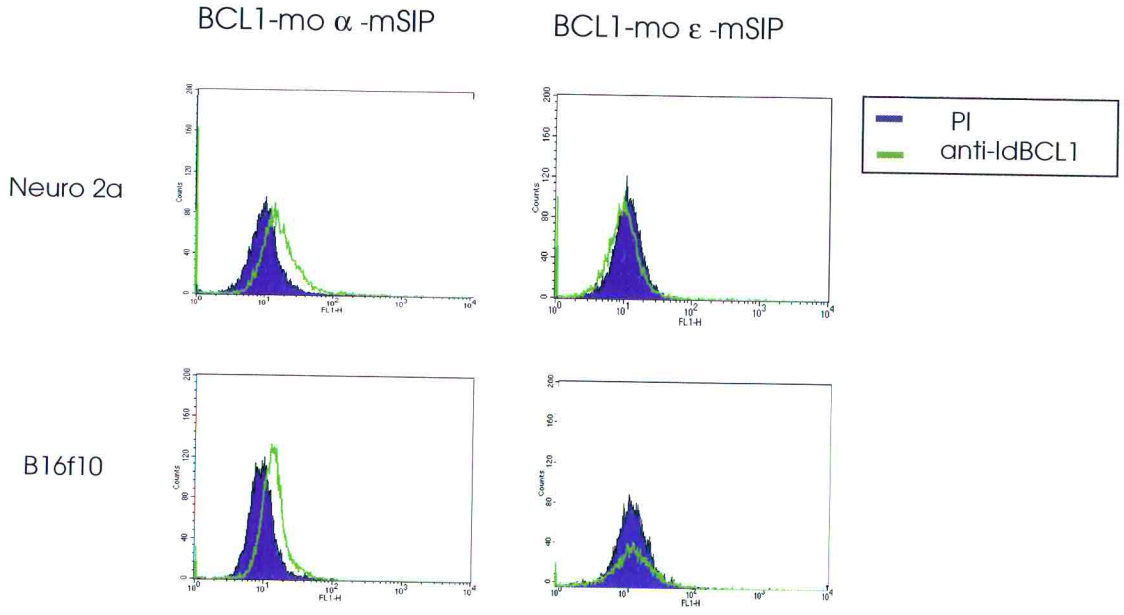


Figure 8. (a) Schemes of mSIP DNA cassettes and expression format. **(b)** Flow cytometry expression analysis with Pre immune sera (PI) from non immunized animals (purple), and anti-Id^{BCL1} sera (green) in 1:200 dilution. FITC conjugated anti human IgG (γ) was used for screening.

Since we have repeatedly failed to generate stable expressing Id^{BCL1} clone, we decided to change the strategy, in order to efficiently display the BCL1 idiotype on tumor cell membranes.

For this purpose, instead of the C-terminal parts of membrane heavy chain immunoglobulins, Id^{BCL1} was conected to the 24 a.a. long peptide from gene encoding a GPI anchor region (Carrasco et al., 2004). This construct was prepared in two different variants:

a) with dimerazing human epsilon4 domain (Batista et al., 1996),

b) without dimerazing domain.

For obtaining control constructs, an irrelevant idiotype (Id^{SS}) was cloned into the same set of expression vectors.(Fig9).

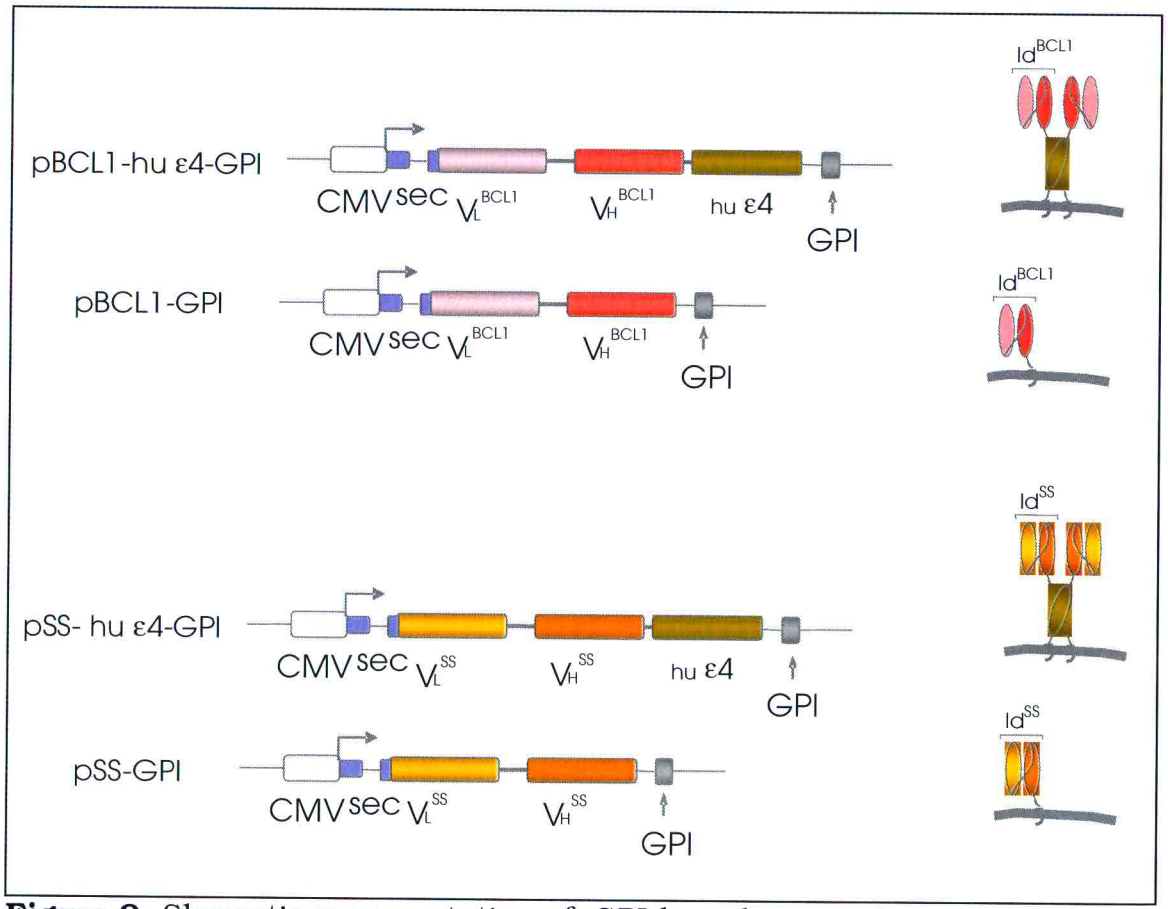


Figure 9. Shematic representation of GPI-based expression constructs.

Glycosylphosphatidylinositol (GPI)-anchored proteins comprise a diverse class of membrane molecules. They protect cells from complement-mediated lysis, control cell to cell adhesion, activate T cells, and play a role in the etiology of slow viral diseases. Despite their functional diversity, GPI-anchored proteins are all attached to the plasma membrane by a common glycolipid anchor (Censullo and Davitz, 1994). For our purpose, this property of GPI signal sequence has been used to efficiently bring the idiotypic proteins to the cell membrane, were it is displayed. Having (or not) human ε4 domain expressed on tumor cell, could eventually demonstrate the requirement for immunogenicity of our challenging tumor cells expressing a xenogenic domain.
These set of constructs were then used for transfection of the MC38 cell line, a different mouse colon adenocarcinoma model.

The transfection of MC38 cells was performed by electroporation, and were maintained under the presence of geneticin as a selection marker. Within 14 days, BCL1 and SS idiotype positive expressing clones were selected by flow cytometry (Fig10a). In order to produce and select the cells with highest degree of expression, we had to introduce another selection round. Several cycles of cell sorting were performed for generating the best idiotypic expressors (Fig10b).

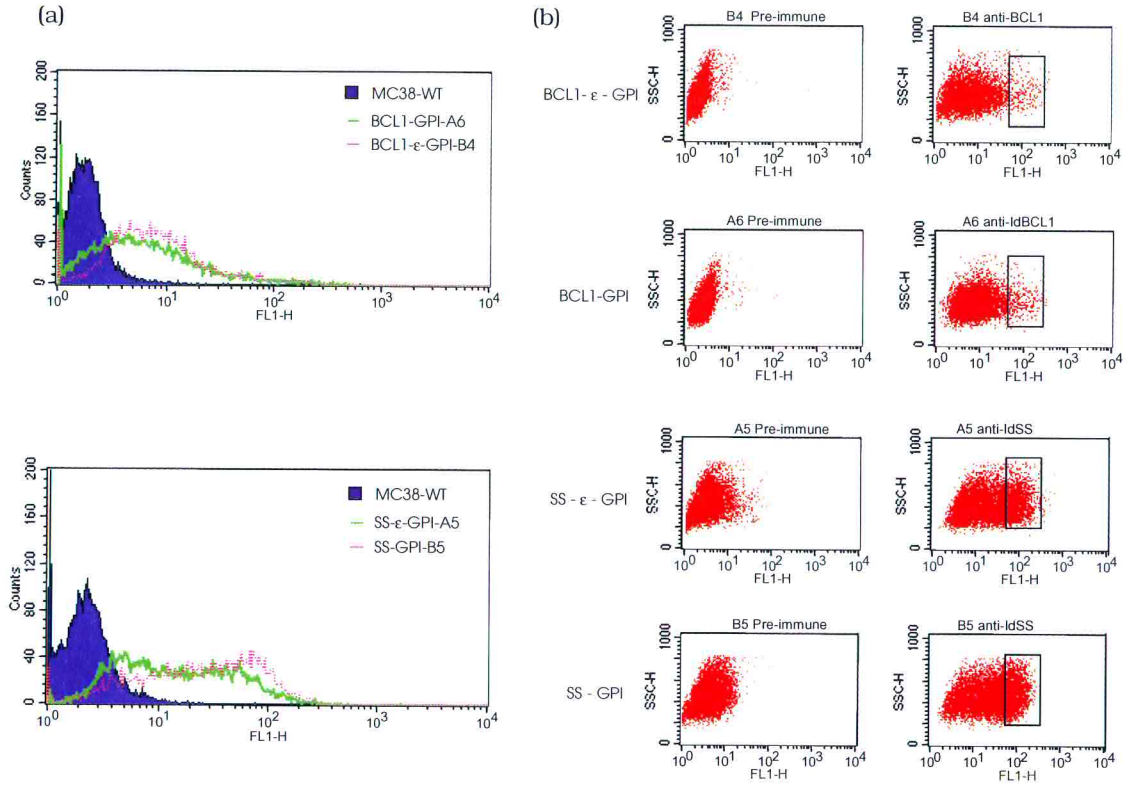


Figure 10. FACSCalibur analysis and sorting of GPI clones. **(a)** Histogram plot of indicated BCL1 (up) and SS(down) idiotype expressing clones prior to sorting. **(b)** Sera of non immunized mice were used as negative controls (Pre-immune). Cells were first incubated with anti-BCL1 sera (1:200) or anti-SS sera, and stained with FITC-anti-mouse IgG. Region of sorted cells is shown in boxes (□).

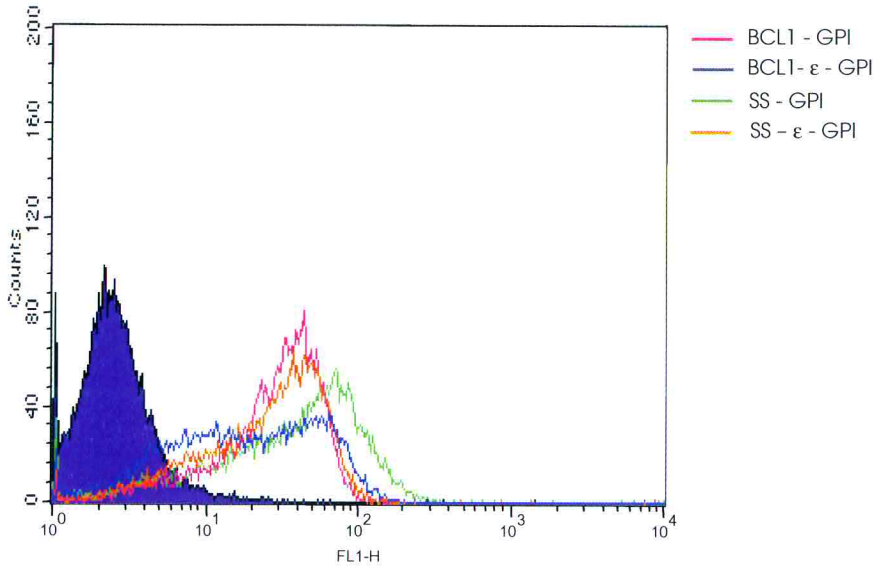
3.2.3. *Ex vivo* analysis for the persistence of idiotypic expression

The selected MC38 clones with highest levels of idiotype expression , were analyzed by FACS prior to *in vivo* inoculation (Fig11a) and the number of cells needed for producing the tumor *in vivo* was determined. We found that subcutaneous inoculation of 5 x 10^5 of idiotype expressing cells into C57BL/6 mice was required for obtaining solid tumor. Growth of tumor was monitored over time.

One month after the inoculation, mice were sacrificed, tumors were collected and cells were analyzed by FACS for the persistence of idiotypic expression.

The result clearly demonstrated that both, BCL1+ and SS+ sets of idiotypic membrane proteins, were still expressed after one month of *in vivo* tumor progression (Fig11b). Although all cells derived from the different clones were still positive, they also showed a different expression profile when compared to cells before inoculation, indicating that there is a partial reversion (loss of expression) of the transgene.

(a)



(b)

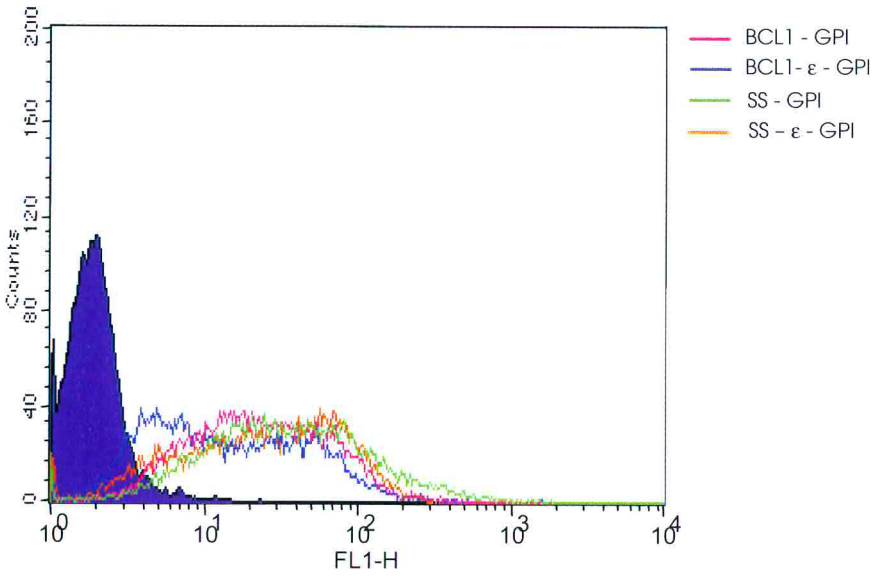


Figure 10. **(a)** Selection of a sorted cell clones before *in vivo* inoculation. Cells used as the tumor implants were administrated by subcutaneous injection. **(b)** *Ex vivo* analysis of MC38-idiotype positive clones. 30 days after tumor implantation mice were sacrificed, tumors were digested with collagenase and cells were analyzed by flow cytometry.

3.2.4. *In vitro* analysis of BCL1 idiotype expressing MC38 clones

It has been previously reported that anti-Id antibodies were able to elicit apoptosis and cell cycle arrest of BCL1 tumor cell *in vitro*, after both, protein (Racila et al., 1995), and DNA immunizations (Cesco-Gaspere et al., 2005).

To address the question of weather anti –BCL1 idiotypic sera have any effect on our MC38-BCL1 expressing clones, cells were incubated with different amounts of DNA-induced anti-idiotipic sera.

As we have expected, MC38 idiotype expressing clones do not go in apoptosis, after incubation with anti BCL1 serum and cross-linking with anti-BCL1 immunoglobulins. This result demonstrated that there is no effect of anti Id^{BCL1} antibodies to trigger tumor cell death on MC38 clones in vitro, thus excluding the possibility of direct apoptotic signaling.

On the other hand, after incubation with 3ul of anti-BCL1 serum, a clear cytopathic effect on BCL.3B3 cells was observed, leading to apoptotic death.

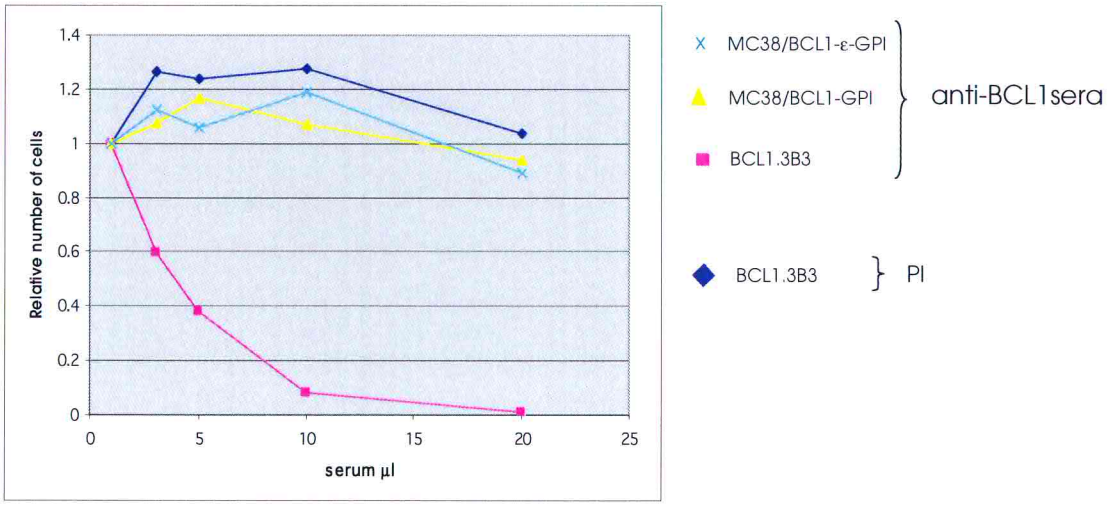


Figure 12. Absence of response of MC38-BCL1 expressing clones and dose dependent response of BCL1.3B3 to O/N incubation with different amounts of non-immune (PI) or anti-BCL1 sera.

3.2.5. MC38 challenging and mice survival

We designed in vivo experiment in the way in which two different groups of mice were immunized with pBCL1 or with pSS and challenged with different idiotype expressing carcinoma cells. As a negative control we used the group vaccinated with pSS and challenged with BCL1 idiotype expressing cells (Table 2).

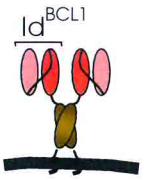
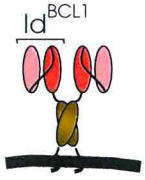
C57BL/6 mice groups:	Immunized with:	Challenged with:
Group 1	BCL1 - γ1-CH3	MC38/BCL1-ε4-GPI 
Group 2	BCL1 - γ1-CH3	MC38/BCL1-GPI 
Group 3	SS - γ1-CH3	MC38/SS-ε4-GPI 
Group 4	SS - γ1-CH3	MC38/SS-GPI 
Group 5 control	SS - γ1-CH3	MC38/BCL1-ε4-GPI 

Table2 Experimental design scheme.

Prior to tumor challenging, we first validated the immune response in vaccinated animals, confirming that all of the vaccinated mice had comparable levels of anti-Id sera (Fig13a). After challenging, animals were followed for survival. All mice died within day 71 after challenging (Fig13). Challenging experiments were repeated 3 times with different number of mice per group, but a significant delay of tumor growth could not be observed in these groups.

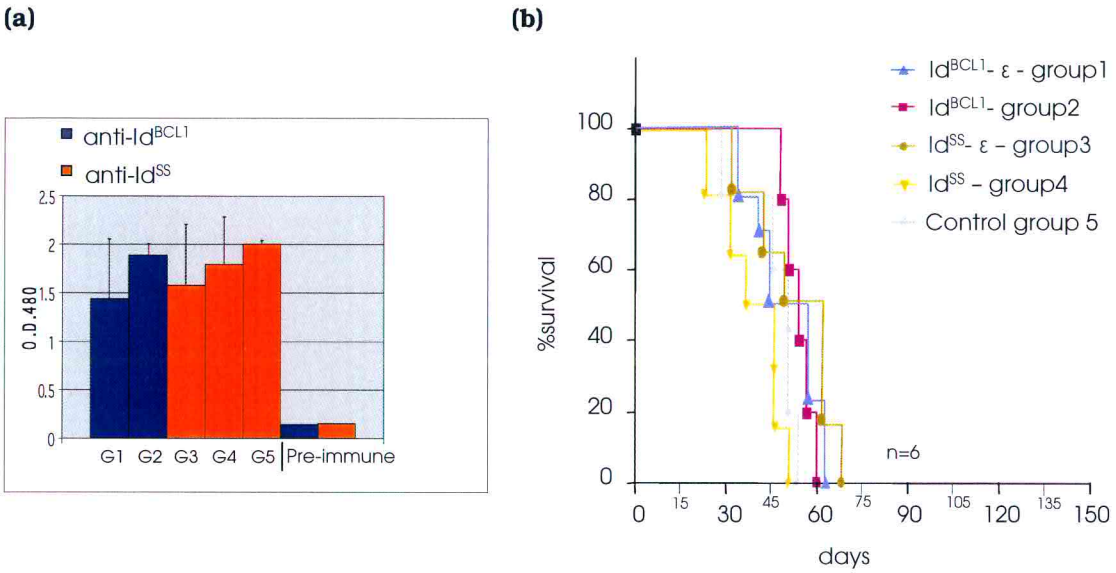


Figure 13. **(a)** Anti Id^{BCL1} and anti^{SS} responses in immunized groups (G1-5) before chellanging **(b)** Survival curve of 5 groups of mice injected with 5x 10^5 of MC38 idiotype expressing cells.

Although the high levels of anti-Id sera were obtained, *in vivo* mice tumor challenging demonstrated that no animals were protected despite the fact that tumor cells were still expressing the exogenous target Id. These results suggested that the BCL1 idiotype, as a protective tumor associated antigen in our B cell lymphoma case, is not transferable to colon adenocarcinoma (MC38) model.

3.3 ENHANCING ANTI-IDIOTYPIC RESPONSE

DNA vaccines have conventionally used the human CMV immediate early promoter and enhancer, due to its ability to generate strong gene expression in a wide variety of cell types (Manthorpe et al., 1993; Montgomery et al., 1993).
Independent of the route of delivery, CMV promoter-driven plasmids are expressed in all cells at the site of immunization. In order for Ag-specific T and B cell activity to be elicited, the expressed protein needs to be processed and presented on MHC Ag by a professional APC that can then stimulate naive Ag-specific T cells resident in lymphoid tissues.
This professional APC could have been directly transfected with the CMV promoter-driven plasmid at the site of immunization, leading to translation of the DNA vaccine-encoded protein and subsequent processing of Ag. Alternatively, the APC could have endocytosed another directly transfected cell or cellular material, shuttling its Ag to both the class I (via cross-presentation) and class II MHC pathways.

3.3.1. Gene targeting strategies

In order to enhance anti-idiotypic response we proposed different DNA immunization strategies. We hypothesized that in vivo, genetic targeting to different cell types could lead to development of divergent quality of immunologic response. Moreover, we aimed to analyze whether the delivery of the Id^{BCL1}-CH3 exclusively to specific cells could result in an enhancement of its immunogenicity.

Thus, to answer these questions the DNA vaccines were engineered with different promoters for cell type-restricted Ag expression.

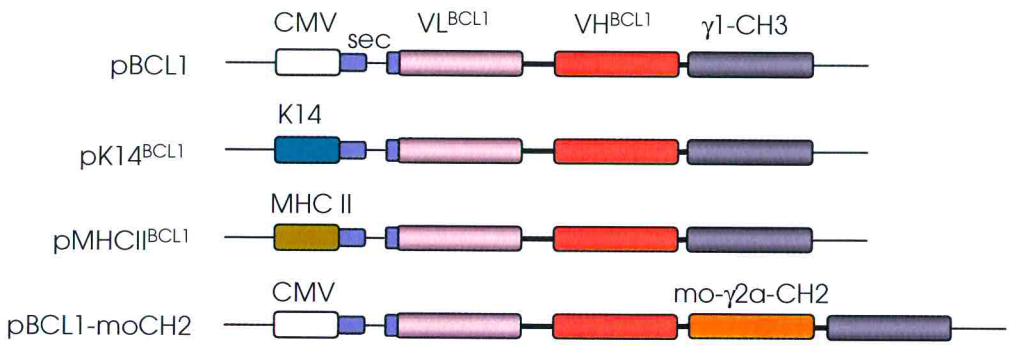


Figure 14. Scheme of different targeting constructs.

For that purpose several DNA vectors were designed and constructed (Fig 14).

Besides of the pBCL1 construct in which the Id^{BCL1}-CH3 protein , driven by CMV promoter, can be expressed in a variety of different tissues and somatic cells, several other constructs were made in order to target specific cells for expression of our idiotype. Efficient cell type specific Id^{BCL1}-CH3 expression could provide information on whether it makes a difference in eliciting better immuno-response, in respect to non-tissue specific, CMV driven vaccine. To answer this question, the CMV promoter in pcDNA3 expression vector, was replaced by both, keratin 14 (K14) and MHC II promoters, for specific targeting of keratinocytes and APCs, respectively. In order to investigate the capacity of APC to be directly transfected upon biolistic immunization we used a promoter specific system (MHC II promoter) to allow expression of the immunizing protein exclusively in APCs (Kouskoff et al., 1993). Unlike the ubiquitously active CMV promoter, the 2-kb MHC II promoter and enhancer, would restrict

Ag expression exclusively to MHC+ APC resident in the skin at the site of gene gun immunization, including Langerhans cells and dermal DCs.

It has been previously shown that the K14 promoter is specific to basal epidermal keratinocytes in the skin, and is not active in DCs (Garg et al., 2003; Lin et al., 2001; Trainer and Alexander, 1997). Furthermore, the frequency of other cells, such as T and B lymphocytes, in the skin is negligible, making direct transfection of these cell types unlikely events.

Apart from a promoter specific, cell type-restricted targeting constructs, we designed and developed the *pBCL1-moCH2* vector, consisting of CMV driven BCL1-CH3 plasmid which contains the CH2 region of mouse IgG2a antibody (Fig14). This domain incorporates the amino acid sequence Leu 234-Glu-Gly-Gly-Pro 238 which stand for a high affinity binding site for the Fc γ receptor (FcγRI). Previously, it has been shown that Fc γ receptors can serve as an interesting targets for enhancing immunological response, as they represent a privileged antigen internalization route for efficient MHC class I and II- restricted antigen presentation by dendritic cells (Regnault et al., 1999; Sallusto and Lanzavecchia, 1994).
These antigen targeting strategies could elicit strong antibody response because of efficient protein secretion from transduced cells (by the CMV promoter BCL1-moCH2-CH3 expression) and enhanced T-helper response (by DC's , through the FcγRI uptake and peptides loaded on MHCII).

Functional expression of our proteins was investigated by transfection of different cell lines in vitro and analyzed by western blotting (Fig15).

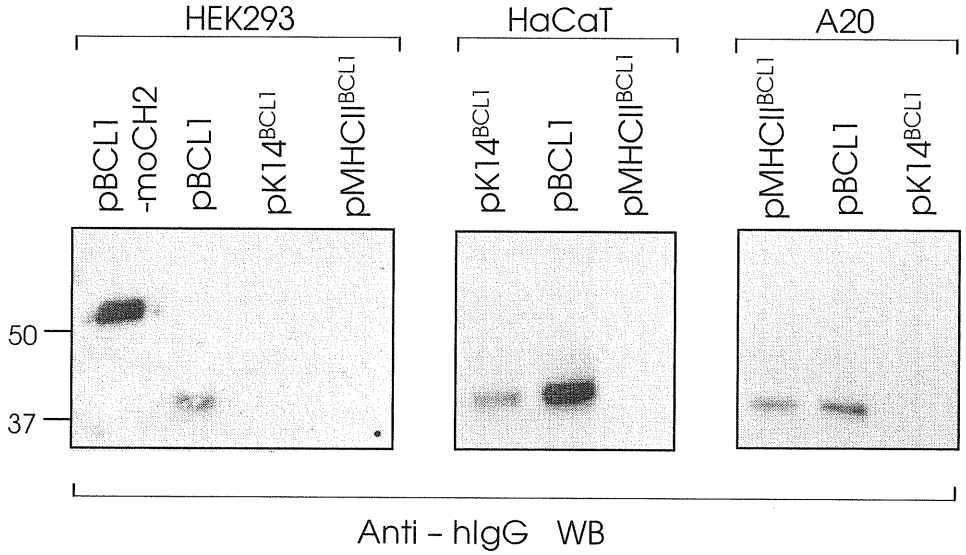


Figure 15. WB of transfected cell lines.

After transfection of human embryonic kidney cells (HEK293), investigated supernatants showed an expression of both CMV promoter driven proteins, BCL1-CH3 and BCL1-moCH2-CH3, but not of those led by either K14 nor MHCII promoters. Also, the keratinocyte cell line (HaCaT) was successfully transfected only by K14 and CMV promoter driven constructs, $pK14^{BCL1}$ and pBCL1, respectively.
Selective protein expression in MHCII+ cells was shown by stable transfection of A20 cells, a murine lymphocytes cell line expressing MHC II molecules. Id^{BCL1}-CH3 was efficiently produced upon transfection of A20 cells with both, pBCL1 and $pMHCII^{BCL1}$, but not with $pK14^{BCL1}$, as expected.

3.3.2. Analysis of *in vivo* response

Using the promoters described above (Fig14a), we investigated whether after gene gun DNA immunization, transfected tissue specific cells are capable of producing a sufficient level of Ags for inducing an Ab response. Female BALB/c mice were immunized at day 0 by one gene gun delivery with gold beads coated with pCMV-, pK14-, pMHCII-BCL1, or with the pBCL-moCH2 plasmid construct. At Day 15 after the first DNA immunization, pooled sera from the immunized mice were analyzed for BCL1-specific Ab responses (Fig. 16a).

As expected, mice immunized with $pK14^{BCL1}$, that directs Id^{BCL1}-CH3 expression only in keratinocytes, produced significant levels of anti-BCL1 IgG responses. These Ab levels were comparable to the ones induced by expression driven by CMV promoter, suggesting that keratinocytes are the major cells that take up the gene gun delivered, plasmid DNA and produce enough Ags to generate Ab responses (Fig. 16).

These data support a previous studies in which non-migratory cells such as keratinocytes influenced the magnitude of Ab responses after gene gun DNA immunization (Klinman et al., 1998)

As keratinocytes do not express appreciable levels of class II MHC or T cell costimulatory molecules, and do not migrate to draining lymphoid tissues, immune responses generated from vaccination with K14 promoter-driven construct would thus represent efficient priming of T cells by DCs or other APCs, which have captured Ag exogenously produced.

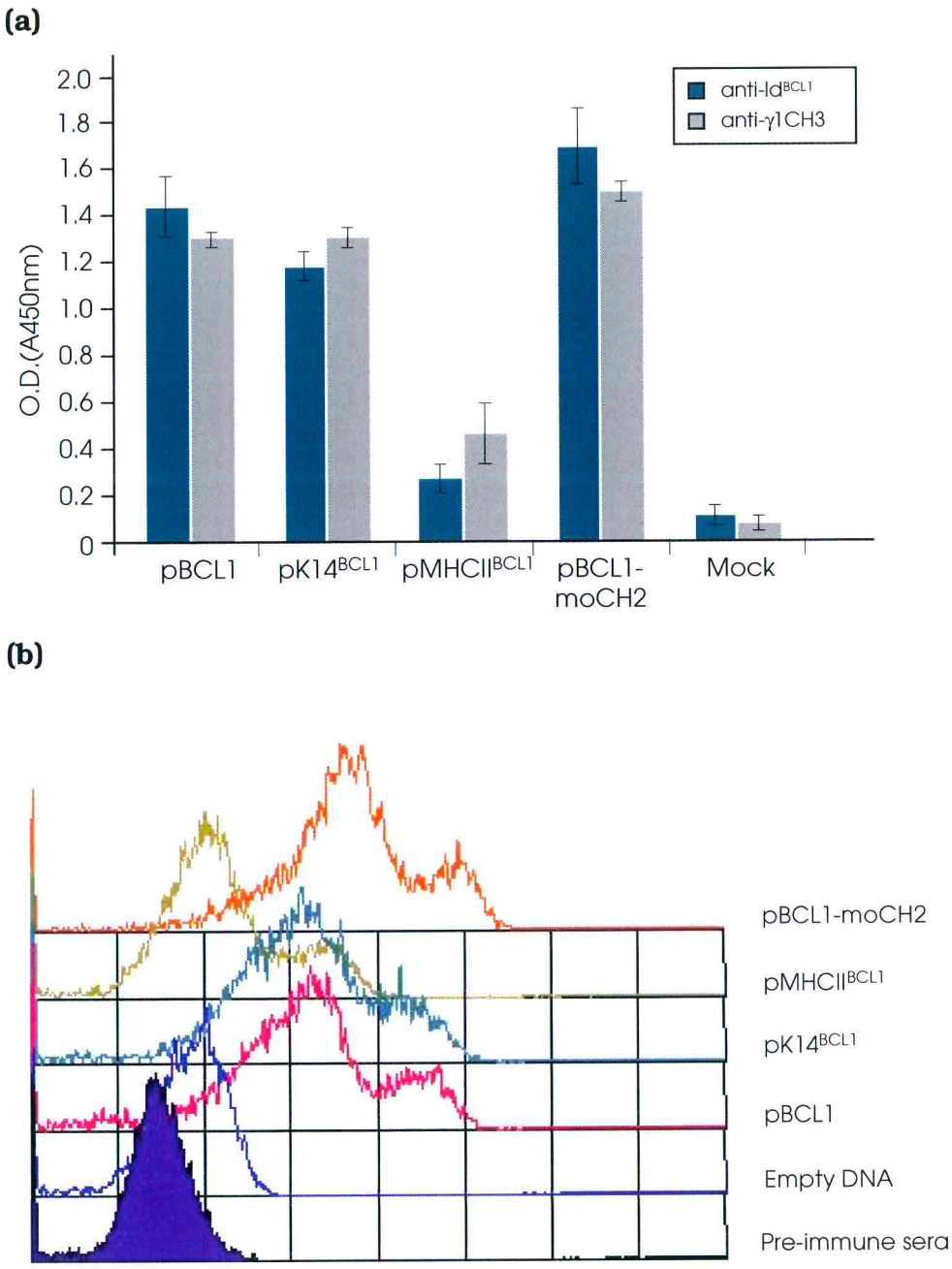


Figure 16. Induction of antibody responses. Mice (six/group) were immunized with different DNA constructs once, and sera were harvested at day 15 after DNA immunization. **(a)** Antibody levels measured by ELISA against g1CH3 or Id^{BCL1} in 1:200 diluted sera. **(b)** FACS analysis on BCL1 expressing 3B3 cells and binding of anti-idiotypic sera (1:200) of vaccinated mice.

In contrast, vaccination with pMHCII^{BCL1} resulted in low anti-Id Ab levels. Given that the relative strength of the MHCII promoter was similar to that of the CMV promoter (Fig. 15), the nature of a poor Ab response in pMHCII^{BCL1} -immunized mice is not likely to result from the weak promoter activities. The results are most likely a consequence of the limited production of Ag by the small numbers of directly transfected MHCII^{+} APCs and DCs at the site of biolistic immunization. In spite of this, the moderate, yet significative response also indicates that gene gun immunization is effective in delivering DNA to APCs. In comparison with the pBCL1 anti-Id response, immunization with pBCL-moCH2 vaccine showed the highest anti -Id^{BCL1} Ab levels. In contrast, there were no anti-BCL1 responses in mock (empty DNA plasmid) immunized mice as a negative control (Fig. 16). The design of pBCL-moCH2 vaccine that include the constant heavy chain domain of murine γ2a immunoglobulin, proved its targeting capability. Differing from pBCL vaccine only in the presence of the moCH2 domain, it shows an increase of anti-Id^{BCL1} response after one gene gun immunization. This difference is even more evident after 3 gene gun immunizations (Fig 17).

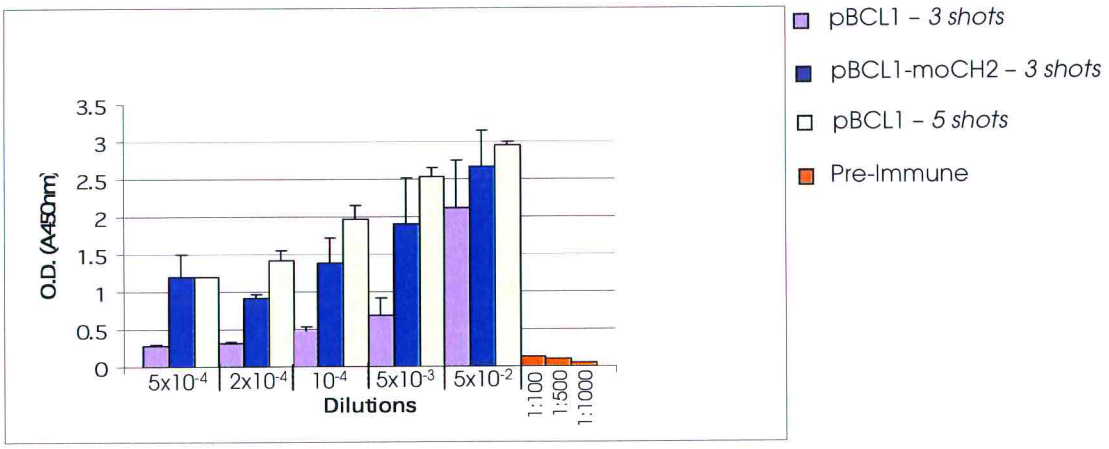


Figure 17. Different dilution of Anti-Id^{BCL1} response measured by ELISA. Mice were immunized 3 or 5 times by gene gun with pBCL1, and 3 times with pBCL-moCH2 vaccine.

The increased anti-Id^{BCL1} antibody levels in animals immunized with pBCL1-moCH2 were probably due to the enhancement of APCs antigen uptake through FcγRs and presentation of peptides by Th cell priming and consequent activation of B cells. Previous reports suggest that Ag presentation by DCs through FcγR-mediated uptake can be increased 100-fold over pinocytosis of soluble antigens (Regnault et al., 1999).

3.3.3. Vaccine enhancement by recombinant adeno-associated virus

3.3.3.1. IdBCL1 delivery trough recombinant AAV

For vaccination purposes, AAV constitutes a combination of the best properties of viral and non-viral vectors. It represents the only available eucariotic non-pathogenic virus. Moreover, recombinant vectors based on AAV have been found able to induce a long lasting expression of the foreign protein in the target tissue (Xiao et al., 1996).
The use of a rAAV for Ag delivering in vivo, could be a reliable way to improve Ag immunogenicity by increasing the number of in vivo transduced cells. Moreover, the long term expression of the Ag could result in a prolonged stimulation of the immune system. We characterized the capacity of recombinant AAV encoding the Id^{BCL1} to induce anti-Id immunity and to test whether it is capable of boosting humoral immune response when rAAV vaccine is applied after gene gun immunization.

3.3.3.2. Construction of rAAV^{BCL1}/CH3 and in vivo immunization

The recombinant AAV vector, expressing IdBCL1-CH3 protein under the control of CMV promoter were previously engineered (Fig. 18a) (provided

by Dr. Michela Cesco-Gaspere). This construct was then used to produce an AAV by a standard packaging system based on co-transfection of AAV and adenovirus helper functions. After purification on a CsCl2 gradient, the viral titers were determined by a competitive PCR assay. The yield was comparable in all viral preparations and estimated around 10^{12} particles/ml (data not shown).

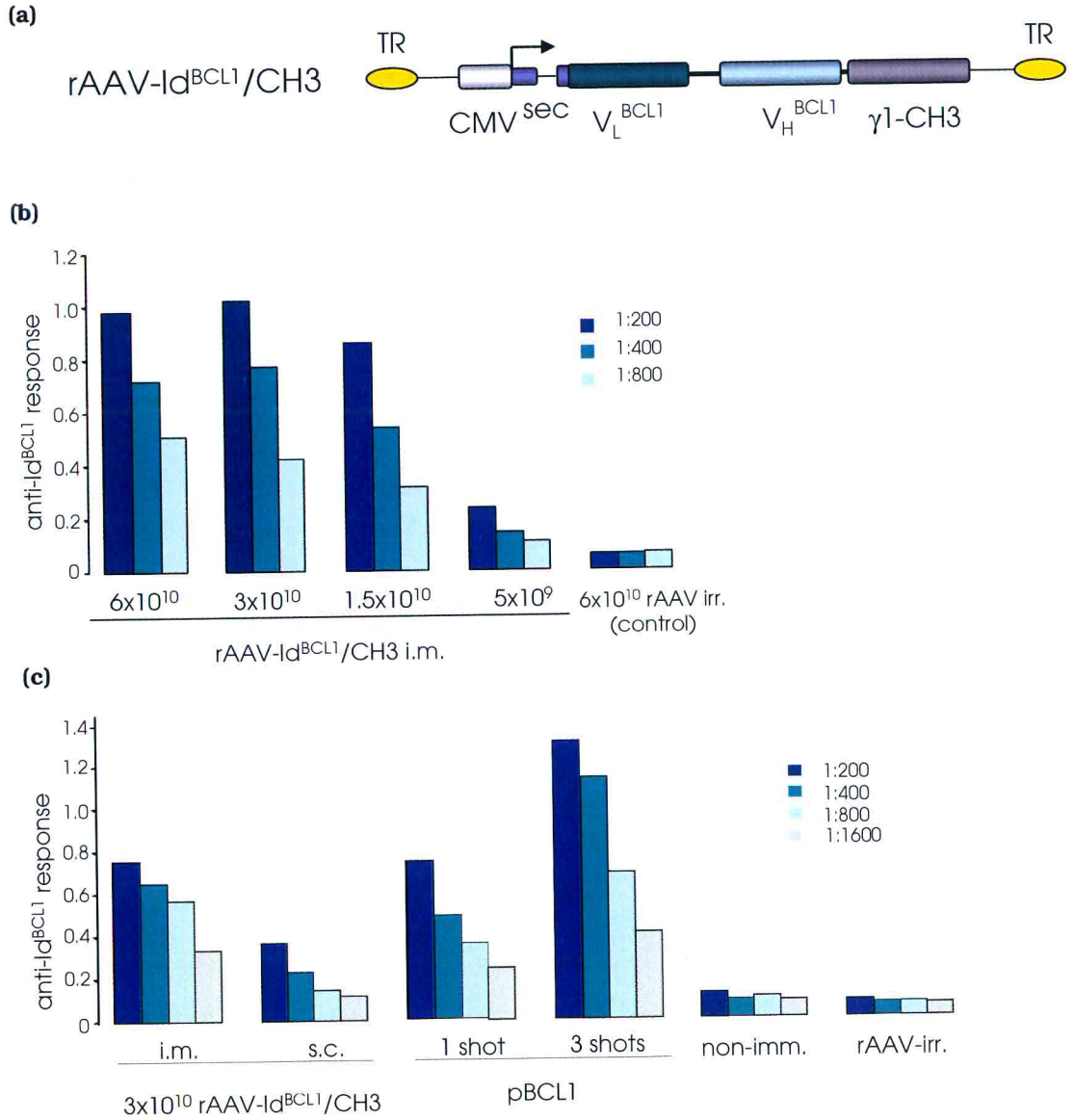


Figure 18.(a) Scheme of designed rAAV Id^{BCL1}/CH3 vector. **(b)** Anti-Id^{BCL1} dose dependent response upon mice injection with rAAV-Id^{BCL1}/CH3 or irrelevant rAAV. **(c)** Mice were vaccinated i.m or s.c. with 3x10^{10} rAAV-Id^{BCL1}/CH3, or immunized by gene gun. Sera were analyzed by ELISA.

We first aimed in establishing the optimal amount viral particles needed for inducing an efficient anti-Id^{BCL1} response. Anti-Id^{BCL1} Ab titers were measured by ELISA against BCL1 IgM one month after administration. Considering well-characterized tropism of AAV for muscle tissue, rAAV was administrated by a single injection in leg's *tibialis anterior* muscle. A significant anti-IdBCL1 response was seen, reaching a plateau upon injection of $3x10^{10}$ of viral particles (v.p.) (Fig 18b). This result suggest that a single AAV mediated delivery of the idiotype in vivo is a reliable way to induce anti-Id immune response. In order to compare the AAV-mediated Ag delivery could result in a comparable levels of anti-Id^{BCL1} Ab response when compared to the DNA gene gun vaccination, we performed the following experiment. The mice were treated by a single intra muscular (i.m.) or sub-cutaneous (s.c.) administration of $3x10^{10}$ v.p. of $rAAV^{BCL1}$-CH3 or a single or three pBCL gene gun immunizations. In all cases mice were bled one month after vaccination (or after the last vaccination in the case of pBCL1 3 shots vaccination), and sera were analyzed for reactivity to Id^{BCL1}. Antibody titers induced by i.m. injection of rAAV were comparable to that raised by a single gene gun shot (Fig 18c). As expected i.m. route of administration was the route of choice, as s.c. vaccination resulted in lower yield of anti-Id^{BCL1} Abs.

3.3.3.3. Boosting effect of rAAV vaccine

Previous results showed comparable anti-Id Ab titers induced after a single gene gun shot of pBCL1 or after a $rAAV^{BCL1}$-CH3 i.m. injection. For vaccination purposes, it would be important to determine proper immunization strategies in order to enhance an immune response. Prime-boost immunization strategies, using sequential administration of different antigen delivery systems encoding the same epitopes or antigen,

have been shown to induce enhanced and persistent levels of CD8+ and CD4+ T cells, which are protective against different diseases. Recently, several studies have demonstrated the efficacy of prime-boost vaccination strategies in generating immunity to a variety of pathogens. These include: malaria (Schneider et al., 1998) , leishmania (Hanke et al., 1999), plasmodium (Gonzalo et al., 2002), *M. tubercuosis* (McShane et al., 2001), *Listeria monocytogenes* (Fensterle et al., 1999), hepatitis C virus (Matsui et al., 2003). This promising strategy involves priming the immune system to a target antigen delivered by one vector and than selectively boosting this immunity by re-administration of the antigen in the context of a second and distinct vector. For our pupuse, in order o explore eventual change in development of anti-BCL1 response, mice were immunized by following immunization regime.

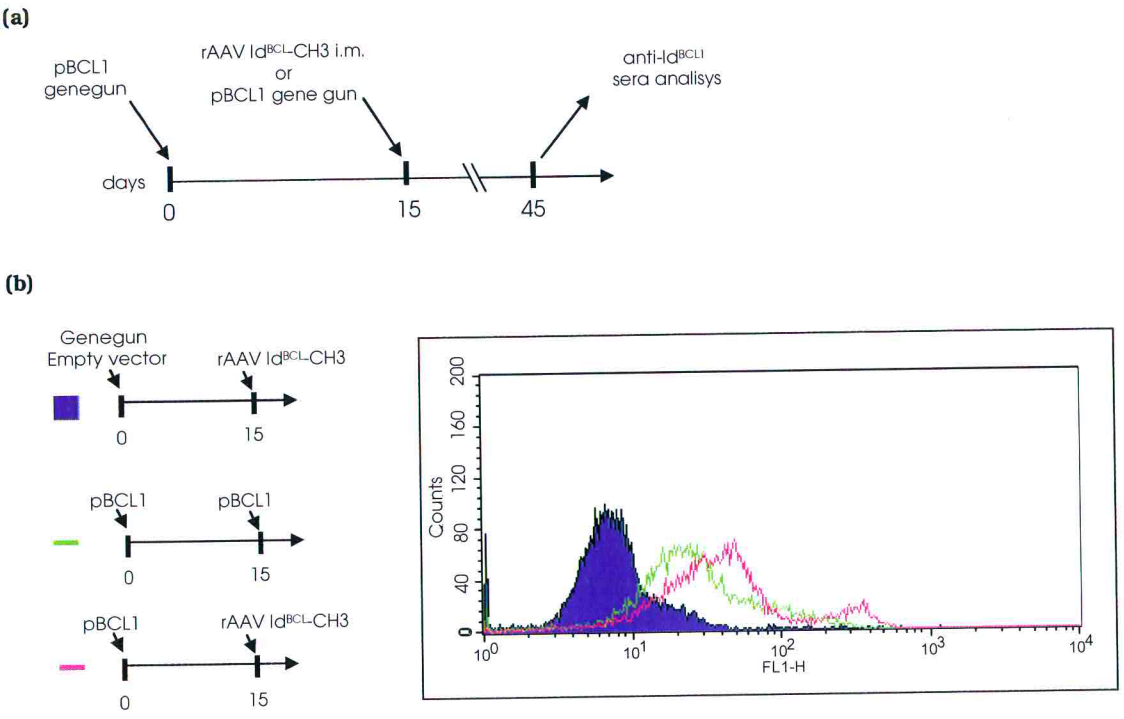


Figure 19. **(a)** Scheme of vaccination protocol. **(b)** FACS analysis of anti-Id^{BCL1} sera binding on BCL1.3B3. cells

15 days after the gene gun vaccination with pBCL1, animals were treated with a single i.m. injection of rAAV^{BCL1} and sera were harvested and analyzed one month after the rAAV vaccine (Fig. 19a). There was an open question of how this strategy may correlate with gene gun, DNA/DNA immunization protocol.

Anti-IdBCL1 titers in DNA/rAAV mice group, were significantly higher, then DNA/DNA immunized animals (Fig.19b). These results suggest that although the Ab levels after a single DNA and AAV vaccines are comparable (Fig.18c), in the case of prime-boost (DNA/rAAV) strategy it seems it does not simply represent the sum of 2 shots , but rather that DNA/rAAV vaccination strategy influence the engagement of immune system in a different way. The animals immunized with empty DNA vector, followed by rAAV^{BCL1} vaccine, served as a negative control.

In order to put rAAV vaccine boosting principle in the context of cell specific promoter strategies, we performed following experiments. First we asked the question of whether rAAV is capable of boosting the response previously obtained by tissue specific Ag expression/cell targeting DNA gene gun shots. We analyzed whether the boosting effect of rAAV is still present, when the virus is administrated 15 days after a different promoter-specific, cell targeting DNA vaccines.

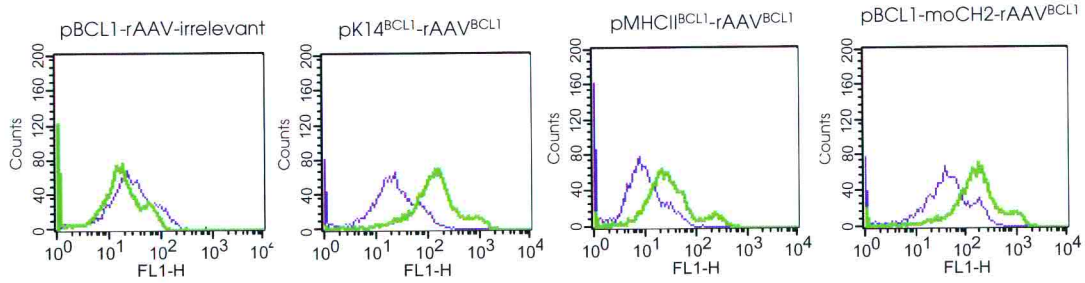


Figure 20. Anti-BCL1 response measured by FACS on 3B3 cells. Animals were vaccinated by gengun, and response was analyzed after 15days (blue line), and with rAAV (green line)

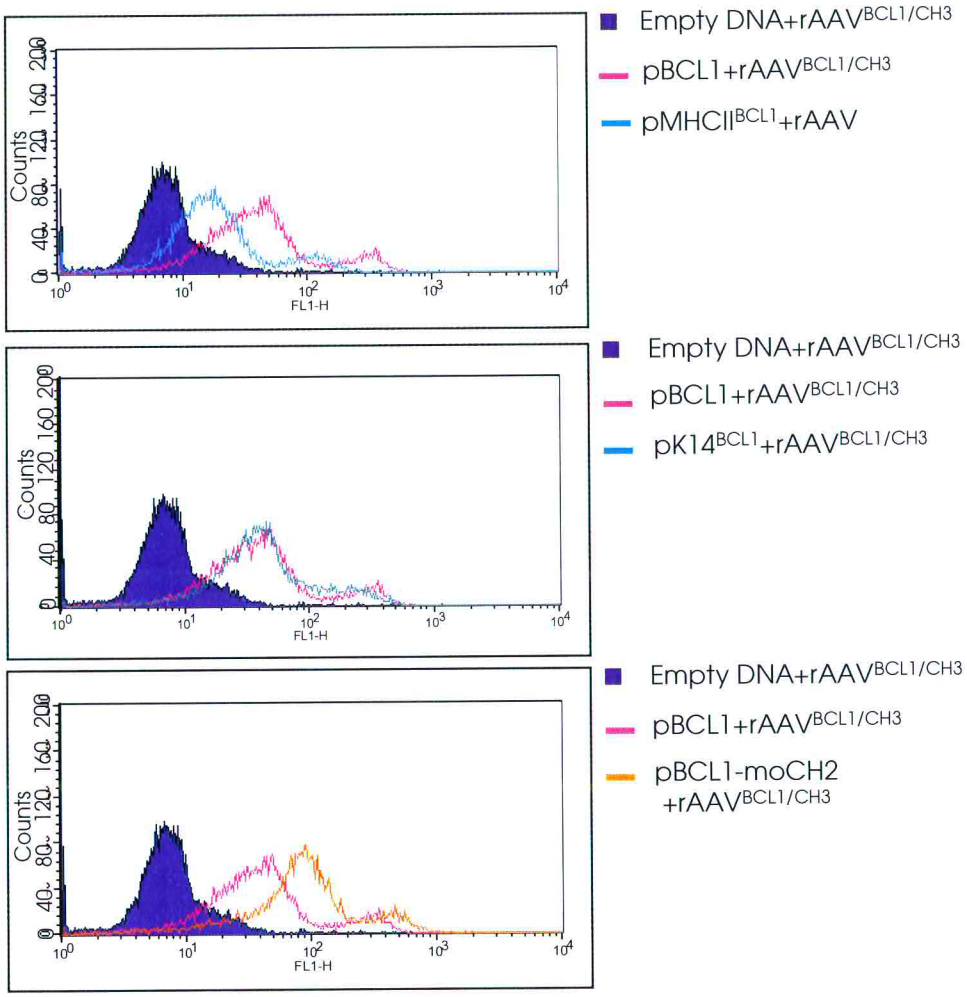


Figure 21. FACS analysis of anti-Id^{BCL1} sera. Mice groups (8/group) were vaccinated once by gene gun with DNA plasmids showed above. 15 day later a single rAAV injection was administrated and sera were harvested, pooled and analyzed one month after viral administration.

As shown in Fig. 20, the rAAV^{BCL1} boosts the response when the mice groups were treated with pBCL1, tissue unspecific vaccine under the control of CMV promoter, but also when the mice were previously vaccinated with pMHCII^{BCL1}, pK14^{BCL1} and pBCL-moCH2 DNA. This boosting effect shows comparable Ab levels increase in all immunized animals, except the group vaccinated with pBCL1 and revaccinated with irrelevant rAAV.

Moreover, we also showed that the gene gun vaccination (priming) effect is crucial in development of Abs, as results showed lower anti-Id response in pMHCII^{BCL1}, similar levels in pK14^{BCL1} group and increased levels upon pBCL1-moCH2, when compared to pBCL immunized animals, all boosted with rAAV^{BCL1} (Fig. 21).

These data suggests that rAAV virus boosting strategy represents an efficient way of increasing anti-Id Ab titers, after a single rAAV i.m. injection, and that initial, gene gun tissue specific produced antigens, were crucial for development of differential profiles of humoral response.

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3.4 TARGETING THE Fc EPSILON RECEPTORS ON APCs FOR CROSS-PRESENTATION

3.4.1. FcεRs and cross-presentation

Professional APCs (which include dendritic cells, B lymphocytes, and macrophages) are specialized to induce acquired immune responses, presenting endogenous peptides on class I MHC and exogenous peptides on class II MHC to stimulate Ag-specific CD8+ and CD4+ T cells, respectively. In addition, DCs and B cells are able to use these exogenous peptides to shuttle them to the class I MHC-processing pathway, a process termed cross-presentation (Ackerman and Cresswell, 2004). Immune responses generated from DNA immunization involve both direct and cross-presentation of Ag (Corr et al., 1999). Delivery of DNA plasmids coated onto gold particles directly transfect DCs and keratinocytes resident in the skin. DCs at the site of immunization, primarily Langerhans cells in the suprabasal epidermis, will mature and migrate to draining lymph nodes (dLNs), where they act as professional APCs to initiate Ag-specific T cell responses (Garg et al., 2003).
Another receptor family - FcεRs were shown to be interesting targets for antigen uptake and presentation. IgE Fc receptors: FcεRI and FcεRII/CD23 are expressed on variety of cells such as mast cells, basophiles, B cells, macrophages and some dendritic cell subtypes.
In humans, FcεRI engagement of DC subtype (Langerhans cell-like dendritic cells – LCDC) is shown to be crucial for induction of chemotatic signals and naive T cell priming (Novak et al., 2004).

Murine low affinity FcERII (CD23) has a restricted cellular expression, and it's isoform has been demonstrated on B cells (Rao et al., 1987) and follicular dendritic cells (FDC) (Maeda et al., 1992).
There are reports that in vitro B cells efficiently take up and present Ag complexed with IgE in a CD23-dependent manner (Bheekha Escura et al., 1995). Therefore, a possible mechanism underlying IgE-mediated enhancement of the Ab response in vivo is Ag presentation to specific Th cells by CD23+ B cells (Getahun et al., 2005).
The objective of our study was to test a model of specific delivery through any of the Fc epsilon receptors to induce cross-presentation.
For this purpose we have used the class I restricted OVA-derived peptide to activate CD8+ T cells.
We addressed the question of cross presentation of a model peptide antigen (OVA) upon specific targeting of Fc epsilon receptors potentially expressed on APCs (B cells, macrophages, DC subsets),and their ability to activate antigen specific CD8+ T cells.

3.4.2. Design and expression of targeting protein

The construct containing two Fcε domains – epsilon 3 and epsilon 4 domains (E34) which suffice for receptor binding, fused to the Class I-restricted OVA derived peptide (LEQLESIINFEKLTEWTSD) was made. Also, a construct containing the OVA peptide fused only to epsilon 4 domain was constructed, which served as a negative control. Both constructs (tagged with sv5) were easily expressed in HEK293 cells (Fig. 22).

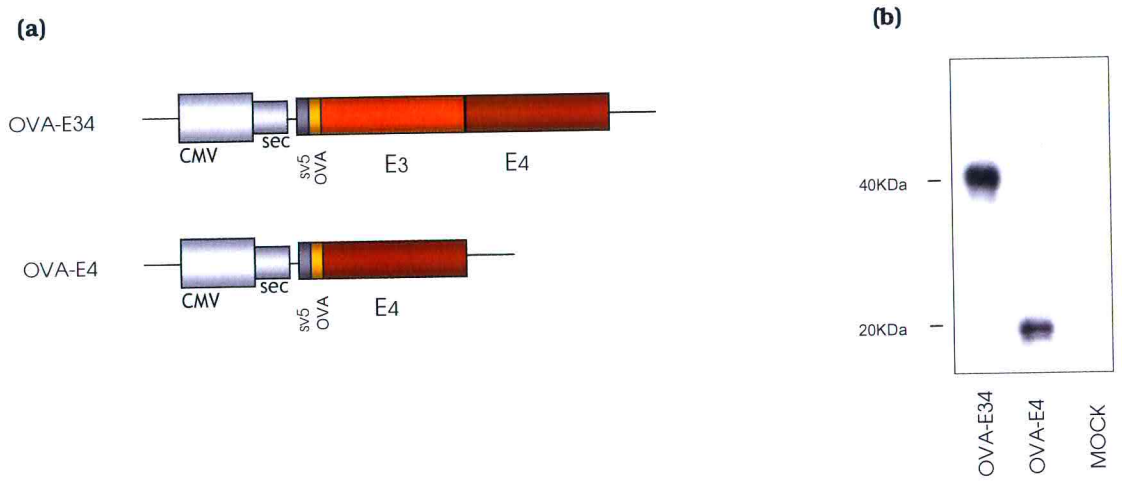

Figure 22. **(a)** Scheme of OVA-E34 and OVA-E4 DNA constructs. **(b)** Expression of OVA-epsilon proteins in HEK293 cells. SDS-PAGE developed with anti-sv5 and anti-mo IgG-HRP.

3.4.3. Functional properties of epsilon domain

In order to study binding characteristics of the epsilon domain, the following strategy was developed. Several constructs of epsilon 3 and epsilon 4 domain containing (or not) the biotin acceptor peptide (BAP) in C- and N-terminal form were made for in vivo biotinylation (Fig 23a). Recently, biotinylation of tags has been utilized in the recovery and characterization of ribonucleoprotein complexes (Penalva and Keene, 2004). This approach utilizes the co-transfection of the cell with two plasmids. One expresses the recombinant binding protein (in our case E34) tagged with the BAP, and the other plasmid expresses the enzyme BirA. The biotinylation of the BAP tag only occurs in the presence of BirA upon the addition of exogenous biotin. Supernatants from HEK293 transfected cells were collected after 48 hours and dialyzed over night against Phosphate Buffer Saline (PBS). Western blot developed with anti-sv5 showed that more than 50% of E34-BAP or BAP-E34 protein was biotinylated.

This is reflected by the amount of protein that get retarded in the gel when incubated with streptavidin, which bind to biotin even in the presence of SDS. The E34 protein not tagged with BAP (deltaBAP-E34) could not be biotinylated and therefore did not bind to streptavidin (Fig. 23b).

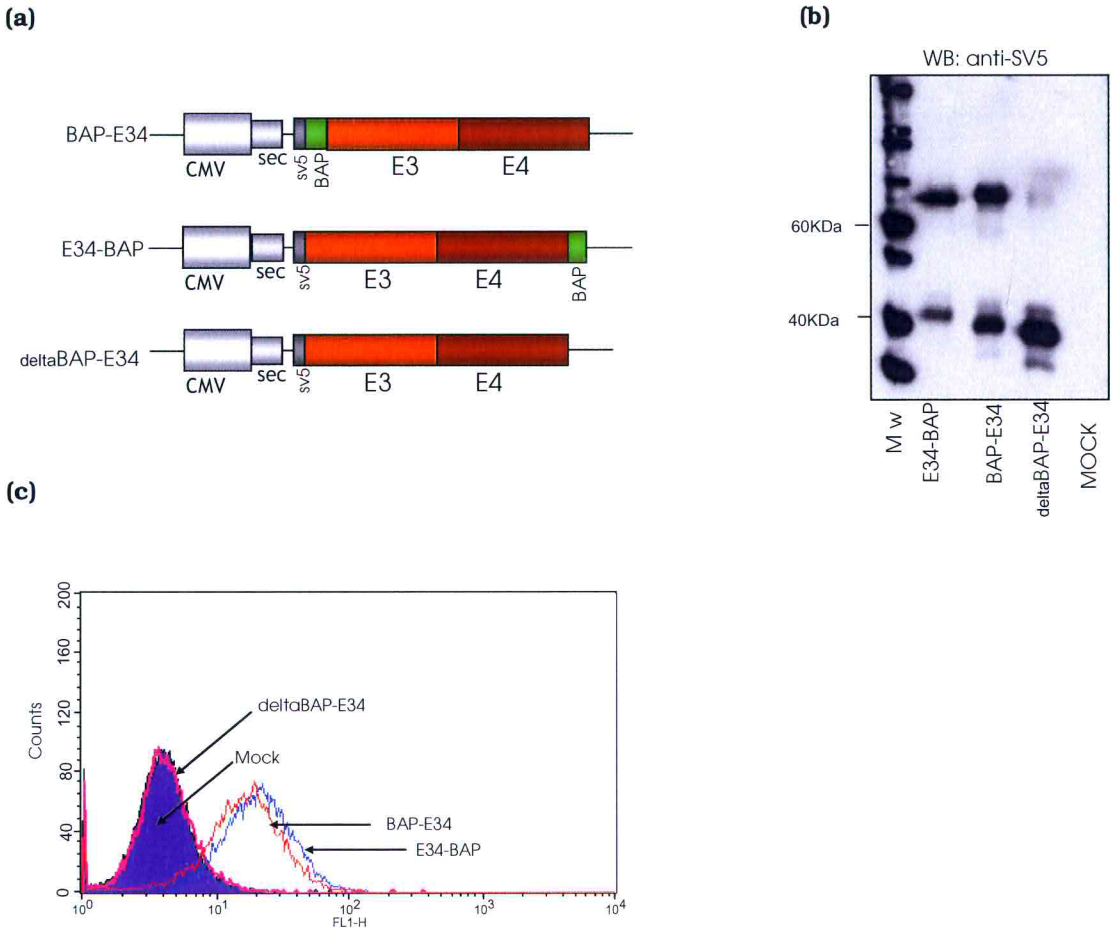

Figure 23. **(a)** Scheme of E34 constructs. **(b)** WB of dialyzed SNs preincubated with streptavidin **(c)** Binding of biotinilated E34 to RBL (huFcε $-\alpha\beta\gamma_2$) cells.

To test the binding properties of our recombinant proteins, dialyzed supernatants of all three formats of E34 and Mock (empty DNA transfection), were analyzed by flow-cytometry for binding to Rat

basophilic leukemia (RBL) cells previously transfected with human FcεRI receptor containing $\alpha\beta\gamma_2$ receptor sub-units. As mouse IgE (unlike human IgE) binds to both human and rodent FcεRI (Blank et al., 1991), our E34 proteins were expected to bind RBL(hu-$\alpha\beta\gamma$2) cells. The binding was visualized by Streptavidin^{FITC} Ab (Fig 23c).

3.4.4. Search for binding partners

Evident expression in HEK293 cells and binding properties of E34 protein to a FcεRI on RBL cells, was followed by the search for binding partners in APCs. For this purpose, bone marrow derived dendritic cells (BMDC) from C57BL/6 mice were produced and cultivated in vitro. DC were generated from bone marrow progenitors as described by Lutz et al. (Lutz et al., 1999).

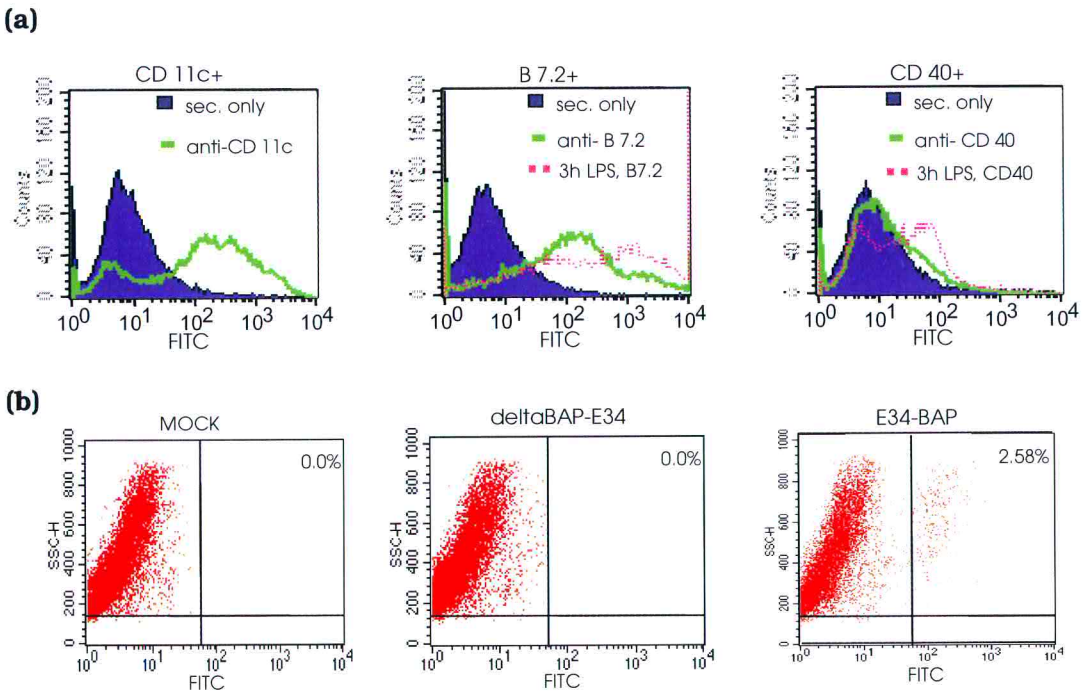

Figure 24. (a) Phenotype of BMDC day 8. Filled histograms - rat anti-mouse IgG (secondary Ab only)**(b)** Binding of indicated SNs (200µl) to the 5x10^4 of BMDCs day 8.

This population of cells at day 8 of differentiation shows phenotype of ~80% of CD 11c+ immature BMDCs, as showed in Fig 24a. Upregulation of maturation marker CD40, but also co-stimulatory receptor B7.2 (CD86) after 3h of incubation with LPS, demonstrate immature state of DCs. First experiments performend on this cell population showed binding of E34-BAP protein to ~2.5% of gated, live BMDCs (Fig24b).

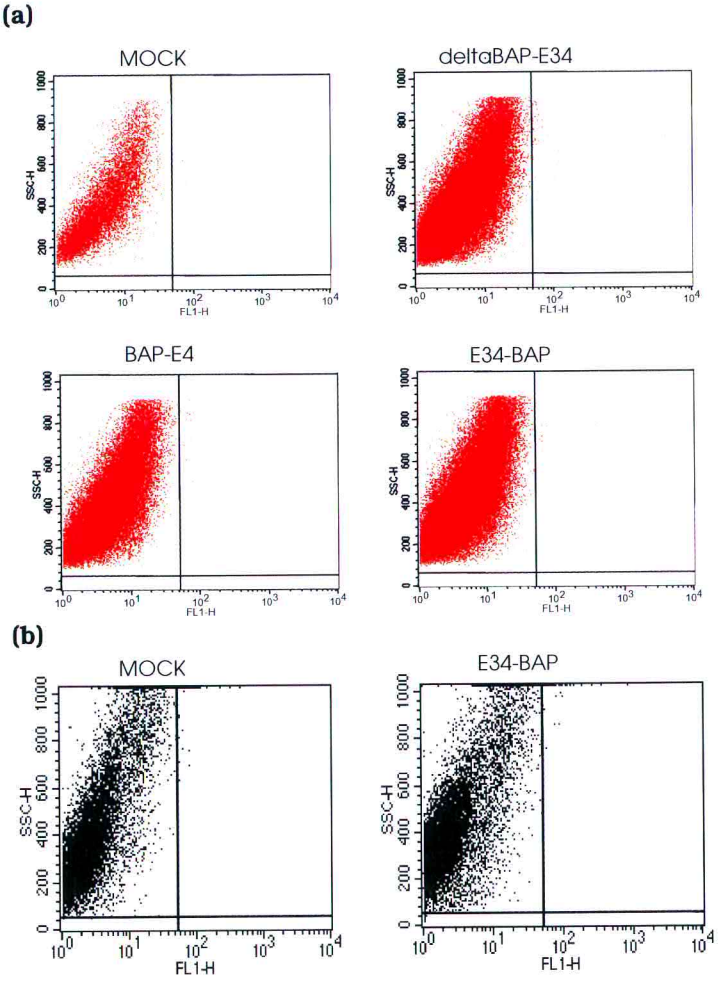

Figure 25. **(a)** Absence of binding to BMDC day 8 and **(b)** BMDC day 4, after preincubation with 600 µl of indicated supernatants.

Unfortunately, the binding of E34-BAP supernatants could not be repeated. Even after various transfection assays and preincubation with increased amounts of SNs no binding could be detected (Fig.25a). Experiments were also performed with not fully differentiated BMDCs day 4, but failed to detect any binding to E34 protein (Fig.25b).

To address the possibility of E34 binding to low affinity Fc epsilon receptor (CD23) experiments were performed on different CD23+ B lymphoma cell lines. Unlike the FcεRI, the FcεRII/CD23 is not a member of the Ig superfamily, but a type II transmembrane receptor which is expressed on a variety of cells of the immune system including B lymphocytes (Delespesse et al., 1991).

In order to test the binding on B cells, the binding capability of OVAp-E34 (containing sv5 tag) was first confirmed by FACS on the RBL cells (Fig.26a). The same batch of supernatants were than used to investigate the binding on 3B3 (Fig.26b) and WEHI231 (Fig.26c) CD23+ B lymphoma cells.

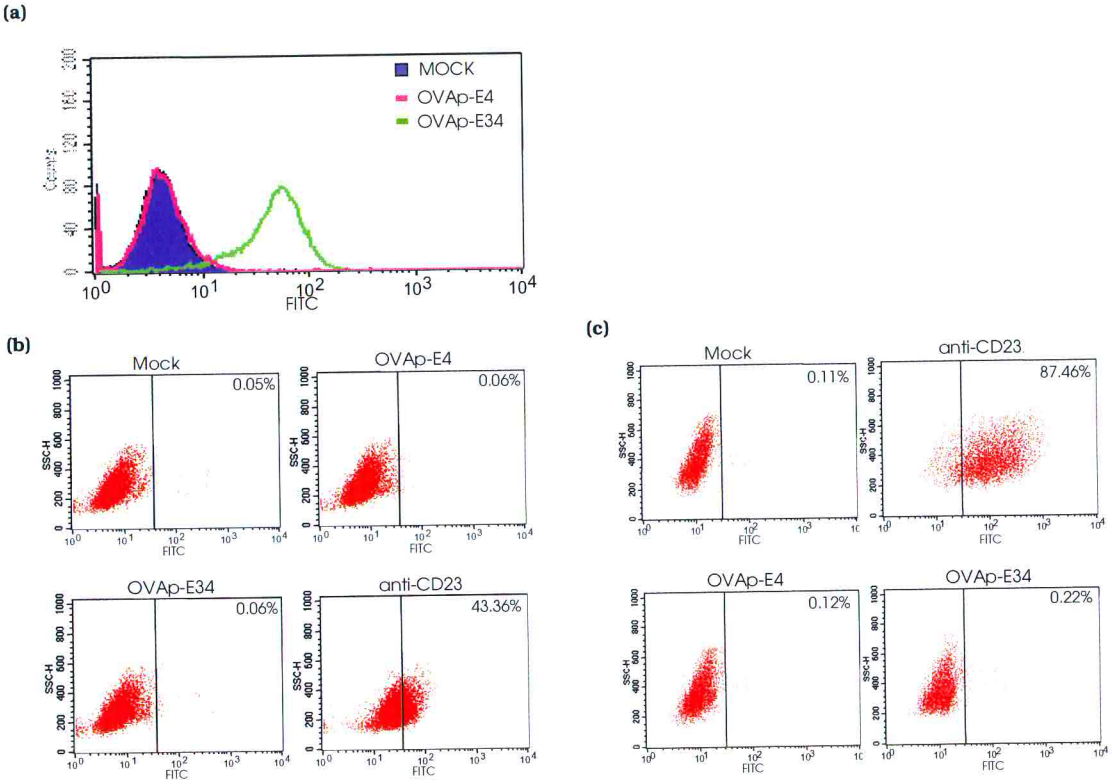

Figure 26. **(a)** Binding of 200µl of OVAp-E34 dialyzed SN to RBL cells. (b) 500µl of Sns were used for incubation with 3B3.BCL1**(b)** and WEHI 231 **(c)** B lymphoma cells. All binding assays where SNs are used, were visualized by mouse anti-SV5, and anti-moIgG^{FITC} Abs.

Unexpectedly, no significant binding could be observed after incubation with OVAp-E34 SNs. Absence of binding to CD23 could be explained by high amount of E34 protein required for binding, considering the difference in receptor affinity of FcεRI, (KA ~10^{10} M^{-1}) , compared to (KA ~10^{7} M^{-1}) of CD23 for binding of IgE. Moreover, although the A-B loop in Cε3 was identified as a critical region for the interaction, deglycosylation of IgE has been shown to expose epitopes on the Cε2 domain (not present in OVAp-E34) (Bjorklund et al., 1999) and a role for N265 in CD23 binding has been postulated previously (Young et al., 1995)

In order to detect and characterize eventual binding partners of OVAp-E34 protein in WT mouse, splenocytes and axillary and inguinal lymphnodes from C57BL/6 mouse were harvested and purified.

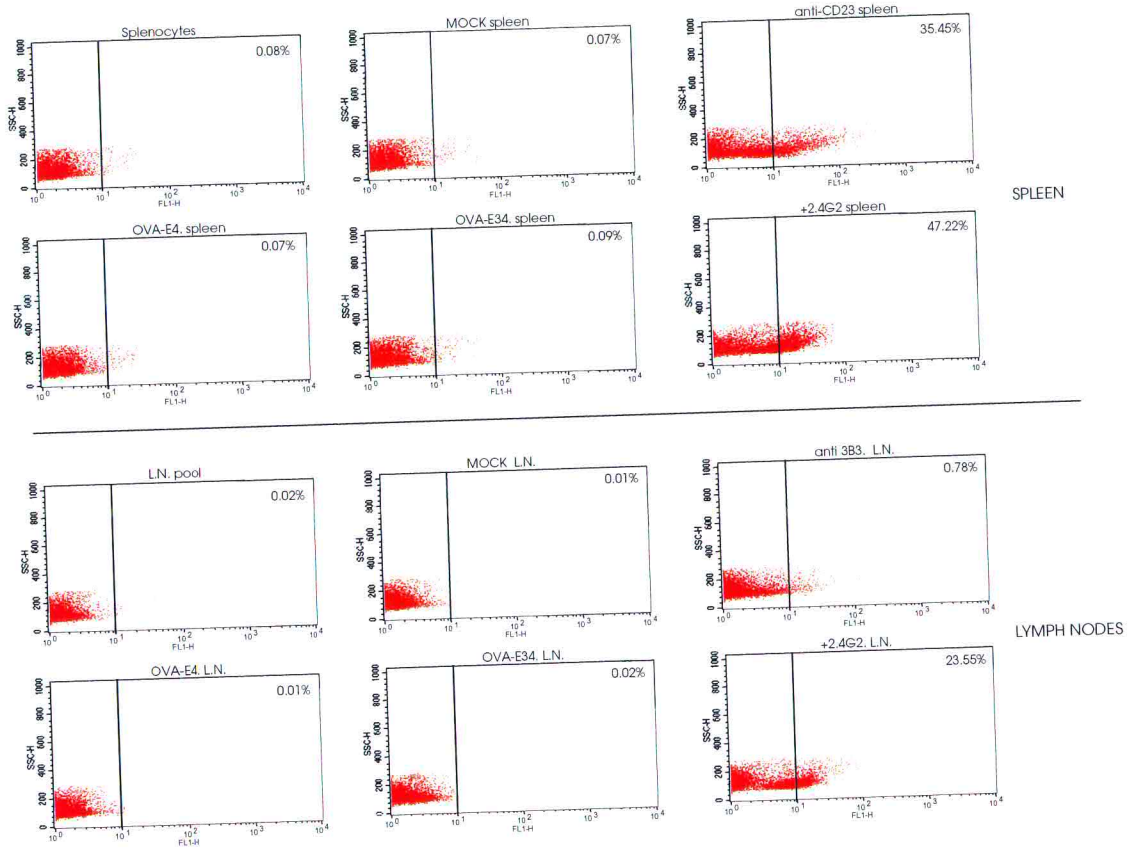

Figure 27. 2x10^5 of splenocytes and pooled axillary and inguinal lymphnodes were purified and incubated with 500µl of indicated dialyzed SNs and Abs for 1h at 4C.

Dialyzed SNs where than tested for binding to mouse splenic lymphocytes and pooled lymphnodes. Unfortunately, no binding partners for OVAp-E34 could be detected (Fig. 27). As a positive controls, the rat MoAb 2.4G2 (anti mouse FcγRII and FcγRIII) and anti mouse CD23 MoAb were used.

3.4.5. In vitro activation of OT1 cells

Despite of the fact that we were not successful in finding the binding partners for OVAp-E34, but being aware of its demonstrated binding capability to FcεRI, we performed in vitro activation assays. For screening the activation we used purified CD8+ T cells from OT1 mice, transgenic for a TCR that recognizes the SIINFEKL peptide of OVA presented on H-2K^b, and have been previously described (Hogquist et al., 1994)

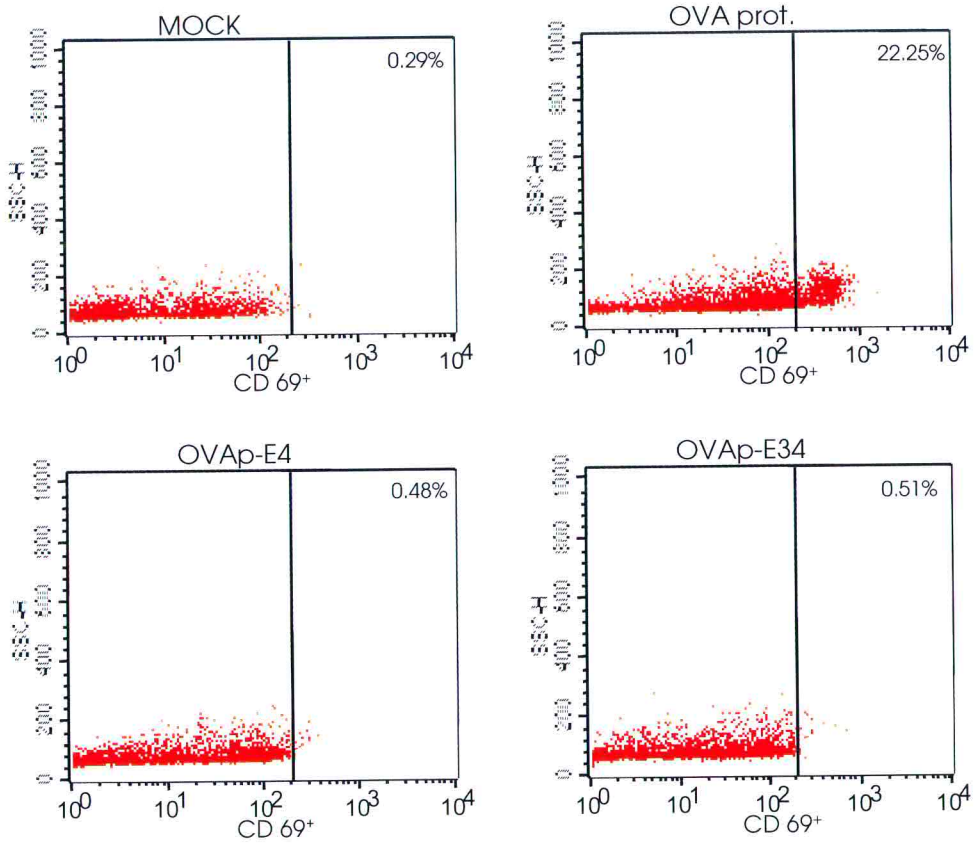

Figure 28. In vitro activation assay of OT1 cells.

Splenic lymphocytes derived from WT C57BL/6 mouse were collected and purified by FICOLL gradient centrifugation. 2x10^5 of cells were pulsed with 200µl of MOCK, OVAp-E4 and OVAp-E34 dialyzed SNs or

OVA protein, for 4h at 37C. Cells were washed and $5x10^4$ of OT1 cells were added and incubated O/N. Next day OT1 cells were gated and labeled for T cell early activation marker CD 69+.
As expected, the activation of a small population of OT1 cells could be induced by pulsing with of OVA protein (1mM). Activation of OT1 cells could not be observed when the cells were pulsed with Mock, OVAp-E4 nor OVAp-E34 proteins (Fig. 28). We repeatedly failed to detect OT1 activation on total spleen and purified lymphocytes, but also in experiments with BMDCs (data not shown). These results were in agreement with previous in vitro binding experiments, since no binding partners could be find in mouse splenic lymphocytes.

3.4.6. In vivo activation of OT1 cells

Since there was a significant number of cells that could not be targeted in our binding and activation experiments in vitro, as they were only involving limited cell types (found in spleen, lymphnodes and cultivated BMDCs) we decided to test whether in vivo FcεRI targeting of OVAp-E34 could result in OT1 cell activation.
Apart of the Mast cells and Basophiles, the epidermal skin Langerhans cells and periferal blood DC subsets that express FcεRI could serve as a potential target. To target these cells we designed and performed in vivo activation assays. First, C57BL/6 females were immunized by gene gun for 3 times each 14 days with constructs containing OVAp-E34, or OVAp-E4 protein coding genes.

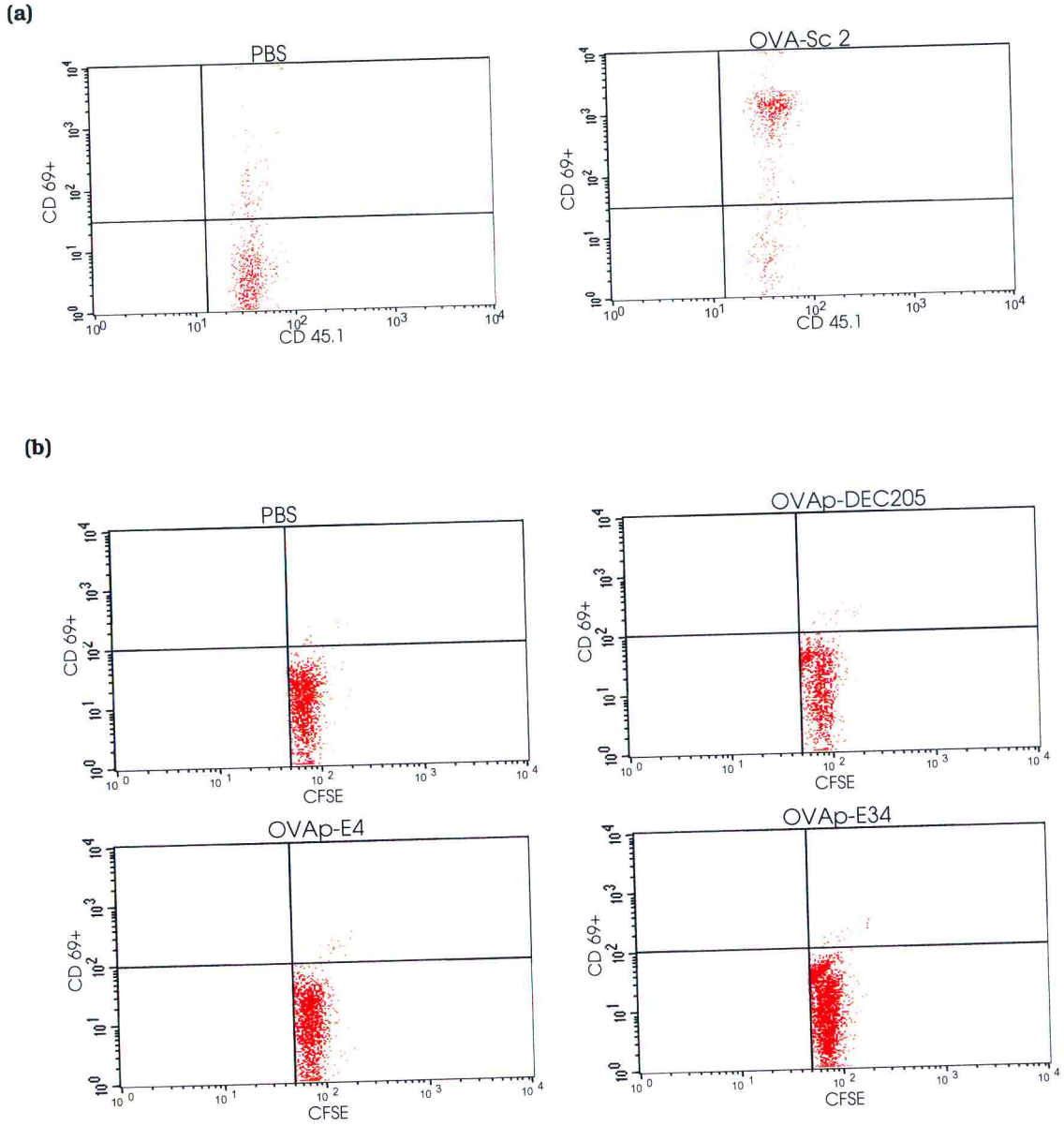

Figure 29. FACS analysis of OT1 cells after the mice were vaccinated with **(a)** OVA-Sc2 protein (positive control) or PBS, OT1 were injected in animals and harvested 16h later and double stained with CD45.1 and CD69+ **(b)** gene gun immunization with indicated DNA. 1-1.3x10^6 of OT1 cells were labeled with CFSE before i.v injection in the tail. 20h after, splenic and L.N. cells were harvested CFSE+ gated and analyzed for CD69+ expression.

Additionally, to demonstrate that cross-presentation and T cell activation has been exclusively made through the targeting of specific receptor, we included the anti-DEC 205 in the ScFv format to target DEC 205 endocitic receptor of DCs, fused to the OVA peptide. It is expressed at high levels on lymphoid tissue DCs (Guo et al., 2000) and greatly enhances the efficiency of antigen presentation (Jiang et al., 1995)
Prior to i.v. injection, OT1 cells were purified from OT1 transgenic mice, stained with CFSE, and counted. 1-1.3x10^6 of cells where than injected in previously immunized mice. Next day, mice were sacrificed, splenic cells , as well as inguinal, axillary and mesenterial lymphnodes were collected, pooled, and analyzed on FACS. CFSE+ cells were gated and screened for CD 69+ expression.
It was expected that OVAp-E34, if bound to FcεRI+ cells, would have been capable of internalizing and presenting OVA peptide on MHC class I, and therefore induce OVA- specific T cell activation. However, both in vitro experiments (on pulsed splenocytes and BMDC) and in vivo experiments (on gene gun immunized animals) showed that such an event does not occur under these settings (Fig. 29b).
Recently, Demangel *et al.* have shown that antigen targeting to DCs via a DEC-205 binding scFv leads to enhanced immunogenicity (Demangel et al., 2005). Surprisingly, in our in vivo experiments, gene gun immunization with OVAp-DEC 205 animals did not result in activation of OT1 cells.

In contrast, in a separate experiment (done by Elisa Tagliani) when the OVA-SC2 protein which targets CD36 scavenger endocytic receptor on DCs , was injected together with a OT1 cells (positive for leukocyte common antigen CD45.1) in C57BL/6 mice (CD45.1-) OT1 cell activation, shown by CD 69+ up-regulation, took place. (Fig. 29a).

Contrary to what we hoped, the strategy of DNA immunization showed to be inefficient as a model for receptor targeted cross-presentation, as it did not result in *in vivo* activation of OT1 cells. To conclude, although the OVAp-E34 protein was shown to be capable of binding to RBL (huFcε $-\alpha\beta\gamma_2$) cells, we repeatedly failed to detect any binding partners in WT mice in vitro, and showed complete absence of OT1 cell activation, both in vitro and in vivo.

4. Discussion

The ability of new technologies to reveal candidate tumor antigens together with the recent advances in understanding of the molecular biology of cancerogenesis and regulation of immunological pathways, provided the opportunity to design vaccines against a wide range of cancers.

The idiotype is a widely accepted tumor marker and a promising therapeutic target for immunotherapy of B-cell malignancies.

Most murine tumor models use artificial tumor antigen, often immunogenic by itself. In contrast, syngeneic murine lymphomas and myelomas express clone-specific tumor Id, which is usually a non-immunogenic self-antigen. It is postulated that immunity elicited in these models using Id-vaccines would closely predict a future clinical outcome in humans. In concordance, data from clinical Id-vaccine trials also supported the reliability of these murine models. In fact, the ability of GM-CSF to enhance potency of an Ig-KLH vaccine and activate the T-cell arm of the immune response,(Kwak et al., 1996) the efficacy of Id/IL-2 liposomal vaccine formulation (Kwak et al., 1998) and the possibility of transferring Id-specific immunity from immunized allogeneic marrow donor to recipients were predicted from preclinical studies in syngeneic murine B-cell tumor models.

Therefore, the vaccine's initial design and its validation for future clinical trials are vigorously tested in murine models of B-cell malignancies of various genetic backgrounds. There are two ways to test vaccine formulations in mice: one is to induce immunity prior to challenge with tumor cells (protection experiment), the other is to treat non-immune naive mice bearing tumor cells with the vaccine (therapy/eradication experiment). Although the importance of therapy to eradicate an

established tumor is often over emphasized since it resembles the clinical situation, it is usually a challenging task by itself even if tumor cells grow slower in mice. For example, it has been reported that Id-vaccine can eradicate a slower growing established A20 lymphoma and induce antitumor immunity (Biragyn et al., 2001).

Our tumor protection experiments showed that mice immunized with Id-BCL1 were protected after challenging with BCL1 expressing lymphoma cells. These results demonstrated that the induction of anti-Id antibodies is a necessary condition for tumor protection.
These findings are in agreement with previous observations reported in this model upon protein vaccination (George et al., 1988). Indeed, passive immunization of SCID mice with protein induced anti-Id antibodies, together with depletion of natural killer (NK) cells were sufficient to induce BCL1 tumor protection (Racila et al., 1995). These Abs were found to be able to trigger tumor cell death and cell cycle arrest upon IgM cross-linking.
Our group have investigated whether DNA- induced antibodies retain the same ability, despite their exclusive reactivity to VL/VH combined epitopes (Benvenuti and Burrone, 2001). Since biological activity of antibodies strictly relates to their specificity, this demonstrates an important issue. Moreover, ex-vivo results indicated that antibodies elicited by DNA vaccination induce both, tumor cell death and cell cycle arrest, suggesting that DNA-induced conformational antibodies retain the functional capacity of protein-induced antibodies. Hence, DNA vaccination was found to be as efficient as protein immunization in inducing tumor protection (Cesco-Gaspere et al., 2005).

Numerous strategies have been reported to reverse the weak immunogenicity of murine B-cell tumor-derived Ig and elicit anti-Id antibody responses. High titers of anti-Id antibodies were elicited by Id immunizations together with a variety of immunologic adjuvants such as SAF, CFA, QS-21 or KLH (Campbell et al., 1990). In our studies, we made immunogenic chimeric molecule by linking the BCL1 idiotype in ScFv format to human γ-CH3 domain. It showed to be an efficient strategy for eliciting strong anti-Id response in mice. However, it should be noticed that in respect to murine lymphoma models, the induction of anti-Id antibodies by DNA vaccination in humans is still a difficult task. In a clinical trial lymphoma patients were immunized with DNA encoding a chimeric Ig molecule, containing VL and VH chain sequences derived from each individual tumor linked to the mouse IgG2a and kappa Ig constant region chains, respectively (Timmerman et al., 2002). However this strategy failed to elicit a significant anti-Id immune response, while the safety profile of the immunization procedure was demonstrated.

Since we showed that anti-Id antibodies play an essential role in tumor rejection of B cell lymphoma, we then studied the possibility of making this model of tumor protection transferable to other tumor type.
We reasoned that anti-Id^{BCL1} response could generate a cascade of immunological events, involving possible engagements of ADCC and CDC, directed to exclusively kill tumor cells displaying the idiotype, and in turn, amplify a T-cell immune response mediated by TAAs released from these cells.
Complement fixation is likely to actively participate in rejecting the tumor, as reported for some tumor models (Syrengelas and Levy, 1999). Similarly ADCC may contribute to prevent tumor growth. Interestingly, DNA vaccination has been shown to mainly raise antibodies of the IgG2a isotype, which indeed have been clearly demonstrated *in vitro* to induce a

superior ADCC anti tumor effect compared to the other isotypes (Kaminski et al., 1986).

After examining different expression strategies in order to display the idiotype on different tumors, we were able to generate stable MC38 clones displaying the Id^{BCL1}. Moreover, these colon adenocarcinoma clones showed to be truly persistent in the expression of the membrane bound idiotype. We saw an efficient expression of our idiotype on MC38 cells, even after 1 month of in vivo tumor progression.

It has been shown previously in our lab, that anti-Id^{BCL1}antibodies were able to provoke apoptosis and cell cycle arrest of BCL1 B lymphoma cells, after cross-linking with anti-Id^{BCL1} sera induced by DNA immunization. In contrast, we showed that preincubation of anti-Id^{BCL1} sera with MC38 cells displaying Id^{BCL1}, doesn't result in apoptosis nor it shows any cytopathic effect.

This result was expected since the BCL1 idiotype on B lymphoma cells is a part of B cell receptor (BcR) including associated molecules and chains, transmitting signaling events to the cell, while in the case of MC38 cells, the BCL1 idiotype represents an artificially displayed membrane protein without associated signaling molecules.

In our tumor protection experiments, vaccinated animals challenged with MC38 Id^{BCL1}+ cells, did not show any tumor rejection nor the delayed tumor progression. We showed that BCL1 idiotype as a protective tumor idiotype, as shown in B lymphoma case, is not transferable to MC 38 model under these settings. Probably the direct signaling events of anti-Id antibodies plays a significant, if not a crucial role in rejecting the B cell lymphoma tumor development and survival of challenged mice.

In this study we also aimed to further characterize the nature of anti-Id response and develop the strategies for its enhancement.
To this end, we used two experimental approaches. First, we used a tissue-specific promoter-directed gene expression system in which DNA-encoded Ags can be expressed specifically by either APCs (MHCII promoter) or non-APCs (K14 promoter) in vivo. This approach allows us to exclude possible artifacts resulting from an in vitro or ex vivo manipulation of APCs from DNA-immunized mice (Girolomoni et al., 1990; Schuler and Steinman, 1985). Second, we performed a receptor mediated internalization process to enhance APC antigen presentation and increase immune responses.
We investigated whether our immunization system with MHC II promoter driven protein was efficient to directly transfect APC. The result showed that poor but significative anti-Id response could be seen 15 days after a single DNA gene gun shot. In other models, DCs have been found to be directly transfected by intramuscular needle injection and to be able to activate naïve T lymphocytes *in vitro* (Chattergoon et al., 1998). Also, a pioneer study on the mechanism by which gene gun vaccination induces Ag-specific immunity identified the presence of bombarded projectiles in the cytoplasm of DCs in the lymph nodes, providing the first demonstration of transfection of DCs (Condon et al., 1996). The activation of CD8+ T cells from immunized mice by directly transfected DCs has also been demonstrated (Porgador et al., 1998).
Similarly, our findings demonstrate that gene gun immunization is effective in inducing a humoral response to a secreted Ag when its expression is restricted to APC. This suggests that even with a low efficacy, biolistic immunization is able to directly transfect APC.

After immunization of mice with DNA vaccines driven by the CMV or the K14 promoter, a high level of Ag expression occurs in the many keratinocytes of the basal epidermis directly transfected by the DNA

coated gold particles. We confirmed this by obtaining similar levels of anti-Id^{BCL1} response in vaccinated mice groups with CMV or K14 Ag-driven expression. Because keratinocytes are incapable of directly priming B- and T-cell responses because of their lack of costimulatory molecules (Akbari et al., 1999), the ability of DNA-transfected keratinocytes to efficiently induce humoral responses appeared to depend on uptake and presentation of secreted Ags, by a local DC population. In fact, in animals immunized by K14 promoter-driven vaccines, the keratinocyte- derived Ag must have been taken up by professional APC that then stimulate naïve T cells in the secondary lymphoid tissues. These results are in line with a study conducted by Cho *et al.* (Cho et al., 2001), who compared T cell responses in mice after gene gun immunization with a APC-targeted vaccine (plasmids driven by the CD11b promoter, which is active in macrophages and a subset of DCs) vs a non-APC-targeted vaccine (plasmids driven by the K14 promoter). They found that non-APC-specific gene expression was markedly better at inducing both T and B cell responses than the APC-targeted expression. They concluded that cross-priming is indeed the predominant mechanism for generation of immunity after DNA vaccination.

Taken together, these findings confirm that DNA gene gun immunization is efficient in inducing Ab response after protein secretion in tissue specific manner. Moreover, levels of anti-Id sera are in direct correlation with the amount of protein secreted.

In the second approach we investigated to enhance APC's antigen presentation by a receptor mediated internalization.

There have been attempts to target an antigen to APCs to enhance the potency of DNA vaccines, such as using CTLA4 molecules (Boyle et al., 1998; Deliyannis et al., 2000). The vaccination strategy we describe relies on Fcγ receptor mediated uptake of the secreted protein, which contains the CH2 domain of the mouse IgG2a. This antibody is usually a product of the adaptive Th1-type immune response, and binds with high affinity to FcγRI. The comparison of normal and FcγRI – deficient mice showed that FcγRI is the major IgG2a receptor and provides most rapid and extensive uptake of immune complexes (Barnes et al., 2002)
This strategy can elicit strong Ab responses because of efficient protein secretion from transduced cells and enhanced T-helper response.
In our experiments we showed significant increase of anti-Id^{BCL1} levels in animals vaccinated with only one shot of pBCL1-moCH2, when compared with response induced by pBCL. The number of cells transfected by gene gun, and the amount of protein produced by these two vaccines, is likely to be identical, considering that they are both driven by CMV promoter, and that in vitro transfection assays showed comparable amounts of protein produced. However, different quality of in vivo response is clearly due to the presence of mouse γ2a domain that direct the protein to APCs.

Efficacy of antitumor antibodies has been attributed to direct growth inhibitory effects of tumor cells, complement-mediated cytotoxicity, or ADCC resulting from FcγR activation on myeloid and NK effector cells (Carter, 2001). Genetic evidence in humans (Cartron et al., 2002) and mice (Clynes et al., 2000) supports a general requirement for FcγRs engagement for the efficacy of antitumor antibodies in vivo, implicating Fc-dependent effector ADCC as a common mechanism underlying tumor-specific humoral immunity. However, a requirement for CD8 cellular cytotoxicity for the efficacy of an antitumor mAb (Honeychurch et al.,

2000) suggests that, in addition to mediation of ADCC, FcγR–mediated enhancement of antigen presentation is another mechanism contributing to tumor immunity.

The data we presented here directly demonstrate that FcγR targeting of APCs results in increased anti-Id humoral immunity, after DNA biolistic immunization.

Recently, it was shown that an additional component of anti-tumor antibody efficacy, may be the induction of tumor antigen– specific T cell responses. (Rafiq et al., 2002) The priming of naïve T cells in vivo by extracellular antigen is orchestrated by DCs, which have multiple pathways for antigen uptake, including macropinocytosis, endocytosis, and phagocytosis (Banchereau et al., 2000). DCs are critical for initiating and modulating B- and T-cell responses elicited by DNA vaccination.

In the future, idiotypic vaccines should be tailored to target preferentially various subsets of immune cells, such as DCs, which would uptake and properly process and present Id, activating both humoral and cellular arms of the immune system. Moreover, the vaccine should induce the production of a milieu of inflammatory cytokines and lymphokines at the delivery site to elicit a T helper type 1 (Th1) immune response.

Components of the inflammatory response can be used to target DCs *in vivo*, activating the so-called *danger* signal for circumventing the poor immunogenicity of selftumor antigens. For example, chemotactic factors of innate immunity are able to deliver Id to APC and render immunogenic, this otherwise non-immunogenic antigen. The strategies developed for Id vaccines can be used as a general strategy for eliciting T-cell immunity to other weakly immunogenic, clinically relevant self-tumor antigens.

Contrary to naked DNA vaccines, virus based vectors allow efficient penetration to cells while mimicking natural infection (Chen et al., 1996). Viral vectors, that lack the genes required for replication in human cells, are good candidates as carriers for genes of therapeutic interest. Among these, non-replicative viruses AAV has been extensively explored for its capacity to infect both dividing and non-dividing cells and to induce a long term expression of the Ag. Moreover, AAV is unique among the viruses currently being used for gene transfer, as it is a native human virus known not to cause any disease, and may suppress the induction of tumors by other viruses (Khleif et al., 1991). A lot of studies has been made to explore the potentiality of this vector for immunotherapeutic proposals (During et al., 1998; Liu et al., 2005; Liu et al., 2000; Xin et al., 2002). Overall these studies demonstrated the reliable capacity of rAAV to induce specific immunity to a foreign Ags upon i.m., s.c., and oral administration. It is reasonable to expect that upon i.m. injection immune response is more easily elicited when Ag is secreted and consequently, allowed to reach resident or lymphatic APCs for processing and T cell priming.

In our model, the employment of rAAV could allow us to take advantage of the exquisite features of this delivering system, such as safety, efficacy of Ag delivery and long term expression. Moreover, we characterized the boosting capability of rAAV.

Taking into consideration the tropism of AAV for muscle tissue, mice were initially injected with escalating doses of rAAV-BCL1/CH3 in the legs Tibialis anterior muscle. The significant anti-Id^{BCL1} titers were detected one month after rAAV injection. The response reached a plateau with the injection of $3x10^{10}$ of rAAV v.p. We also showed that i.m. route of administration resulted in inducing the highest yield of anti-Id antibodies. Moreover these Ab titers, raised by rAAV injection, were

comparable with anti-Id Ab levels obtained after one DNA gene gun shot. This suggests that APC direct transfection and/or stimulation through unmethylated CpG motifs induced by gene gun may prevail over the higher efficacy of cell targeted viral transduction obtained by rAAV administration. In this line, it was of our particular interest to use DNA vaccination to prime a T cell response, that may be later boosted by rAAV immunization.

Generically referred to as 'prime– boosting,' this strategy is effective at generating high levels of T-cell memory (Ramshaw and Ramsay, 2000). Although much of the early work using this strategy was driven by efforts to develop vaccines to control malaria, it was subsequently applied to vaccine development against a variety of pathogens (Newman, 2002). The increased numbers of T cells 'push' the cellular immune response over certain thresholds that are required to fight specific pathogens (Seder and Hill, 2000). Furthermore, the general avidity of the boosted T-cell response is enhanced, which presumably increases the efficacy of the available T cells (Estcourt et al., 2002).

Our studies have shown that rAAV boosts the response previously induced by DNA gene gun immunization. Moreover, anti- Id^{BCL1} titers were increased in all vaccinated animals, independently of the promoter tissue specificity of a DNA vaccine and type of cells that are producing the protein. These data confirm that heterologous boosting using rAAV represents an efficient strategy for improving the humoral response, as showed by increased levels of anti-Id antibodies.

To speculate about the mechanism of enchanced anti-Id response, in our model using this strategy we probably induced a sustained CH3-specific CD4+ T cell response, which gets subsequently boosted by the production of large amount of Ag by rAAV injection. It remains to be established whether rAAV boosting could result in the activation of CD8+ T cell responses. In fact induction of Ag specific CTLs by rAAV

immunization has been reported previously, after an i.m. (Liu et al., 2000) and s.c. or i.p. (Brockstedt et al., 1999) administration.

As mentioned before, specialized immune cells, including dendritic cells, can capture proteins from other cells and direct them into their own MHC I pathway. This unusual ability to transfer exogenous proteins into the MHC I pathway has been referred to as cross-presentation. The mechanistic basis for cross-presentation is not well understood, but several pathways have been described (Heath et al., 2004; Yewdell et al., 1999). It has been recently discovered another pathway, by which cells obtain peptides from their neighbours though gap junctions. Neijssen *et al.* also show that Langerhans cells, one of several subtypes of dendritic cells (Heath et al., 2004), form gap junctions with surrounding skin cells (keratinocytes). This provides the Langerhans cells with a potential method of sampling keratinocyte peptides before migrating to the lymph node, where they initiate immunity to skin infections. In this line it has been reported that Langerhans cells can cross-present peptides expressed by keratinocytes (Mayerova et al., 2004)

High affinity Fc epsilon receptor-FcεRI and low affinity- FcεRII-(CD23) receptor have shown to be an interesting candidates for gene targeting and Ag presentation in humans as they are expressed on a variety of cells, such as mast cells, basophiles, B cells and several DC subtypes. In humans, FcεRI on monocytes and DCs was shown to mediate small calcium fluxes (Maurer et al., 1994), and to promote IgE-mediated antigen presentation (Maurer et al., 1995). This antigen presenting function by FcεRI is even more effective on circulating dendritic cells (Maurer et al., 1996). More recently, the FcεRI engagement of DC subtype (Langerhans cell-like dendritic cells – LCDC) has shown to be crucial for induction of hemotactic signals and naïve T cell priming (Novak et al., 2004).

Consistent with our DNA immunization protocol we aimed to design a vaccine which could efficiently target Fc epsilon receptors, eventually found on mouse APCs, and specifically activate TCR transgenic OVA specific CD8+T cells, by cross presentation of OVA peptide.

We showed that Cε3-Cε4 domains in both, biotinylated- E34-BAP and OVAp-E34 forms were able to bind RBL (huFcε-αβγ2) cells. However, we failed to detect any binding partners for E34 in vivo and in vitro, neither in spleen and lymph nodes of WT mouse, nor in BMDCs.

This could be explained by the difference in FcεRI in human and rodents. While rodent FcεRI has an obligatory αβγ2 tetrameric structure, human FcεRI can be expressed as both trimeric (αγ2) and tetrameric (αβγ2) structures. Moreover, all human APCs express αγ2 trimeric complexes. Therefore it is possible that this αγ2 structure serves the Ag-presenting function by specifically targeting the antigen-IgE-FcεRI complexes to the intracellular antigen presenting compartment.

Efficient uptake and presentation by B cells of Ag complexes with IgE, in CD23 manner, has been shown before (Bheekha Escura et al., 1995). Recently, the enhancement of the antibody response was shown to be due to the Ag presentation to Th cells by CD23+ B cells (Getahun et al., 2005). In our case, the binding to low affinity IgE receptor-CD23, could not be detected. CD23+ B lymphoma cell lines (3B3.BCL1, WEHI123) did not bind to E34 protein even when increased amounts of protein was used.

Potential OVAp uptake and cross presentation on class I was screened through OT-1 cell activation in vitro and in vivo. DNA immunization of animals with OVAp-E34 was unable to activate the exogenous OT-1 cells in vitro and in vivo. These results have shown that DNA immunization

was not efficient in this model of receptor targeting. This could be explained by several observations.

First of all, as mentioned above, the difference in FcεRI receptors between human and rodents, and the fact that αγ2 trimers couldn't be found in mouse could be crucial for OVAp-E34 uptake and cross-presentation.

Second, in humans, healthy donors often show low or no surface FcεRI on APCs, depending on the cell type, whereas atopic donors display high levels (Gosset et al., 2001; Reischl et al., 1996). The difference in the expression of FcεRI between the WT mice and the atopic mice, still has to be determined. Since increased levels of IgE in the blood are required for the FcεRI upregulation, probably the experiments on the atopic mouse models would be of a particular interest.

In any case, the FcεRI-expressing cells supporting Ag presentation are not seen in rodents and for that reason, it has not been easy to asses the overall biological importance of the FcεRI mediated Ag presenting function. Future studies should be performed in the human FcεRI knock in mice, since the work of Dombrowicz *et. al.*, showed that the human FcεRIα 9.5kb transgene recapitulates not only the appropriate cell specificity of expression of human FcεRI, but also the appropriate cell type/structure observed in human cells (Dombrowicz et al., 1996; Dombrowicz et al., 1998). The "FcεRI-humanized" mice may be a useful model in this regard.

5. Conclusions

In conclusion, we explored possibility to transfer BCL1 model of tumor protection to colon adenocarcionoma. Moreover, we characterized and developed DNA vaccination strategy to enhance anti-idiotypic immune response. We showed that Id vaccine based on rAAV was able to induce anti-Id response and that rAAV revaccination of DNA-immunized animals can efficiently boost this response. Finally, we addressed possibility of cross-presentation of OVA peptide after targeting the Fc epsilon receptors on APCs.
We were able to demonstrate that:

1. The BCL1 idiotype, as a protective tumor associated antigen in the B cell lymphoma case, is not transferable to colon adenocarcinoma (MC38) model, despite the fact that tumor cells were efficiently expressing the exogenous target Id in vivo.
2. Gene gun immunization is effective in delivering DNA to APCs, as shown by anti-Id titers raised by MHC II promoter driven vaccine.
3. The increased anti-Id^{BCL1} antibody levels in animals immunized with the vaccine containing the constant heavy chain domain of murine γ2a immunoglobulin, showed its targeting capability.
4. A single injection of the rAAV containing BCL1 idiotype linked to human CH3 domain was efficient in inducing anti-idiotypic titers.
5. The rAAV clearly enhance the response of DNA vaccinated animals. The rAAV boosting strategy represents an efficient way of increasing anti-Id Ab titers, even after a single rAAV i.m. injection. Moreover, these Ab titers were increased in all immunized animals, independently of the promoter tissue specificity of a DNA vaccine.
6. We failed to detect any binding partners of Fc epsilon domain in APCs of WT mice and mouse BMDCs. Consequently, no cross-presentation of OVA peptide resulting in activation of OT1 cells could be seen, in vitro nor in vivo.

6. References

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