Acta virologica **56**: 271 – 282, 2012 doi:10.4149/av_2012_04_271

Novel approaches to antiviral and anticancer immunotherapy

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Received June 27, 2012; accepted November 5, 2012

Summary. – In this review we discuss existing as well as new approaches to immunotherapy directed against infected or cancerous cells. These approaches traditionally exploit either natural components of immune system (such as cytokines, chemokines, co-stimulatory molecules and adjuvants), or monoclonal antibodies designed to target foreign agents and/or diseased cells through their molecular markers. Additional strategies in development include therapeutic vaccines, oncolytic viruses and T-cell therapies. In addition, we briefly describe a novel strategy called ReDIT (Re-Directed ImmunoTherapy), based on re-orienting the existing long-lasting immune responses (e.g. induced by measles vaccination or natural infection) towards new target molecules on the surface of infected or malignant cells. This can be principally achieved by using bi-functional protein constructs that contain an antigen carrier component and a re-directing component. The antigen carrier component can consist of the ectodomain of the measles hemagglutinin that can be recognized by antibodies and memory cells generated during previous infection or vaccination. The re-directing component consists of the specific virus- or tumor antigen-binding molecule. The fusion constructs are expected to boost existing anti-measles immunity and re-direct it against a new target, engaging the existing anti-measles immunity as an effector mechanism. Thus, ReDIT is a promising novel approach that may represent a valuable addition to immunotherapy of difficult to treat infections and tumors, as it exploits a mechanism distinct from other available therapies.

Keywords: re-directed immunotherapy; bi-functional protein; anti-measles immunity

Introduction

The immune system is a complex collection of organs and cells that protects the body against disease. As a part of immunological surveillance the immune cells respond to foreign substances, such as infectious agents or cancerous cells. A number of non-specific and specific defense mechanisms developed during the evolution. Long periods of coexistence and interactions of microorganisms and higher organisms led, however, to the development of mechanisms by which pathogens can under certain circumstances evade host immune responses or hijack and modify cellular machinery in ways advantageous to the pathogen survival. Similarly, in a significant proportion of individuals cancer will develop at some stage during their lifetime, suggesting that tumor surveillance often cannot identify and deal with the re-programmed cancerous cells. We have at our disposal a number of tools to fight infectious diseases and cancer, such as antibiotics, antivirals, radiotherapy or chemotherapy, with varying degree of success and side effects. As our knowledge of immune system components and interactions progresses, it is increasingly possible to harness the power of immune response developing novel immunotherapeutic approaches.

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Abbreviations: APC(s) = antigen-presenting cell(s); CAR(s) = chimeric artificial antigen receptor(s); CA IX = carbonic anhydrase IX; DC = dendritic cell; EBV = Epstein-Barr virus; GITR = glucocorticoid-induced TNFR-related protein; GM-CSF = granulocyte-macrophage colony-stimulating factor; HPV = human papillomavirus; HSV = herpes simplex virus; IFN(s) = interferon(s); MAb(s) = monoclonal antibody(ies); MeaH = measles hemagglutinin; PBMC = peripheral blood mononuclear cell; ReDIT = Re-Directed ImmunoTherapy; RSV = respiratory syncytial virus; TAA = tumor-associated antigens; TCR = T-cell receptor; TERT = telomerase reverse transcriptase; TLR = toll-like receptor; Tregs = T regulatory cells

Immunotherapy (or biological therapy) is commonly defined as any form of treatment that uses body's natural abilities that constitute the immune system to fight infection and disease or to protect body from some of the side effects of treatment. Majority of immunotherapy treatments are directed at inducing or enhancing/modulation of immune response. In some cases, such as allergy and the prevention of graft rejection the opposite effect of the immune response suppression is required instead. A number of different approaches have been developed to achieve these goals. In this review, we will focus on therapeutic applications in individuals where the targeted disease process has been initiated, rather than immunoprophylactic effects of vaccination. This includes period after a contact with infectious agent or ongoing oncogenic process.

According to some classifications, we can distinguish passive immunotherapy, active immunotherapy, which can be either nonspecific or specific, and adoptive immunotherapy. Examples of promising new approaches in various categories of immunotherapy are discussed in this review (Table 1).

Cytokines, chemokines, co-stimulatory molecules and adjuvant systems

Cytokines are secreted by various types of cells as signals for other cells to stimulate or inhibit certain types of effector activities. Use of interferons (IFNs) and interleukins (IL) represents one type of "non-specific immunotherapy". Type I IFN (various IFN-alpha subtypes, IFN-beta, IFN-epsilon, IFN-kappa and IFN-omega) exhibit antiviral, immunomodulatory, and antiproliferative activities during the viral infection (Gibbert and Dittmer, 2011). All type I IFNs have very similar structure, consisting of 161–167 aa and having up to 70% of amino acid homology. Despite this structural similarity and binding to the same receptor, they all exhibit different activities and expression profiles depending on the type of pathogen and the infected cell type. This can apparently be explained by different affinities of individual type I molecules to the same receptor (Jaks *et al.*, 2007). IFN binding to receptor induces JAK-STAT signaling pathway leading to the gene expression of numerous IFN-stimulated genes. Type I IFN is used for the treatment of chronic viral hepatitis B and C, commonly in combination with small molecule antivirals,

Table 1. Overview of immunotherapeutic approaches against viral infections and/or cancer

Type of immunotherapy	Principle	Effector cells/molecules/compounds	Reference
Cytokine-based	antiviral, immunomodulatory, antiproliferative activities	type I IFN, various subtypes IL	Gibbert and Dittmer (2011)
Chemokine-based	boosting immune cell trafficking to the tumor and lymphoid tissues	CCL-21-CCL19-CCR7 or CXCL12-CXCR4-CXCR7	Lechner *et al.* (2011)
T-cell co-stimulation	stimulation of T-cell activation using artificial co-stimulatory antibodies or immunoligand fusion proteins; blockage of the natural inhibitor of co-stimulation	CD137L (co-stimulatory TCR ligand), OX40L (CD134 ligand), CD40L ligand, GITR and its ligand	Gough and Weinberg (2009) Lechner *et al.* (2011) Goulding *et al.* (2011)
Toll-like receptor-based	CTL-response induction by TLR-ligand-derived adjuvants	TLR ligands and agonists (CpG oligonucleotides, imiquimod)	Akira *et al.* (2006) Tse and Horner (2007) Morse *et al.* (2011)
Monoclonal antibody-based	antibodies with intrinsic effector functions (virus-neutralizing, ADCC, death-inducing, anti-proliferative) or as carriers of therapeutic compound/radionuclide	humanized or fully human monoclonal antibodies (Cetuximab, Palimizumab, Rituximab, Trastuzumab, Bevacizumab etc)	Law and Hangartner (2008) Reichert (2011)
Therapeutic vaccines	immune response stimulation by antigens, antigen-derived peptides or peptides/antigens-loaded dendritic cells	viral or tumor antigen-derived peptides and proteins and loaded DC cells, co-administered with cytokines or adjuvants	Mellman *et al.* (2011) Kantoff *et al.* (2010) Monie *et al.* (2009)
Oncolytic viruses	modified viruses with tropism for tumor cells and lytic/cytotoxic properties	oncolytic adenoviruses H101, ONYX-015	Kelly and Russell (2007) Wennier *et al.* (2011) Rudin *et al.* (2003)
Adoptive T-cell therapy	lysis of tumor cells and cytokine excretion induced by genetically modified T-cells	T-cells transduced by TCRs, or chimeric artificial antigen receptors, cytokine-induced killer cells, TERT-immortalized T cells	Schmitt *et al.* (2009), Morgan *et al.* (2006) Biagi *et al.* (2011), Sangiolo *et al.* (2011) Barsov *et al.* (2011)
ReDIT	re-directing existing antiviral immunity to new therapeutic targets	bifunctional fusion proteins composed of antigen-carrier part and re-directing component	Petrik (2001)

such as ribavirin. High dose of IFN-alpha is known to block cancer growth and is used in the treatment of hairy cell leukemia, chronic myelogenous leukemia, non-Hodgkin lymphoma or cutaneous T-cell lymphoma. Improvements in formulation, such as IFN pegylation, facilitate dosing – they can be administered weekly.

Chemokines are regulators of immune cell trafficking and homeostasis. They consist of a group of small proteins, sharing the structural homology. Unlike type I IFNs with one common receptor, the chemokines mediate signaling using a group of 20 receptors (Lechner *et al.*, 2011). Their effects are often pleiotropic. They regulate cell trafficking throughout the body acting in gradients (Stewart and Smyth, 2009). Immunotherapeutic applications (e.g. networks of CCL21-CCL19-CCR7 or CXCL12-CXCR4-CXCR7) focus on boosting immune cell trafficking to the tumor microenvironment and lymphoid tissues (Lechner *et al.*, 2011).

T-cell co-stimulatory molecules. The optimal T-cell priming requires two signal cooperation: T-cell receptor (TCR) recognition of MHC-presented antigen (signal one), and ligation of T-cell CD28 with antigen-presenting cell (APC) B7.1 (CD80) or B7.2 (CD86) (signal two) (Hathcock *et al.*, 1994). However, in cancer patients the immature APCs often fail to express T-cell co-stimulatory ligands, leading to poor T-cell activation and possibly weak immunogenicity of tumor antigens. The immunotherapeutic approaches include agonist antibodies or immunoligand fusion proteins as artificial co-stimulatory signals. A parallel approach is the inhibition of CTLA-4, the natural inhibitor of B7 co-stimulation. Other co-stimulatory molecules targeted in immunotherapy are CD 137L, the APC-expressed ligand of the T-cell co-stimulatory receptor CD137 (41BB); OX40L, the APC-expressed ligand of OX40 receptor (CD134), following activation by Toll-like receptor agonists and CD40-CD40L signaling (Gough and Weinberg, 2009); glucocorticoid-induced TNFR-related protein (GITR) and its ligand. Similar to OX40, GITR expression is upregulated on CD4+ and CD8+ effector cells upon TCR-CD28 interaction effecting the signaling related to the strength of initial TCR stimulation (Lechner *et al.*, 2011). Interestingly, OX40 and OX40L (CD252) are key co-stimulatory molecules involved in the generation of protective CD8+ T-cell responses at mucosal surfaces such as lung, and may be potentially used as immunological adjuvants to enhance poxvirus-based CD8+ T-cell vaccines (Goulding *et al.*, 2011).

Toll-like receptor (TLR)-related immunotherapies. TLRs play a significant role in innate responses to microbes. TLRs are important part of pattern recognition receptors, which interact with the components of microbes designated pathogen-associated molecular patterns (PAMPs), although they are not always derived only from pathogenic microbes. At least 11 mammalian TLRs have been identified, interacting with different structures such as peptidoglycan (TLR2), ds RNA (TLR3), lipopolysaccharide (TLR4), flagellin (TLR5), single-stranded viral RNA sequences (TLR7, 8), DNA sequences common in microbial genomes, but rare in mammalian (TLR9) (Tse and Horner, 2007). TLR activated signaling pathways lead to NF-κB and MAPK activation, cytokine and co-stimulatory molecule (CD40, B7) expression. There are differences in signaling from different TLRs, and only ligands for some TLRs (3, 4, 7, 9) induce type I IFN production (Akira *et al.*, 2006). There are a number of ongoing studies evaluating TLR ligands as adjuvants for hepatitis viruses, human papilloma viruses, anthrax, influenza virus and HIV. Although alum is the only widely approved adjuvant for vaccine use, TLR ligands-derived adjuvants seem to be superior in CTL response induction. Monophosphoryl lipid, CpG oligonucleotid and some other ligands are being investigated in phase 1 and 2 clinical trials (Tse and Horner, 2007). Similarly in cancer the TLR agonists are being studied as vaccine adjuvants (Morse *et al.*, 2011), or as monotherapy (CpG oligonucleotide or imiquimod). Generally, the aim is to boost Th1 immunity, increase the number of anti-tumor CTLs or inhibit T regulatory cells (Tregs) activity. On the other hand, endogeneously produced TLR ligands seem to have a role in the pathogenesis of autoimmune diseases, leading to a search for TLR antagonists as potential therapeutics (Tse and Horner, 2007).

Monoclonal antibodies (MAbs)

MAb therapy represents passive specific immunotherapy where large amounts of MAbs can be produced in the laboratory. Hybridoma technology (Kohler and Milstein, 1975) produced a hybridoma cell, a long-lasting tool for the production of epitope-specific Ab. However, because of their mouse origin the therapeutic application in human medicine was problematic due to immune responses sometime causing allergic reactions. Researchers over time developed a number of ways to overcome this problem. First they learned to replace about two thirds of mouse sequences with human (chimeric MAbs), later humanized antibodies were developed. The mouse complementarity-determining regions were grafted into closely related human framework, with subsequent amino acid changes stabilizing the constructs (Jones *et al.*, 1986). The alternative approach was developed using humanized transgenic mice to develop functional human MAbs. The MAb production and selection has been later made more efficient by bypassing the immunization altogether. PCR-produced libraries of antibody genes displayed on the P3 protein of M13 phage were derived from B-cells or hybridomas (McCafferty *et al.*, 1990). A number of strategies to produce huge gene repertoire libraries were described. More recently, another interesting aspect of therapeutic MAb structure became apparent. Glycosylation at conserved

Asn297 of Fc CH2 domain is essential for stabilization of the domains and for optimal effector functions, including Fcg receptor and complement activation. It has been shown that the effector function changes, depending on the oligosaccharide composition: IgG-Fc with non-fucosylated oligosaccharides enhanced ADCC, while that with sialylated oligosaccharides modulated antibody-induced inflammation (Kaneko *et al.*, 2006).

The therapeutic applications of MAb differ somewhat for infectious diseases (focusing on viral diseases) and cancer and they are considered separately below.

MAb immunotherapy for viral diseases. Neutralization is a dominant mechanism in antibody protection against most viruses representing direct function of antibody, not requiring a participation of other components of immune response. However, the extent of Fc-dependent and complement-dependent mechanism contribution varies for different viruses. These are indirect functions leading to activation of complement and complement-dependent cytotoxicity and lysis of virus or infected cell. Alternatively, viruses can be removed by phagocytic mechanisms after immune complex-mediated APC activation and antigen processing and presentation to the adaptive immune system. Despite numerous studies the precise mechanisms of the antibody protection against viruses are not fully understood (Law and Hangartner, 2008). More recent studies aimed therefore to address quantitative and structural aspects of antibody – virus interactions. The multiple hit antibody neutralization model, requiring multiple antibody molecules for virion neutralization is now generally accepted. It has been shown that every functional HIV-1 Env trimer should be occupied by an antibody molecule to neutralize the virion, requiring some 10 antibody molecules (Yang *et al.*, 2005). The neutralization stoichiometry was different for West Nile virus, requiring antibody binding only to 30 out of 180 Env protein molecules for virion neutralization (Pierson *et al.*, 2007). Studies like these are clearly necessary for prophylactic and therapeutic antibody design, especially considering MAb cocktails against multiple neutralizing epitopes.

Currently, the polyclonal antibodies are widely used in several areas such as post-exposure prophylaxis for at risk individuals (hepatitis A and B, respiratory syncytial virus (RSV), varicella zoster, measles), prevention of congenital HBV infection or post-transplant infections due to immunosuppression. General trend is, however to replace polyclonal antibodies by better characterized and more specific preparations of MAbs which can be further engineered in respect of their effector functions or serum half-life, as described for anti-RSV MAb Numax-YTE. Palivizumab, a humanized MAb against RSV F protein, was the first MAb reagent for infectious disease treatment (Pollack and Groothuis, 2002). MAbs can also be conjugated to prodrugs (e.g. gemtuzumab ozogamicin) or toxin to enhance their effects (Law and Hangartner, 2008). This approach is more common for MAb cancer treatment described in the next paragraph.

MAb cancer immunotherapy. MAbs are the most widely used form of cancer immunotherapy (Yamada, 2011). As mentioned earlier the MAbs are used either un-conjugated (naked) or conjugated to a drug, radioactive particle or a toxin. Most naked MAbs either mark cancer cell for destruction after binding to them, or act as activation blockers, binding to and inactivating the antigens of cancer or supporting cells (targeted therapies). Well known examples of the MAbs marking the cell for destruction are Rituximab (Rituxan) and Alemtuzumab (Campath). Rituximab is anti-CD20 MAb used to treat B-cell non-Hodgkin lymphoma, chronic lymphocytic leukemia and some other diseases (Pescowitz, 2006; Masood *et al.*, 2011), while Alemtuzumab is anti-CD52 MAb used to treat patients with B-cell chronic lymphocytic leukemia. The examples of MAbs blocking the activation of targets include Trastuzumab (Herceptin), Cetuximab (Erbitux) and Bevacizumab (Avastin) (King *et al.*, 2008). Trastuzumab is a MAb against HER2/neu protein which after activation promotes the growth of certain tumors. MAb is used to treat breast and stomach cancers (Hudis, 2007). Cetuximab is directed against EGFR, a protein with similar growth-promoting activity. It is used to treat colorectal and some head and neck cancers. Bevacizumab is anti-VEGF protein antibody. VEGF attracts new vessels to tumor and supports their growth. Bevacizumab is used in combination with chemotherapy to treat colorectal, lung, breast, kidney cancers and glioblastomas (Ferrara *et al.*, 2004). Examples of conjugated antibodies include Tositumomab (Bexxar) and Brentuximab vedotin (Adcetris). Tositumomab is a radiolabeled MAb used to treat non-Hodgkin lymphomas which no longer respond to Rituximab or chemotherapy. Brentuximab vedotin consists of anti-CD30 antibody conjugated to monomethyl auristatin E. It is the only approved chemolabeled MAb and is used to treat Hodgkin lymphoma and non-responding anaplastic large cell lymphomas.

Despite some promising studies, there are currently no approved immunotoxin-conjugated MAbs.

Approximately 30 therapeutic MAbs have been approved around the world, with over 250 undergoing clinical trials (Reichert, 2011). Undoubtedly, we will see much wider therapeutic use of MAbs in the near future.

Therapeutic vaccines

Unlike prophylactic vaccines, the development of therapeutic vaccines is proving more difficult. Numerous initial attempts to generate cancer vaccines used short peptides without an effective dendritic cell (DC)-activating adjuvant,

leading to DCs remaining in steady state and promoting tolerance rather than immunity. Recent co-administration of IL-2 with melanocyte differentiation antigen-derived peptide showed more promise, as did the use of longer (~20-mer) peptides rather than 10-12-mer peptides binding to MHC class I molecules. Full-length proteins represent another alternative, as they contain more epitopes, potentially presented by DCs. An example is a phase III trial of a recombinant fusion protein encoding testis cancer antigen (MAGE-A3). It is administered together with an adjuvant consisting of saponin/lipid-A emulsion combined with TLR4 and 9 agonists (Mellman *et al.*, 2011). Other strategies rely on strong immune responses against viral antigens and use viral vector-encoded tumor antigens. One phase II trial involved a recombinant vaccinia virus encoding prostate-specific antigen and adhesion molecules B7.1, intercellular adhesion molecule (ICAM) and lymphocyte function-associated antigen-3 (LFA-3). Subsequently, a fowlpox vector with similar configuration was administered (prime-boost), together with granulocyte-macrophage colony-stimulating factor (GM-CSF) (Kantoff *et al.*, 2010a). Cell-based approaches focus mainly on DC-based vaccines, where DCs are isolated from a patient, loaded with antigens (peptides, lysates, etc), activated and returned into the patient. Despite numerous problems of such development, Sipuleucel-T (Provenge) achieved a success as approved vaccine. It is made up of autologous APCs and a fusion protein composed of prostatic acid phosphatase (PAP) and GM-CSF. The results of early phase I/II clinical trials showed increases in T-cell responses to the vaccine antigen, serum PSA decline of greater than 50% in 10% of patients, and limited toxicity. Despite initial assumption of being an autologous DC-based vaccine, it is a complex mixture of peripheral blood mononuclear cells (PBMC) with a cytokine and tumor-derived differentiation antigen (Kantoff *et al.*, 2010b). It has been approved by Food and Drug Administration in 2010.

In antiviral therapeutic vaccines, a considerable effort has been made to develop a therapeutic vaccine against human papillomavirus (HPV), associated with several human cancers. Despite several prophylactic anti-HPV vaccines being available, a therapeutic vaccine is needed to facilitate the control of HPV-associated malignancies. The choice of antigen is important, and unlike prophylactic vaccines, HPV therapeutic vaccine developments focus on constitutively expressed E6 and E7 proteins, necessary for transformation and co-expressed in HPV-infected but not normal cells (Monie *et al.*, 2009). Peptide-based vaccines while stable, easily produced, and safe, exhibit low immunogenicity and require adjuvant cytokines, chemokines, co-stimulatory molecules and TLR ligands (see above). They are also MHC restricted which is not the case with protein vaccines, although they are weakly immunogenic and induce better antibody than the CTL response. This can be circumvented by the use of vectors, there are, however safety concerns, especially in immunocompromised patients (Lin *et al.*, 2002). DCs pulsed with E7 peptides or transduced with DNA, RNA or viral vectors encoding E7 represent a promising, although labor-intensive approach for circumventing the tumor-mediated immunosuppression. In this respect the DNA vaccines are easy to prepare on a large scale, easier to administer and relatively safe (Monie *et al.*, 2009).

Oncolytic viruses

The idea of tumor destruction by virus is over a hundred years old. A number of clinical trials with naturally oncolytic viruses were conducted in 1950s and 1960s. However, the progress was slow and disappointing outcomes led to almost an abandonment of the field. Advances of genetic engineering started a new chapter and past two decades saw revival and fast progress of this field. A culmination of these efforts was the approval of genetically modified oncolytic adenovirus H101 by the Chinese regulatory agency in 2005 (Kelly and Russell, 2007).

An ideal oncolytic virus should have a tropism for cancer cells but not normal cells, should kill the cancer cell upon infection (oncolysis), and should be able to spread within tumor microenvironment as well as intertumorally (Wennier *et al.*, 2011). Initial efforts focused on the direct lysis effect, using either naturally oncolytic viruses, or targeted towards the tumor by genetic manipulation. The great potential of viruses was evident, but so was a clear need for viruses to be manipulated in order to target cancerous cells more specifically. First successful example was a complete removal of the thymidine kinase gene from herpes simplex virus (HSV) genome for malignant glioma treatment, based on the observation that thymidine kinase-negative HSV replicated in dividing cells but replication was inefficient in non-dividing cells (Martuza *et al.*, 1991). Numerous single-stranded and double-stranded RNA and DNA viruses were tried in animal oncolysis experiments, but only a few were further developed as potential virotherapeutics. The mechanisms, apart from above-mentioned natural or engineered oncolysis, include also a synthesis of death proteins, toxic to the cells, such as adenovirus E3 11.6 kDa and E4ORF4 protein, and initiation of specific or non-specific anti-tumor immune responses (Meerani and Yao, 2010). There are a number of ways to effect immune responses, including the change in a balance of produced cytokines, activation of DCs and generation of adaptive antitumor immunity (Prestwich *et al.*, 2008). The ongoing oncolytic virotherapy clinical trials are based mostly on modified adenovirus and herpes simplex virus. ONYX-015 adenovirus lacks E1B protein. Without it the virus cannot replicate in cells with functioning p53 pathway, but this pathway is inactive in many tumors due to muta-

tions, allowing the virus replication and cell lysis. Trials for wide range of cancers including squamous cell carcinoma of the head and neck or even preventative treatment of precancerous oral tissue were reported using ONYX-015 (Rudin *et al.*, 2003; Meerani and Yao, 2010). HSV-1-derived NV1020 and G207 vectors have various mutations, including a thymidine kinase under a control of new promoter, which sensitizes cells to ganciclovir, in addition to cell lysis in NV1020, and inability of G207 to replicate in non-dividing cells. It is, however, the second generation HSV-1-derived OncoVEX GM-CSF encoding human GM-CSF, which is most interesting from the immunotherapy point of view. The expression of GM-CSF in local tumor environment induces local inflammation, DCs activity, HLA II expression, and is angiogenic. Combination with standard chemoradiation regimen and cisplatin led to impressive 94% of squamous cell carcinoma of head and neck patients tumor-free at surgery (Harrington *et al.*, 2010).

Oncolytic virotherapy is not without challenges. Apart from an obvious problem of antivector immune responses, two incidents of death during clinical trials led to more strict restrictions of patients' inclusion. Questions of vector production rates, which should prevail over growth rates of cancer cells, improvements in delivery methods and limited immunogenicinity need to be addressed. However, the selective character of this approach should bring the benefits, especially in combination with standard anti-tumor treatments (Meerani and Yao, 2010).

T-cell therapy

T-cell therapy is directed predominantly against cancer, although closely linked also to viral infections during post-transplantation delayed immune reconstitution. The cancer often develops and escapes surveillance because the changes in tumor-associated antigens (TAA) against their normal cellular counterparts are small and often insufficient to induce significant immune response. DCs play important roles, bridging the transition between the innate and adaptive immune responses, maintaining self-tolerance and directing other cells towards immunogenic or tolerogenic reactions. Some of the DC immunotherapies were discussed in the section "Therapeutic vaccines". In this section we focus on novel adoptive T-cell therapies.

Allogeneic bone marrow transplantation and the infusion of donor lymphocytes are effective established immune treatments for some leukaemias and lymphomas. Further improvements are represented by lymphodepletion before the T-cell infusion and engineering of new T-cell specificities (Mellman *et al.*, 2011). The conditioning regimens include *in vivo* T-cell depletion as well as *ex vivo* depletion of the graft. Unfortunately, the removal of alloreactive T-cells is accompanied by the removal of T-cells with anti-viral and anti-tumor activities (Leen *et al.*, 2010). An increasing number of viruses have been implicated in complications after human stem cell transplantation. Epstein-Barr virus (EBV), human cytomegalovirus (HCV), adenovirus, HSV, HHV-6, BK virus, metapneumovirus, RSV and some others lead to significant complication due to the immunosuppresion. Several trials of *ex vivo* CD25 immunotoxin-mediated depletion and using recipient PBMCs or EBV-transformed lymphoblastoid cell lines as a source of alloantigen for stimulation, led to T-cell produced reconstitution of antiviral immunity without inducing graft-versus-host disease. The time necessary to produce EBV-transformed lymphoblastoid cell lines and the clinical grade immunotoxin instability represent current limitations of the approach (Leen and Heslop, 2008). Apart from generation of CMV-specific or EBV-specific CTLs, the multivirus-specific (human cytomegalovirus, EBV, adenovirus) CTLs were successfully produced (Leen *et al.*, 2010). In addition, a good manufacturing practice methodology for the simultaneous selection of T-cells with multiple viral specificities and regulatory T-cells was recently described (Lugthart *et al.*, 2012).

As mentioned earlier, poor immunogenicity of TAA is a major obstacle in development of T-cell immunotherapy. Lymphodepletion is important also for removal of host suppressor cells such as myeloid suppressor cells or Tregs. Tregs, which express CD4, CD25 and transcription factor Foxp3, represent up to 10 % of CD4+ T-cells and play a critical role in immune system by suppressing aberrant T-cell activation leading to chronic inflammation and preventing autoimmune disease (King *et al.*, 2008). Their clinical application has been limited mostly due to inability to identify their antigen specificity and problems with the expansion of these cells. However, a recent finding that transforming growth factor induces CD4+ Foxp3+ Tregs from naive CD4+ T-cells both *in vitro* and *in vivo* opens up new possibilities for immunotherapy of autoimmune diseases (Chen, 2011). In cancer, however the Tregs seem to exhibit immunosuppressive effects.

TCR gene transfer. Despite demonstrated effects of adoptive T-cell therapy in allogeneic or autologous setting, particularly for melanoma and acute myeloid leukemia, there are limitations to this approach such as the inability of transferred T-cells to persist at high levels after infusion, difficult isolation of high-affinity TAA-specific T-cells, and long time required for their isolation and expansion (Schmitt *et al.*, 2009). The antigen specificity, lineage selection, effector function and survival are all critically affected by TCR. The promising technique, which can circumvent some of the mentioned obstacles, is TCR gene transfer into primary T-cells. The technique involves the isolation of the T-cell clone expressing a TCR with high affinity for the target antigen. This can be achieved by culturing PBMCs or tumor-infiltrating lymphocytes in the

presence of APCs pulsed with a dominant epitope peptide and selection of high affinity clones by tetramer staining or lysis of target cells. TCR and TCRs chains are cloned, sequenced and inserted into gammaretrovirus or lentivirus vectors, tested and a clinical lot of a vector is transduced into T-cells purified from patient PBMCs and expanded (Schmitt *et al.*, 2009). The trial of autologous T-cells transduced with MART-1-specific TCR, expanded *in vitro* and infused into lymphodepleted melanoma patients revealed a promise as well as challenges of this novel therapeutic approach (Morgan *et al.*, 2006). The challenges include lower avidity of TCR-transduced T-cells than donor cell, often as a result of suboptimal expression of one or both of TCR chains or inefficient pairing, mispairing of introduced TCR chains with endogeneous chains, the affinity of the introduced TCR, the maintenance of TCR expression over time and the persistence of the TCR transduced T-cells *in vivo*. There are a number of practical steps being developed to overcome the identified problems with a level of expression (promoter choice, codon optimization etc), TCR chain mispairing (modifying the constant domain), delivery systems (increasing use of lentiviruses over gammaretroviruses, alternative use of a transposon), avidity (*in vitro* maturation, removal of glycosylation) and target cell choice (King *et al.*, 2008; Thomas *et al.*, 2010).

Chimeric artificial antigen receptors (CARs). Further development in this field is demonstrated by the design of CARs. They consist of antigen-recognizing extracellular domain and T-cell-triggering domain. The antigen-recognizing domain originates in heavy and light chain variable domains of a monoclonal antibody, expressed as a single-chain fragment variable molecule. It is joined to an intracellular signaling domain (zeta chain of TCR/CD3 complex, gamma-chain of Fc-epsilon RI receptor) to achieve a specific lysis of tumor cells and cytokine secretion when exposed to the target antigen (Biagi *et al.*, 2011). Such a construct can avoid some limitations of TCR gene transfer, being non-MHC restricted, independent of antigen processing and potentially targeted to non-peptide molecules, such as carbohydrates or glycolipids. An example of such target molecule is the CAR directed to diasialoganglioside G (D2a), a TAA expressed by human neuroblastoma cells. The infusion of EBV-specific CTLs expressing G (D2a) specific CAR was shown to be associated with tumor regression or necrosis in half of the subjects (Pule *et al.*, 2008).

More than 10 clinical trials using 2nd or 3rd generation CARs are currently ongoing. However, similarly to other new therapeutic approaches an extreme caution needs to be exercised when designing, developing and testing new constructs and therapies. This is documented by two serious adverse events resulting in a death of two patients undergoing separate clinical trials (Biagi *et al.*, 2011).

Cytokine-induced killer (CIK) cells. A treatment of solid tumors non-responding to conventional therapies requires continuous design and development of new therapeutic approaches. CIK cells exhibit certain properties marking them as a potential new tool in adoptive immunotherapy. CIK cells are a heterogeneous subset of *ex vivo* expanded T lymphocytes characterized by a relatively easy and inexpensive *ex vivo* expansion, reduced alloreactivity and MHC-unrestricted tumor killing (Sangiolo, 2011). The expansion, taking 3–4 weeks, starts from PBMCs, bone marrow or umbilical cord blood precursors and requires timed addition of IFN-gamma, anti-CD3 antibody and IL-2. The expansion was standardized under good manufacturing practice conditions, allowing their use in clinical trials, although the end population is heterogeneous (CD3+CD56+ and CD3+CD56-), where the double-positive fraction is considered responsible for the MHC-unrestricted tumor killing (Lu and Negrin, 1994). Reduced alloreactive potential across MHC could lead to a reduced graft-versus-host disease risk, when infused after allogeneic human stem cell transplantation. Several clinical trials were conducted and one of the important messages is low toxicity. One of the trials on 12 patients with advanced non-Hodgkin lymphoma, metastatic renal cancer or hepatocellular carcinoma, resulted in three complete responses and two disease stabilizations, with other trials showing improvements in outcomes (Olioso *et al.*, 2009).

Telomerase reverse transcriptase (TERT) "immortalization" of T-cells. Mammalian somatic cells lose the proliferation ability when reaching the terminally differentiated state and entering the replicative senescence phase. This process is characterized by progressive shortening of telomeres with each DNA replication cycle until the critical length is reached, triggering upregulation of p53, leading to the induction of an irreversible cell-cycle arrest and onset of senescence. Telomeres, the specialized repeats present at the end of chromosomes, can be stabilized or restored by TERT, the enzyme capable of extending telomere repeats on the template of telomerase RNA. Telomerase RNA and TERT are associated with several other proteins, forming a complex at the telomere ends. Unlike other somatic cells, the TERT expression in T lymphocytes (and B-cells) can be briefly reactivated during their stimulation by external stimuli, and viral infections. Not surprisingly, the TERT is upregulated in the majority of cancers (Barsov, 2011). One of the limitations of earlier described T-cell therapies is a limited survival and maintenance of the expanded cells after infusion. It is easy to imagine that mechanisms overcoming this limitation or at least significantly expanding their survival would be extremely valuable. T-cells can be activated *in vitro* and proliferate in response to specific antigen stimulation with concomitant sharp elevation of TERT activity. These cells can be expanded and maintained in the culture for extended periods of time. The mechanisms of immortalization of human T-cell lines and clones are being developed using TERT overexpression. Based on animal model studies these

cells can maintain antigenic specificity and full set of effector functions *in vivo*, making them potentially promising tool in anti-tumor and anti-viral adoptive immunotherapy (Verra *et al.*, 2004; Andersen *et al.*, 2007). TERT can significantly extend a replicative lifespan of T-cells and does not seem to directly lead to malignant transformation. It can provide a valuable source of well-characterized T-cells and potentially develop them into an adoptive immunotherapy tool. However, no studies in humans were published yet, and there are at least two problems, potentially hampering their use: TERT can be recognized by the immune system as TAA, and extended lifespan can lead to the development of genetic instabilities (Barsov, 2011). Before this promising approach can be applied to immunotherapy, these and some additional questions need to be answered.

Re-Directed ImmunoTherapy (ReDIT)

We have been developing yet another approach based on harnessing the existing immunity towards genetically stable viruses in majority of population by its re-directing to difficult therapeutic targets. It is known that long-lasting immunity can be induced by genetically stable viruses, such as measles, mumps, rubella or polio. It is not the case for viruses having antigens as variable as surface glycoproteins of HIV, hepatitis C virus or influenza. This makes vaccination difficult for seasonal epidemics of influenza, targeted each season at particular variants, which are probable to appear based on epidemiological studies. But for serious chronic infections caused by viruses such as hepatitis C virus or HIV, there is a problem of treating millions of individuals already infected. Great efforts put into the development of the antivirals against these viruses brought a degree of success for combination therapies. However, the basic mechanisms of high mutation rates these viruses use to escape immune responses creates a need to continuously produce new antivirals, as the resistance develops to those in use. Perhaps the mechanisms, which could ease the mutation pressure on the effector molecule, could help this situation. A separation of key events – the recognition/binding and the neutralizing functions of antibodies is one example of such approach. This could be achieved if the immunity existing within the majority of the world population as a result of natural in-

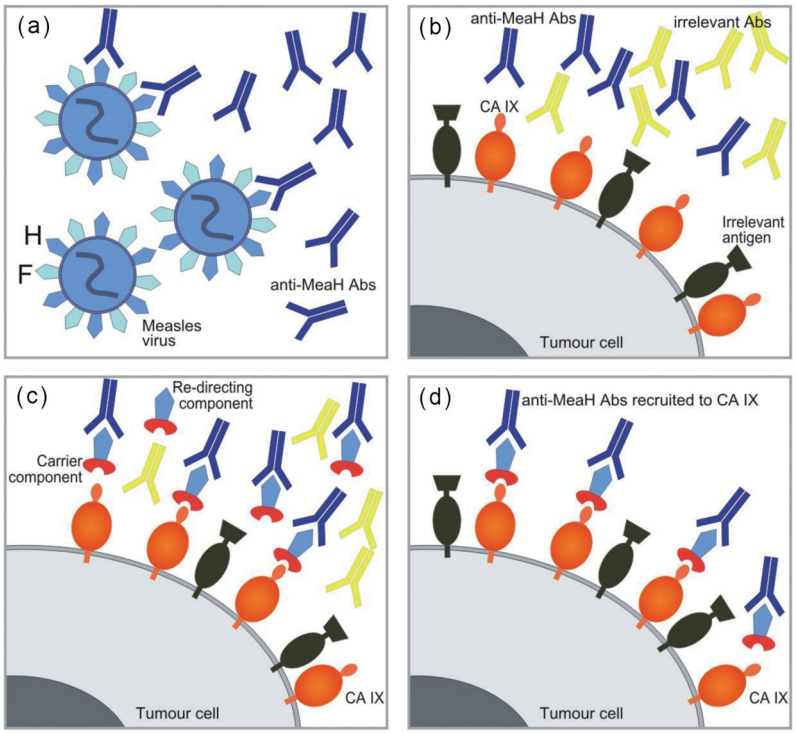

Fig. 1

Schematic illustration of the ReDIT principle

(a) Infection or immunization with measles virus induces antibodies against the virus hemagglutinin (MeaH). (b) These antibodies (or the related memory cells) persist in the human body and their level increases after addition of a bi-functional chimeric molecule (c) composed of the engineered MeaH as an antigen carrier component (blue) and the re-directing component (red). The chimera binds to CA IX through the re-directing CA IX-binding peptide (d) and is recognized by anti-MeaH antibodies.

fection or mass vaccination against genetically stable agent was re-directed against new, genetically variable target. In other words, the host would be tricked into using already present immunity for a dominant antigen such as measles hemagglutinin (MeaH) to eliminate HIV or other genetically variable infection agent (Petrik, 2001). Since measles vaccine worldwide coverage is estimated between 72 and 84% and measles virus is the next WHO eradication target it was considered an ideal candidate for this approach. HIV is clearly a major global health problem but the approach is a platform, which can be used for a wide range of infectious agents and extended to cancerous cell expressing a specific TAA. As mentioned earlier, the immunotherapy approaches can have significant advantages over conventional treatments of surgery, radiation therapy, chemotherapy, in prevention of metastatic and recurrent disease, especially in the extent of the side effects. Alternatively, the combination of immunotherapeutic and conventional approaches may be optimal for particular applications.

Measles vaccine strains induce broadly cross-reactive antibodies with MeaH the major target of these antibodies. It is a glycoprotein as is the second surface protein fusion protein (F). Both of them are required for a fusion of cell membranes, but the sequence of events starts with MeaH binding to the cell receptor CD46. MeaH is a membrane-anchored protein with aa 1–34 proposed to form a cytoplasmic domain, while aa 35 to 58 comprising a transmembrane domain. Residues 59 to 181 are thought to form a stalk, part of which (aa 135 to 181) forms the hinge of a molecule (Sato *et al.*, 1995). Spikes of MeaH on virion surface consist of tetramers (dimers of disulfide bridge-linked homodimers). Cysteins 139 and 154 were suggested to participate in intermolecular disulfide bond between monomeric MeaH glycoproteins. Soluble forms resulting from endoproteinase digestion of measles virus particles all reacted with monoclonal antibodies suggesting the preservation of antigenicity/reactivity (Sato *et al.*, 1995). MeaH domain required for hemadsorption and hemagglutination activities was mapped between residues 451 and 505 and additional region implicated in receptor interaction was between aa 244–250 (Hummel and Bellini, 1995; Lecouturier *et al.*, 1996; Fournier *et al.*, 1997).

Therapeutic bifunctional protein constructs we are developing, contain an antigen carrier part of the molecule represented by the ectodomain of MeaH engineered in such a way that it does not bind to the receptor or cause hemadsorption or hemagglutination, but retains its antigenicity and can be recognized by patients' anti MeaH antibodies and memory cells resulting from previous infection or vaccination. The second part of the fusion protein composition consists of a re-directing molecule (or a fragment), capable of binding to new target. This can be the surface structure of a virus such as multimers formed by their envelope glycoproteins. Stoichiometric relations discussed in the section "Monoclonal antibodies" are very important for our constructs, since similar requirements of binding to sufficient number of the surface structures applies. These constructs should be capable of boosting the existing anti-measles immunity and at the same re-directing it against new target (Petrik, 2001). The cancer model we use is a tumor-associated cell-surface carbonic anhydrase IX (CA IX), (Fig. 1) which is currently evaluated as a marker of tumor hypoxia and therapeutic target in pre-clinical experiments as well as in MAb immunotherapy-based clinical trials (Zatovicova *et al.*, 2010; Reichert, 2011). CA IX belongs to the family of carbonic anhydrases that participate in physiological processes based on pH balance and ion transport. The expression and activity of this protein is associated with hypoxic tumor environment. CA IX expression is common in many types of tumors, and its high levels were detected in the renal, brain, cervical, colorectal, esophageal, pulmonary, and breast tumors. Under physiological conditions it is expressed only in healthy cells of the gastrointestinal tract. CA IX expression indicates a poor prognosis in many types of tumors (except renal cell carcinoma) (Pastorekova *et al.*, 2006, 2008). The re-targeting ligand used is a peptide that binds to a CA IX epitope for monoclonal antibody M75 (Zavada *et al.*, 2000). Alternatively, anti-CA IX antibody fragments can be used (Zatovicova *et al.*, 2003). Preliminary results indicate successful re-targeting of our constructs to CA IX-expressing tumor cells (Trnkova *et al.*, unpublished data).

Re-DIT could develop into a valuable addition to immunotherapy of difficult to treat infectious agents and tumors, as it represents a different mechanism of action from other available therapies. It could be used on its own, or in combination with other agents. Possibly the most important feature of this approach is that it can form the basis of a new platform, with multiple applications depending on the availability of the specific re-directing ligands to new targets.

Conclusion

There are currently numerous immunotherapeutic approaches at our disposal (Table 1), promising "more natural", less toxic therapeutic regimes. Many of the approaches discussed above can serve as stand-alone therapies but in majority of applications they could improve also current commonly used surgical, chemotherapeutical and radiation-based therapies. Whatever the preferred way of treatment, most of these approaches promise significant improvements in the outcomes of difficult-to-treat chronic infections and cancers.

Acknowledgement. The authors' research is supported by grants from the Slovak Scientific Grant Agency (VEGA 2/0072/10) and the Research & Development Operational Program funded by the ERDF (ITMS 2624012007).

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