Volume 59(Suppl.1):69-82, 2015
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

REVIEW

Current approaches of tumor immunotherapy

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ABSTRACT Our immune system, fine-tuned by a long evolution, has a near-infinite capacity to recognize potential pathogens and mutant self proteins. It has also got a varied arsenal of killing mechanisms to battle intruders and mutant cells. Since malignant transformation involves 1) mutations of proteins of various classes and 2) over-expression of non-altered genes, either related or unrelated to the oncogenic process, the adaptive immune system has the potential to recognize and clear malignantly transformed cells. Immunotherapeutic interventions might 1) trigger an immune response to otherwise tolerated tumor antigens, 2) enhance the existing, but insufficient anti-tumor immune response, add new receptors, recombinant antibodies or T cell receptors, to the system, or 4) rely on the transfer of *ex vivo* expanded immune effector cells. Although tumor immunotherapy is decades-old, immune checkpoint inhibitor therapy, probably the most significant breakthrough, is a development of the last few years. Reaching its maturity, tumor immunotherapy is just now becoming an integral part of tumor therapy.
Acta Biol Szeged 59(Suppl.1):69-82 (2015)

KEY WORDS

antibody therapy
CTL
dendritic cells
immunotherapy
NK cells
tumor immunology

Immune surveillance, pro and contra

Immune surveillance is the body's ability to recognize and eliminate neoplastic cells. Although it seems to be self-evident, this notion is less than sixty years old. The concept of immune surveillance was raised by Burnet (1957). An obvious argument prompting the original hypothesis was that immunosuppressed and immunodeficient individuals have an increased incidence of certain types of tumors, mostly skin cancers and leukemia. The situation is, however, more complex than this simplified theory would suggest.

Quite a few immunologists have always been criticizing the concept as controversial. Numerous observations point out that immunosurveillance might not be an utterly effective, neither universal, anti-tumor mechanism. Immunodeficient mice, for example, are not known to have a particularly elevated frequency of malignant tumors (except for tumors of the hematopoietic system). Since the life span of a laboratory mouse is less than two years, one can argue that this model is not relevant when compared to long living mammals, including humans.

Human epidemiological data are also intriguing: although the examples of skin tumors and leukemias are frequently cited, other types of tumors do not seem to be more frequent in immunosuppressed patients. For many years, no synthesis of these conflicting theories was provided.

Submitted Jan 1, 2015; Accepted June 9, 2015
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The only large-scale meta-analysis of the field compared tumor incidence (relative risk) in normal controls and two immunosuppressed patient groups: organ transplant recipients who are treated with Cyclosporine A, an immunosuppressive drug that prevents T cell activation, and HIV infected individuals with overt AIDS, *i.e.*, clinically manifest immunosuppression (de Visser et al. 2006). The effect of immunosuppression on tumor incidence varied, depending on the tumor type. Some types of tumors, like cutaneos tumors, non-Hodgkin lymphoma, or cervical carcinoma, indeed become much more frequent (relative risks: 16-70, 24-30 and 5-9). Importantly, these tumor types are well known examples of highly immunogenic, most frequently virus (typically HPV) induced malignancies. Surprisingly, other cancer types, as breast, prostate-, bladder-, ovarian or uterine cancers, had a drastically decreased incidence (relative risks between 0.28 and 0.8). These results point to a paradoxical, tumor supporting role of the immune system (de Visser et al. 2006).

Tumor immunotherapy, however, provides an indirect proof of the importance of immune surveillance. By enhancing the existing, but weak T cell response by CTLA-4 or PD-1 inhibitors, a "latent" immune response is revealed in a considerable fraction of different malignancies (Weber 2010).

On the other hand, immunoediting, the phenomenon when the anti-tumor immune response shapes the evolution of cancer, is another example when the latent anti-tumor response becomes visible (Dunn et al. 2002).

In conclusion, immune surveillance might protect us from some types of neoplasms, but it is neither universal, nor infallible. On the other hand, manipulation of the immune system by immunization or biological response modifiers might induce an effective immune response even in cases when the effectiveness of the „natural" immune response is insufficient. Adoptive transfer of *ex vivo* activated or expanded immune cells, recombinant immunoglobulins or further, new generation, immune recognition molecules, as bi-specific antibodies, likewise provides new tools for cancer immunotherapy.

Danger signals and tumors

One of the central concepts in modern immunology is danger hypothesis. The term was coined in the nineties by one of the most influential immunologists of the recent years, Polly Matzinger (1994). The adaptive immune system is able to recognize even single amino acid differences, effectively differentiating self- and non-self molecules. However, the adaptive immune system, too dangerous because of its inherent potential of auto-reactivity, is under the control of the more dependable innate immune system. The first step of any immune response is the „validation" of the presence of a noxious intruder, *i.e.*, „danger".

Phagocytes, the first line of defense against intruders, can be triggered by different mechanisms. Particles that have a diameter similar to viruses or bacteria trigger phagocytosis independently of their surface properties. According to a recent seminal paper, the deformation of the cell membrane, as detected via the membrane anchored cytoskeleton, provides the decisive signal (Champion and Mitragotri 2006). Phagocytosis alone is not an „immunogenic" procedure; the processes that destroy the phagocytosed particle must be activated by pattern recognition receptors, otherwise, phagocytosis is immunologically "silent". This recognition might take place either on the outer surface of the cell membrane, or on the inner surface of the phagosome.

Several arrays of phagocytotic and pattern recognition receptors play role in the process. Mannose receptors are C type lectins that detect carbohydrates absent in the mammalian cell membrane, but frequently present on the surface of microorganisms. Scavenger receptors are a distinct set of cell surface proteins of unusual trimeric structure. Beside their defining target structures, oxidized or native low density lipoprotein (LDL), they recognize a wide array of pathogen associated molecular patterns. The most important „danger" receptors are, however, toll-like receptors (TLR). TLRs cover a wide array of pathogen- or cell-stress signals (Janeway and Medzhitov 2002). The double stranded RNA of viruses is recognized by TLR3, the lipopolysaccharide (LPS) cell wall of Gram-positive bacteria by TLR4, the lipoteichoic acid of Gram-negative bacteria by TLR2, the cilia and flagellae of bacteria by TLR5, the non methylated CpG sequences of bacterial DNA, as well as the mannanes of fungi by TLR9. A further distinct family of danger receptors is the nucleotide-binding oligomerization domain receptor (NLR) family. They are intracellular pattern recognition receptors that cooperate with TLR in detecting danger (Maekawa et al. 2011).

Since they are mostly analogous to normal tissues, at first sight, tumors do not seem to present danger signals to the innate immune system. Fortunately, this statement is not entirely valid. The abnormally high cell division rate and protein over-expression, typical for most tumors, can result in cell stress. Chaperones induced by cellular stress then may act as danger signals. In addition, angiogenesis can not always keep pace with the rate of proliferation of the malignant cells, resulting in anoxia, metabolic acidification, and, eventually, necrosis inside the tumor mass. An important feature of malignant tumors is genetic heterogeneity. The genetically different tumor sub-clones then compete with each other for the limited resources, so some of the sub-clones starve, others thrive (Merlo 2006).

IL-1α and its homologue IL-18 are major regulators of the inflammatory immune response. They are stored in the cytoplasm of diverse cell types as inactive precursors lacking signal peptide. Proteolytic enzymes of dying cells release active IL-1α and IL-18, triggering a signal pathway overlapping with those of TLR (Watanabe and Kobayashi 1994; Franchi et al. 2009; van de Veerdonk et al. 2011).

Heat shock proteins, *i.e.*, chaperones overproduced in tumor cells stressed by disturbances of the regulation of protein synthesis, or by "starving" inside an overgrown tumor mass, are also TLR targets activating the immune system. The best example of this type of interaction is recognition of HSP70 by TLR4 on immune cells (Juhasz et al. 2013).

Since they are normally buried inside the cells, otherwise "physiologic" hydrophobic intracellular structures ("Hyppos") exposed to immune cells in case of cell damage, including necrosis inside the tumor mass, are also stress signals (Seong and Matzinger 2004).

NK-T cells and cell stress

NK-T cells share the characteristics of the two cell types. Along with receptors typically associated with NK cells, they express a low diversity T cell receptor (V 24-J 18:V 11) that detects a single target, a stress signal. This stress signal is a membrane lipid component, α-GalCer, presented on a (practically) monomorphic MHC family member, the CD1d molecule. Besides their effector function, NK-T cells are major source of cytokines, acting as key regulators of the immune response. Their therapeutic manipulation, either by their natural ligands, or by other methods, offers new possibilities for modulating the immune response in cancer (Sil et al. 2004).

Effector functions of immunoglobulin isotypes

Neutralization is a function shared by all immunoglobulin isotypes, and it is the only effector function of the IgG4 isotype. Monoclonal antibodies may behave as receptor agonists or antagonists. Receptor antagonist antibodies may block the signals of cell surface growth factor receptors of tumor cells. On the other hand, they may likewise block the signals of negative immune response regulators of immune cells. Both possibilities are exploited in tumor therapy. On the other hand, receptor agonist antibodies may be used to activate co-stimulatory immune cell receptors. As we see later, this approach failed in the first Phase 1 clinical trial, however, theoretically it might still be viable. An important aspect of the receptor agonist therapy is that the targeted immune cells should survive the contact with the antibody. Because of that, IgG4 antibodies, which do not bind Fc receptors, nor activate the complement system, are the best choice in these cases.

Antibody Dependent Cellular Cytotoxicity (ADCC) is a function of NK cells recognizing cell-bound IgG molecules via Fc receptors. NK cells might be able to kill malignant cells recognizing cell stress markers or gene products of oncogenic viruses. NK killing, however, is much more effective if the target cells are „marked" with Fc receptor binding antibodies. ADCC is a major mechanism of action of therapeutic antibodies (Cooley et al. 1999). Phagocytosis of cells "opsonized" by cell surface IgG is also triggered by Fc receptors of macrophages. Importantly, IgG1 is the most efficient IgG isotype for triggering both ADCC and phagocytosis. Complement dependent cell lysis (CDC) can also kill antibody-marked tumor cells. From the IgG isotypes, IgG1 is the most effective in eliciting the classical pathway of complement activation as well.

Taken together, IgG1 is the best choice if the goal is tumor cells' elimination, while IgG2 or IgG4 are the right choices if the goal is immune cell activation. Importantly, not only the isotype, but glycosylation pattern of the immunoglobulins influences the target killing, with non-fucosylated IgG1 antibodies being the most efficient (Jefferis 2007).

Multi-step carcinogenesis provides multiple targets for tumor immunotherapy

Carcinogenesis is not the result of a single mutation; it is rather a multi-step process (Hanahan and Weinberg 2000). Loss of genetic stability is one of the necessary genetic alterations, enabling accelerated "collection" of harmful genetic changes. Until genetic stability is lost, only mutant oncogenes and the up-regulated differentiation antigens provide targets to the immune system. From the point when genetic stability is lost, „bystander mutations", *i.e.*, mutations which are not necessary for maintaining the malignant phenotype, keep collecting. As a result, a considerable part of the genome is altered, providing a rich array of potential immune targets. Some of these targets are unique to the individual tumors, like individual point mutations in signal proteins; others are either tissue-specific or specific to a class of tumors. These „shared" tumor-associated antigens are the most promising targets of anti-tumor immunotherapy. Besides the lack of danger signals, tumor-induced immunomodulation is the main obstacle hindering the generation of an effective anti-tumor immune response (Zou 2005; De Visser 2006; Marton et al. 2012; Sica and Mantovani 2012).

T and B cell receptor epitopes of tumor associated antigens

The evolutionary role of MHC-I is „exposing" the internal composition of the cell to the T cells searching for intruders. Together with the "normal" intracellular peptide repertoire, MHC-I presents viral proteins, as well as mutant self proteins, including potential tumor antigens. MHC-II, on the other hand, presents peptides derived from phagocytosed proteins by digestion in the phago-lysosome. The evolutionary role of MHC-II is „sampling" the internal environment for signatures of eventual pathogens, and transporting them to the lymph node, where the information exchange between the phagocytes (sentinels) and naive and activated effectors takes place. Cross-presentation is the process when not only endogenous, but phagocytosed proteins are presented on MHC-I of professional antigen presenting cells. The main mechanism of cross-presentation is ER-phagosome fusion occurring shortly after the phagosome formation (Heath and Carbone 2001). While only leukemias/lymphomas express MHC-II, all tumors express MHC-I, therefore, they may all be targets of CD8+ cytotoxic T cells.

Cell surface molecules of tumors may serve as targets of antibodies; therefore their detection is relatively straightforward. More complex efforts are required for the discovery of T cell antigens of tumors, requiring primary tumor cell culture, primary T cell culture, creation of tumor cDNA banks, transfection and high throughput functional screening of immune recognition. The established T cell clones can be used to screen tumor antigen libraries, *i.e.*, MHC-identical non-malignant antigen presenting cells transfected with cDNA banks from the tumor cells used to generate the T cell clones (Boon et al. 1997). The read-out might be cytotoxicity, T cell proliferation or cytokine production. The discovered tumor antigens belong to several classes. An exhaustive list of the known tumor antigens is presented in the review of Novellino et al. (2005).

Mutant oncogens, such as the P53 tumor suppressor protein point mutants, are frequent in certain types of tumors. Mutant P53 was indeed shown to be targeted by tumor-specific CTL.

Differentiation antigens overproduced in specific tissue

types, as well as tumors originating from the same tissue might also serve as targets of an anti-tumor immune response. For example, tyrosinase and other enzymes of the melanin biosynthesis are potential targets of an anti-melanoma immune response. In accordance with that, vitiligo due to melanocyte loss might be associated with a successful anti-melanoma immunotherapy.

Cancer cells always express gene product accidentally up-regulated due to global gene transcription inbalances.

In the case of virus-induced tumors, viral antigens might also be expressed. HPV induced tumors are well known targets of anti-tumor (in this case, anti-HPV) immune response. High immunogenicity of the viral antigens provides an obvious target to immunostimulator-based tumor therapy.

The most valuable tumor associated antigens are shared tumor antigens that may form the basis of tumor therapy in the case of many independent tumors in different patient. The future, however, might bring personalized immunotherapy directed against tumor clones of individual patients.

The T cell-NK cell functional complementarity may help to control MHC-I loss tumor mutants

Cancer cells, by definition, always carry an array of potential tumor antigens. The constant selective pressure of the immune system favors mutant cancer cell sub-lines that lose MHC-I expression (the same phenomenon is also known in the case of virus infections). One of the major functions of NK cells is the elimination of „MHC-I-loss" mutants. A basic experimental strategy of tumor immunology is vaccination against tumor associated antigens. The T cell response induced that way increase the selective pressure on the tumor cells, so NK activity eliminating the MHC-I loss mutants becomes crucial. Importantly, interferon gamma, the defining cytokine of Th1 cells, simultaneously increases 1) MHC-I expression, 2) CTL activity and 3) NK activity, thus facilitating the elimination of both MHC-I+ tumor cells, and MHC-I- tumor escape mutants. MHC-I loss, due to either lost MHC-I expression or beta2 microglobulin expression (Bernal et al. 2012) is general phenomenon hindering efficient anti-tumor vaccination (Slingluff 2007).

The principles of tumor therapy

Beside surgical resection, malignancies are typically treated with radiotherapy or chemotherapy. Radiotherapy, introduced in clinical practice in the beginning of the twentieth century, uses ionizing radiation to damage the DNA of the tumor cells, eventually destroying them. The first generation chemotherapeutic agents were alkylating compounds that damage the tumor cells' DNA. Later several classes of chemotherapeutical agents were discovered, including anti-metabolites, *i.e.*, compounds that interfere with DNA synthesis, mitotic inhibitors of plant origin, like vinca alkaloids or taxol, DNA intercalating agents, as doxorubicin, platins, *i.e.*, platinum compounds that behave mostly like alkylating agents, and topoisomerase inhibitors (DeVita and Chu 2008). From 1965, chemotherapeutic agents have been used in combinations, rather than alone. These „classical" chemotherapeutic agents are not strictly selective to tumor cells; they act on dividing cells, thus causing a wide array of side effects mainly involving fast-renewing tissues, such as skin, gut epithelium, bone marrow, and the immune system. Since immune response is mostly beneficial, at least in the early phase of tumorigenesis, this last side effect is especially worrisome.

Both radiotherapy and most chemotherapeutical agents target rapidly dividing cells, such as bone marrow cells and peripheral immune cells. Via inducing massive tumor cell necrosis, chemotherapy might also provide danger signals, *i. e.*, may also stimulate a type 1, inflammatory, and therefore anti-tumoral immune response. In addition, the „immunosuppressive" effects of chemo- or radiotherapy target not only the effector arm of the immune response, but also the negative regulators of the immune effectors, like Treg cells. In conclusion, immunosuppression may, paradoxically, also stimulate the anti-tumor effector functions. The literature of this highly controversial field is summarized by Zitvogel and coworkers Zitvogel et al. (2008).

Targeted therapy is a new generation therapy that acts on molecular processes typical to cancer cells, rather than all dividing cells. The most recent developments led to the rather artificial category of biological therapy that targets tumor cells more specifically. Gleevec® (imatinib mesylate) is an enzyme inhibitor of a tumor specific tyrosine kinase. Iressa® (gefitinib) targets the epidermal growth factor receptor (EGFR). Sutent® (sunitinib) is a multi-targeted kinase inhibitor inhibiting, among others, the vascular endothelial growth factor (VEGF) receptor (Sawyers 2004). Anti-angiogenic therapy is an especially promising option, since its targets are genetically stable endothelial cells responding to the major pro-angiogenic factor VEGF, among others, by monoclonal antibodies (Sitohy et al. 2012). A different class of antibody-like proteins is immunoglobulin Fc region containing recombinant proteins. The Anti-VEGF molecule Ramucirumab contains a VEGFR domain fused with the human IgG1 Fc domain, functioning like a VEGF-neutralizing IgG1 (Fuchs et al. 2013).

Personalized tumor therapy, including tumor immunotherapy, can now rely on a new generation of minimally invasive diagnostic tools, as analysis of circulating tumor cells collected from blood samples as "liquid biopsy" (Ligthart 2013).

Importantly, classic cytotoxic tumor therapy may also induce immunogenic cell death (ICD). Tumor cell necrosis/apoptosis may trigger an effective anti-tumor immune response, contributing to the success of the therapy (Kroemer

et al. 2013). In other words, anti-tumor immune response is inseparable from the therapeutic effect of cytotoxic chemotherapy.

Organizing rendezvous between activated dendritic cells and tumor antigens

Vaccination and adjuvants

A century old observation is that soluble proteins or peptides alone are not strong immunogens. Immune adjuvants, *i.e.*, immunostimulatory components administered together with the protein antigen, may substantially increase the effectiveness of immunization. The textbook experimental adjuvant is Freund's adjuvant, discovered by the Hungarian immunologist Jules Freund (1890-1960). The main component of Freund's adjuvant is mineral oil, a mixture of petroleum-derived alkenes related to paraffin that contains heat-inactivated mycobacteria, providing classic danger signals. Freund's adjuvant, however, induces an exceedingly violent inflammatory reaction, unacceptable in clinical setup; therefore in humans it must be substituted with less aggressive compounds. The most widely used vaccine adjuvant, used in some of the human clinical trials of tumor antigen immunization, is alum (aluminum phosphate and aluminum hydroxide). Earlier supposed to be a simple slow release depot, alum is now known to activate the NLRP3 inflammasome, resulting in inflammatory cytokine production (Eisenbarth et al. 2008; Franchi et al. 2009). Further alternatives include squalene, a natural triterpene, or saponine (*Quillaja saponaria* extract). They are the main components of the experimental adjuvants MF59 or QS21, respectively. Experimental adjuvants may also contain synthetic, experimental TLR agonists. Alternatively, the antigen can be „packed" in empty influenza virus envelopes (virosomes), which act as a „natural" delivery vehicle targeting the immune system. Experimental cancer vaccines, including antigens and adjuvants, are reviewed by Finn (2003).

One of the first adjuvant ever used, the *Mycobacterium tuberculosis* vaccine strain (Bacillus Calmette-Guérin, BCG), has been successfully used in the therapy of bladder cancer for decades (Gsponer et al. 2012).

Interestingly, a "new generation adjuvant", the TLR-7 agonist Imiquimod (Aldara), when applied directly to skin tumors, results in immune activation and tumor rejection. Imiquimod, first approved for medical use in 1997, is now widely used as a patent applied treatment for basal cell carcinoma, squamous cell carcinoma and melanoma (Beutner et al. 1999).

Since quite a wide array of tumor-specific antigens is known today, selecting a target antigen is generally not a problem. Any kind of immunization/vaccination may work in this strategy, and, indeed, several different approaches are being tried. The simplest approach is administration of a protein or peptide antigen plus an adjuvant. In this case, the encounter of danger signal, dendritic cell and antigen takes place at the site of injection and the draining lymph node of the area. Peptide antigens are likewise useable, either as more extensive stretches of tumor antigen peptides containing several potential epitopes, or shorter peptides directed to the (pre-determined) MHC alleles of the patient.

One of the most efficient experimental immunization approaches is infection with recombinant vaccinia viruses carrying tumor antigens. Genetically engineered pox viruses have several advantages. The most important is that the large vaccinia virus, engineered to express tumor antigens, might also express immunostimulatory cytokines. These possibilities are extensively being studied in mouse models. The different modalities of vaccine delivery were compared by Bolhassani and coworkers Bolhassani et al. (2011).

Besides the various experimental approaches, peptide based vaccines are also licensed for human clinical use. The first clinically approved vaccination strategy (Vitespen) is based on the use of gp96 heat shock protein (HSP90B1)-peptide complex purified from resected autologous tumors (Wood et al. 2009).

Ex vivo dendritic cell therapy

According to the literature, activated dendritic cells pulsed with tumor antigens *ex vivo* are more effective in inducing anti-tumor CTL response than the irradiated tumor cells, either with or without adjuvant. In accordance with these literature data, we obtained similar results using mouse spleen dendritic cells matured in the presence of the inflammatory cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) and labeled with an MHC I binding peptide derived from the TRP-1 melanoma differentiation antigen. Mice immunized with these "peptide-pulsed" dendritic cells triggered a more robust CTL response against the B16 mouse melanoma cell line than mice vaccinated by the tumor itself (Vizler, unpublished). Analogous approaches are also being tested in clinical setup.

Surprisingly, the first FDA approved approach is much more complex. Sipuleucel-T (trade name Provenge) is an *ex vivo* dendritic cell therapy for advanced prostate cancer. In the first step, dendritic cells are obtained from prostate carcinoma patients by leukapheresis. The dendritic cells are then treated with a fusion protein composed of a frequently expressed prostate tumor antigen, prostatic acid phosphatase (PAP) and the pro-inflammatory cytokine GM-CSF. The cells are then re-injected in the patient. The company has several similar products, based on different tumor antigens, under development (Kantoff et al. 2010).

Adding new receptors to the patient's immune system

Antibody therapy

Tumor specific monoclonal antibodies administered systemically, as our own antibodies, rely on effector mechanisms of the natural immune system. From its introduction in 1997 (Maloney et al. 1997), antibody therapy is a widely used therapeutic option for cancer (Scott et al. 2012). Monoclonal antibody therapy is generally based on the use of the most "aggressive" immunoglobulin isotype, IgG1. Among the human IgG immunoglobulin isotypes, IgG1 is the most effective in 1) binding to Fc receptors of phagocytes, thus inducing phagocytosis of the antibody-labeled cells, 2) binding to Fc receptors on NK cells, thus inducing antibody-dependent cellular cytotoxicity (ADCC), and 3) triggering the classical complement cascade, resulting in both opsonization of the tumor cells by membrane-bound complement factors and killing by membrane attack complex formation. Until the last few years, therapeutic antibodies were created by immunizing animals, typically mice, with the tumor antigen, then hybridomas were created, and monoclonal antibodies of IgG1 isotype were selected. Today, the majority of monoclonal antibodies are genetically engineered; therefore the variable part may be generated by different methods, starting from different species, while the human Fc part is added in the last step. The recombinant antibody is then produced industrially by fermentation.

Various therapeutic antibodies are directed against the relatively well known cell surface tumor associated antigens, as carcinoembryonic antigen (CEA), epithelial cell adhesion molecule (EpCAM), CD19, CD20, and CD22. IgG1 type monoclonal antibodies are then able to mark their targets for killing by complement dependent lysis, phagocytosis or ADCC. An interesting experimental approach relies on preactivation of the patients' NK cells by treatment with agonistic antibody directed to the killer cell activating receptor CD137, resulting in an enhanced ADCC activity when a subsequent antibody treatment is directed to tumor antigens, as CD20 (Korth et al. 2011).

The effectiveness of these effector mechanisms is, however, not always sufficient. The killing efficiency of antibodies can be enhanced by conjugating them with a lethal "payload" (Teicher and Chari 2011). Some examples of these „armed" antibodies are Oportuzumab monatox (EpCAM-specific single chain variable fragment fused with *Pseudomonas aeruginosa* exotoxin A), Yttrium (90Y) clivatuzumab tetraxetan (humanized anti-MUC1 antibody conjugated with a radioactive isotope), and Nacolomab tafenatox (mouse monoclonal antibody fused with *Staphylococcus aureus* enterotoxin A).

Antibodies may also be conjugated with cytokines, providing a targeted delivery of the immunomodulating molecules. Since the action of cytokines is mostly autokrine and parakrine, rather than systemic, this targeting may increase the efficiency of the therapy, simultaneously preventing side effects of the pleiotropic systemic cytokines. The example for this approach is Tucotuzumab celmoleukin (humanized anti-EpCAM-IL-2 conjugate).

A further possibility being explored by a number of pharmaceutical companies is the use of antibodies conjugated with chemotherapeutic drugs. This approach might increase the local drug concentration, resulting in higher effective dose and lower side effect than in the case of classical chemotherapy (Zolot et al. 2013).

The main classes of anti-tumor antibodies – the example of therapeutic antibodies directed to the CD20 tumor associated antigen

First generation monoclonal anti-tumor antibodies were typically produced in mice. The variable domains of antibodies, especially the complementarity determining regions (CDR) where the genetic variability concentrates, are, by definition, foreign structures that may trigger a host immune response. If the antibody is from another species, not just the variable region, but the whole IgG molecule can be the target of a humoral immune response. Host antibodies recognizing the therapeutic antibodies may then eliminate them. Even more serious problem is the potential systemic hypersensitivity reaction triggered by the repeatedly administered foreign antigen.

The solution of this problem can be recombinant antibody technology, *i.e.*, swapping of the immunogenic "foreign" part of mouse antibody to a much less immunogenic human sequence. Some residual immune reactivity might be induced even in this case, due to minor differences between the immunoglobulin genes of different individuals (immunoglobulin allotypes). The non variable part used in these scenarios is generally that of the human IgG1 molecule. IgG1 is the antibody isotype that triggers host effector mechanism most robustly, as this isotype induces the classical pathway of complement activation, opsonization and ADCC.

CD20, a B cell surface molecule frequently expressed by B cell leukemias, was the target of the first therapeutic antibody approved by the FDA (Maloney et al. 1997). Today it is an example of tumor-associated antigens targeted by therapeutic antibodies of different generations (Lim et al. 2010).

When the Fab domains of the resulting hybrid antibody are from the original mouse sequence, the Fc domains are from human IgG, the product is a chimeric antibody (Example: Rituximab).

When only the essential parts of the mouse antibody, the CDR domains are retained, the result is a humanized antibody (Example: Ocrelizumab).

The best solution is the use of genetically modified "humanized mice" for immunization and then creation of hybridoma cell lines. In these humanized mouse strains, the mouse immunoglobulin gene cluster is replaced with a complete human immunoglobulin gene cluster. Immunization of these mice results in rearrangement of human, rather than mouse immunoglobulin gene segments, resulting in fully human immunoglobulins (Green 1994; Lonberg 1994) (Example: Ofatumumab).

According to the standardized WHO pharmacological nomenclature, fully mouse antibodies contain the "-tumo-" subtems (e.g., Tositumomab), chimeric antibodies are called "-tuxi-", (Rituximab), humanized antibodies are called "-tuzu-" (Ocaratuzumab, Ocrelizumab, Obinutuzumab /a „glycoengineered" antibody, where even the glycosilation pattern is human-like/), and fully human antibodies contain the "-tumu-" subtem (Ofatumumab).

A further variation of the last technology is based on an *in vitro* selection system, rather than on immunization of an animal. These methods involve creation of a library of variants (mutants) of the (preferably human) CDR region sequences, followed by a high throughput selection for the (extremely rare) high binding affinity variants. The selected sequences are then built in the cloned human IgG1 gene. The method most widely used for selecting high affinity recombinant immunoglobulin variants is phage display.

TcR therapy

A more sophisticated approach is adding T cell receptors, rather than immunoglobulins (B cells receptors) to the patient's immune system. A direct approach of T cell therapy is *in vitro* activation/expansion, then re-administration of tumor-infiltrating lymphocytes (TIL) (Restifo et al. 2012). A more complex approach is based on isolating T cells, then "arming" them with a tumor-specific T cell receptor by transfection, typically using retroviral vectors (Kershaw 2005; Restifo et al. 2012). The transfected cells, expressing both the original and a transgenic T cell receptor, are then infused into the patient. The *ex vivo* manipulation and genetic modification of the – potentially immortal – patient T cells carries the risk of malignant transformation, so the genetic modification must include the insertion of a suicide gene, typically the herpes virus thymidine kinase, which sensitizes the cell to the cytotoxic action of the drug acyclovir. Unlike the human enzymes, the herpes virus thymidine kinase incorporates the chain-terminating nucleotide analog acyclovir in the DNA of the transfected cell, thus killing it.

T cell receptors recognize the target epitopes bound to MHC molecules; therefore, they are strictly MHC specific. The use of TcR transgenes would not be possible if none of the (extremely numerous) MHC-I alleles would be over-represented in the populations. Luckily, an MHC-I allele, HLA-A1, is present in 10-25% of the European population, so typically HLA-A1-restricted TcR transgenes are used in the experimental therapy, naturally, only in patients pre-screened for the presence of this allele. Importantly, one of the immunodominant peptides of the melanoma-associated antigen Melan-A/MART-1 seem to be presented by multiple HLA-A alleles (Fleischhauer et al. 1996).

Hybrid immunoglobulin-TcR approaches

An interesting „hybrid" approach is being developed by the English pharmaceutical company Immunocore. In their system, the tumor antigen-TcR contact is „mimicked" by a hybride molecule bridging the MHC-peptide complex on tumor cells with the TcR of T cells. The hybrid molecules, termed immune-mobilizing monoclonal TCRs against cancer (ImmTACs), activate host T cells independently of the specificity of their endogenous TcR. ImmTACs are comprised of a tumor-specific monoclonal TcR fused to a humanized CD3-specific single-chain antibody variable fragment (scFv). CD3 is a part of the T cell receptor complex, and its activation by agonist covalently coupled Ab mimics the activation through the T cell receptor (TcR) by its cognate ligand (Lyddy et al. 2012).

A similar approach, based on bispecific antibodies, is being developed by AMGEN. The Bispecific T Cell Engager (BiTE®) Antibodies, analogously to the ImmTACs, recognize tumor cell surface antigens and the CD3 molecule of the TcR complex, thus redirecting T effector cells (Topp et al. 2014).

Fine-tuning the existing immune response

Administration of immune activating cytokines

Cytokine therapy as cancer therapy has been explored from the eighties, and IL-2 and type 1 interferons are licensed for clinical use for more than 20 years. Beside IL-2 and interferons, much effort was concentrated on colony stimulating factors (Heberman 1987). Today, type I interferons are the most widely used cytokines in tumor therapy, including melanoma therapy (Mocellin et al. 2013). Type I interferons were first described as anti-viral defense molecules, and the first clinical trials with interferons were done in diseases of validated, or, at least, hypothesized viral origin (Kirkwood and Ernstoff 1984). The mode of action of type I interferons in tumor therapy is still not fully understood.

IL-2 has a complex biological role. It activates T cells and is also produced by them; therefore it supports T cell proliferation by positive feedback. On the other hand, it is indispens-

Table 1. The main targets of tumor immunotherapy

Immune mechanism	Defect in cancer	Potential therapy
Phagocytosis	Lack of triggering signals	Cytotoxic chemotherapy for inducing immunogenic cell death (ICD). *Ex vivo* tumor cell phagocytosis by autologous dendritic cells. *Ex vivo* transduction of dendritic cells by tumor antigens.
Dendritic cell activation via pattern recognition receptors	Lack of danger signals	Local application of danger signals (natural or synthetic TLR agonists). *Ex vivo* dendritic cell activation by danger signals or inflammatory cytokines. Infection with bacteria driving Th1 differentiation.
Co-stimulatory ligand expression, dominant over inhibitory ligand expression	Tumor-induced immunosuppression	Agonistic monoclonal antibodies targeting stimulatory T cell co-receptors (failed attempt). Blocking monoclonal antibodies targeting inhibitory T cell co-receptors (the most promising approach of our days).
Primary T cell activation	Low abundance of tumor antigens and/or co-stimulators	Vaccination with tumor antigen. Vaccination with tumor antigen-presenting dendritic cells. Vaccination with genetically modified viruses expressing tumor antigens.
T cell expansion, CTL killing	Insufficient number of tumor-specific CTL	Immunostimulatory cytokine administration. Adoptive transfer of *in vitro* expanded T cells. Relieving tumor induced immunosuppression by cytokine-neutralizing antibodies. "Hybrid" TcR activating molecules.
Tumor-specific antibody production	Insufficient amount or killing efficacy of antibodies	Tumor-specific IgG1 monoclonal antibody administration. „Armed" tumor-specific monoclonal antibody administration.
NK killing of MHC-I negative tumor escape mutants	Insufficient NK activity	NK cell activation by TLR ligands, killer activating receptor agonists, or HSP induction. Adoptive transfer of *in vitro* expanded (allogeneic) NK cells.

able for the activity of T regulatory (Treg) cells, the main negative regulators of cellular immune response (Coventry 2012). In spite of this pleiotropic effect, IL-2 is successfully being used in tumor immunotherapy (Rosenberg 2001).

As a „master regulator" of cellular immune response, IL-12 also seemed to be a good candidate for tumor immunotherapy. Since it induces interferon- and activates both cytotoxic T cells and NK cells, it seemed to be obvious that its effect is based on an enhanced anti-tumor cellular immunity. The situation, however, seems to be more complex. IL-12 induces the release of high amounts of interferon-γ, mostly from NK cells. The produced interferon-γ then induces further cytokines, including IP-10 (CXCL-10). IP-10, in turn, is an effective inhibitor of angiogenesis. In accordance with that, the IL-12 dose required for an anti-tumor effect is frequently above the optimal CTL activating level, and tumor regression does not necessarily correlates with an increased anti-tumor CTL or NK response (Vizler et al. 1998). On the other hand, animal studies showed that the effectiveness of IL-12 treatment relies on the production of type I interferons. Clinical studies on its systemic use were not encouraging, but its application for local combination therapy is still under investigation (Lasek 2014).

Beside systemic treatments with recombinant proteins, cytokine gene therapy, typically based on transfection of the tumor cells *in vitro* or *in vivo*, has also been tested in animal models, and later in clinical setup. Although several (or maybe most) cytokines were able to induce rejection of transfected tumor grafts via activating different anti-tumor effector mechanisms, the treatments did not prove to be effective against established tumors or metastases, so this approach has been mostly abandoned (Nanni et al. 1999; Nagy et al. 2003).

T cell activation via agonistic antibodies targeting co-stimulatory molecules

Although, on a purely theoretical basis, application of agonistic monoclonal antibodies seemed to be a promising option, the first trial ended with a disaster, probably hindering further similar studies. The rationale was that TGN1412, a humanized agonist antibody directed to the major T cell co-stimulatory molecule CD28, will trigger an effective immune response against tumors. Unfortunately, some of volunteers participating in the phase I study suffered life threatening side effect, probably because of systemic immune hyper-reactivity causing edema and multi-organ dysfunction. Retrospective studies suggested that the side effects, absent in animal experiments, might be due to the activation of memory T cells, much more frequent in humans that in laboratory animals raised under „sterile" conditions (Suntharalingam 2006).

Relieving tumor-induced immunosuppression by antibodies blocking negative regulators of the T cell response

Checkpoint inhibitors targeting negative regulators of T cell responses, including the anti-tumor T cell response, *i.e.,* the

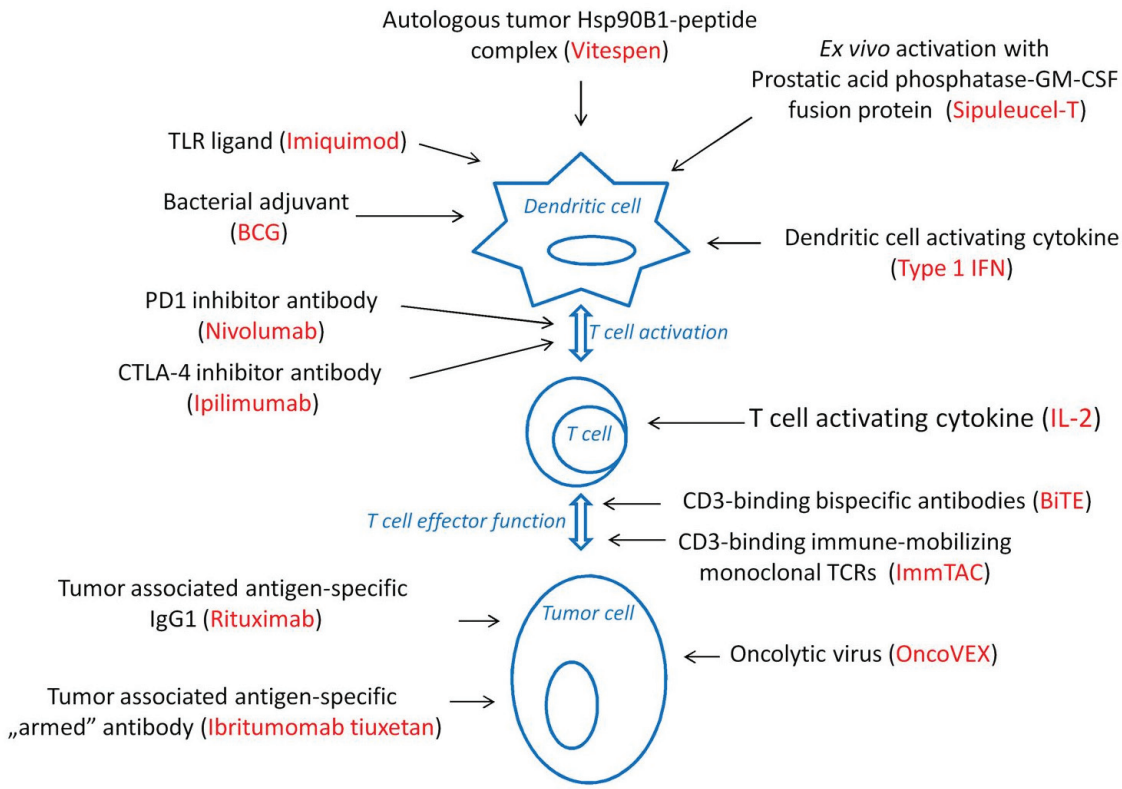

Figure 1. A brief summary of immunotherapy approaches licensed for clinical use or currently undergoing Phase III clinical trials. The trade names or, in some cases, scientific names of example therapeutic agents are given in parentheses. Classification of the current immunotherapies in clinical or experimental phase is provided by Galluzzi et al. (2014). The immune checkpoint blocking approaches are summarized by Kyi and Postow (2014). The known tumor antigens are classified by Novellino et al. (2005).

CTLA-4 and the PD-1 receptors, might be the most promising of all the tested approaches.

The T cell surface molecule CTLA-1 is one of the major negative feedback regulators of T cell response. Activation and inhibition of the CTLA-1 signal offer therapeutical possibilities in autoimmunity and cancer, respectively. Ipilimumab (Yervoy), a fully human IgG1 monoclonal antibody, has been successfully used in both cases (Hodi et al. 2010). In addition to melanoma, Ipilimumab is under clinical trials for non-small cell lung carcinoma, small cell lung cancer, bladder cancer, and prostate cancer.

Another T cell surface protein of the CD28/CTLA-4 family of T cell regulators, PD-1, has been the target of successful immunomodulatory antibody therapy (Topalian et al. 2012). The monoclonal antibody treatment, disrupting the contact of PD-1 with its ligands PD-L1 and PD-L2, has been shown to be more effective than dacarbazine, the first choice drug in melanoma chemotherapy (Robert et al. 2014). The PDF1 blocking antibodies, Pembrolizumab or Nivolumab, function by relieving immunosuppression in cancer. Since PD-L1 and PDL2 are widely expressed in tumors of different histological origin, this is one of the most interesting discoveries of the last years. The antibody is fully human monoclonal antibody. In these cases, in contrast with treatments targeting the tumor cells themselves, the goal is receptor blocking without killing the immune cells carrying them, so IgG4 antibodies are optimal.

The high efficacy of these immunostimulating antibodies is mirrored by their immune activation-related side effects, including meningitis, pneumonitis, vitiligo, colitis, hepatitis, hypophysitis, and thyroiditis (Topalian et al. 2012; Voskens et al. 2013). The cost-benefit balance of the treatments, nevertheless, remains positive.

Ex vivo expansion of anti-tumor cytotoxic T cells and NK cells

Another, decades old, but still experimental strategy for enhancing the anti-tumor immunity is *in vitro* expansion of effector cells. Although the proper type protective host immune response is frequently induced by malignant cells, the magnitude of the response is not always sufficient for

elimination of the tumor. In such cases, collection and *ex vivo* expansion of the immune cells offers a solution. This type of therapy generally starts with the isolation of autologous T cells from the circulation, or from excised tumors or biopsies (tumor-infiltrating lymphocytes, TIL) (June 2007). The cells are then expanded *in vitro* using the proper cytokine cocktail, then re-infused into the patient, either alone, or together with immune-activating cytokines. Importantly, immunotherapy with *in vitro* expanded T cells gave rise to even more complex approaches, like those based on T cells transfected with a tumor-specific T cell receptor (Rosenberg et al. 2008).

As T cells, NK cells can also be expanded *in vitro* for adoptive transfer. While clinical studies with autologous NK cells typically gave negative results, allogeneic NK cells had a therapeutic effect (Miller et al. 2005).

Interestingly, while the killer inhibitor receptor (KIR) repertoire of NK cells of individuals seem to be fixed (*i.e.*, dictated by the genetical background, especially MHC-I alleles), the killer activating receptor (KAR) composition seems to be determined by environmental factors ("education"). This fact raises the possibility of a therapeutic exploitation of the potentially tumor-specific KAR inducibility and variability (Vivier et al. 2012; Horowitz 2013).

Harnessing infection-related immunomodulation

Since infections provide danger signals, a natural assumption is that infection might help to enhance the otherwise weak anti-tumor immune response, thus fighting cancer. The seminal discovery of William Coley opening the way for immunotherapy predated the birth of immunology. Following case reports of patients who recovered from cancer following erysipelas (caused by *Streptococcus pyogenes* infection), Coley reproduced the phenomenon by injecting cancer patients with *S. pyogenes* and *Serratia marcescens*, first in living form, later as dead bacteria (Coley's toxin, or Coley's vaccine, from about 1893). Although the treatment has some success, it was replaced by the more reproducibly effective radiotherapy. Nevertheless, from time to time, the concept resurfaces in different forms.

Another interesting (and robust) support for this theory comes from epidemiological data. In 2003, a large clinical study performed by EORTC (European Organization for Research and Treatment of Cancer) provided unexpected evidence for a positive effect of microbial infection in cancer. Krone et al. showed that febrile infections or vaccinations with Bacillus Calmette Guerin or vaccinia virus in early childhood significantly decreased the incidence of malignant melanoma (Krone et al. 2003; Krone et al. 2005).

The third line of evidence comes from clinical data of patients treated with chemotherapy. According to these data, chemotherapy decreases the resistance to opportunistic pathogenic bacteria that, in turn, induce a classic Th1 type, inflammatory immune response. This „re-education" of the immune system by pathogens, diverted by the immunomodulatory effect of tumors, is beneficial. The hypothesis is supported by experiments where elimination of opportunistic pathogens compromised the effectiveness of chemotherapy (Iida et al. 2013).

In addition to their immunological effect, different bacteria were shown to display specific homing into tumors (Wei et al. 2008). Several attempts are made to harness this effect, either for tumor killing, typically by *Clostridium* strains (Zwagerman 2014), or for tumor specific delivery of a pharmacological payload, typically by *Salmonella* strains (Kazmierczak et al. 2015).

Besides bacteria, different oncolytic viruses are under Phase II or Phase III clinical trials, including GM-CSF expressing herpes simplex virus Talimogene laherparepvec or OncoVEX GM-CSF (Hu et al. 2006); Oncorine (H101), a genetically modified adenovirus targeting P53 deficient cancer cells (Garber 2006); the reovirus-based Reolysin (Sei et al. 2009); or JX-594, a GM-CSF expressing vaccinia poxvirus (Heo et al. 2013).

Conclusions

The main types of immune mechanisms deficient in cancer, as well as the therapeutical strategies targeting them are summarized in Table 1. Immunotherapy relies on manipulating the delicate balance of immune regulation, therefore, its use might have a "price" to pay, in the form of side effects related to immune reactivity (Caspi 2008). Nevertheless, immunotherapy is now coming to age (Galluzzi et al. 2014). This promising field is in constant growth, with a wide array of strategies that are routinely used in medical practice, or are at the threshold of clinical use. The most important approaches in clinical use or in Phase III clinical trials are summarized in Figure 1.

Acknowledgements

This work was supported by the TÁMOP-4.1.1.C-13/1/KONV-2014-0001 program entitled „Practice-oriented, student-friendly modernization of the biomedical education for strengthening the international competitiveness of the rural Hungarian universities". The related experimental work was supported by FP7-HEALTH-2012-INNOVATION-1 (proposal No: 305341-2; CTCTrap) and HUSRB Cross border cooperation HUSRB/1203/214/230.

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