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Review

Immunotherapy of Cancer through Targeting of the p53 Tumor Antigen

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Abstract. The expression of the p53 tumor suppressor protein is frequently increased in a great variety of human cancers, making this antigen an attractive candidate for targeting therapeutic T cell immunity. However, potential complications as a result of immunological tolerance or autoimmune pathology must be taken into account when exploiting this ubiquitously expressed auto-antigen for the immunotherapy of cancer.

Key words: cancer immunotherapy; p53 tumor antigen.

Introduction

Despite the development of several alternative strategies to eradicate malignant cells, surgery remains the main option for the curative treatment of solid cancers. It has, however, long been known that adjuvant therapy, following surgery, can prolong life expectancy in human beings with various types of malignancies. Until recently, experience with adjuvant therapy was solely based on chemotherapy or irradiation. In the last decades the potential use of immunotherapy has attracted the attention of researchers. The major advantage of immunotherapy over chemo- and radiotherapy is that it may permit selective elimination of cancer cells in the absence of damage to somatic tissues that are not affected by the disease. T cell-mediated immunotherapy would be particularly suited for this purpose, because T lymphocytes have been shown to specifically recognize and eliminate aberrant tumor cells *in vitro* and *in vivo*. Furthermore, T cells have the capacity to deeply invade tissues, a property which is important for attacking solid cancers such as colon carcinoma.

Tumor Antigens

Recognition of tumor cells by T lymphocytes requires the expression of tumor-associated antigens and the processing of peptides derived from them into MHC molecules at the tumor cell surface. At present, an impressive number of such tumor-associated antigens has been described[38]. Several of these antigens are exclusively expressed by tumors and can therefore be exploited as truly tumor-specific targets for anti-tumor T cell immunity. For example, tumors induced by oncogenic viruses (e.g. human papillomavirus type 16-induced cervical carcinoma) express viral antigens that can serve as targets for the T cell responses. However, the majority of human cancers are not associated with the expression of viral antigens. Nevertheless, most

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tumor types do exhibit expression of auto-antigens that are not, or only in a very restricted fashion, expressed by non-transformed somatic cells. This difference may create a therapeutic window that allows T cells to attack tumor cells without killing normal cells. Several studies show that the tumor-associated antigen p53 is aberrantly expressed in approximately 50% of all human malignancies[23, 74]. Therefore, p53 is a particularly attractive candidate antigen for the targeting of therapeutic anti-tumor immunity.

p53 and Cancer

Wild-type p53 is a tumor suppressor protein that is ubiquitously expressed in normal tissue cells[35]. Its main function is to guard the structure of the genome, thereby preventing cells from becoming malignant. Under normal conditions p53 is present in an inactive conformation that is highly susceptible to degradation through the action of proteins such as Mdm2[24, 39]. As a result, the inactive wild-type p53 has the very short half-life of approximately 20 min[45] (Fig. 1A). Due to various mechanisms, involving radiation or chemical stress, single- or double-stranded breaks can be introduced in the DNA. Sensory molecules, including DNA-dependent protein kinase (DNA-PK), can subsequently trigger functional activation of p53[57], causing blockade of the cell cycle and, if the DNA damage is not readily repaired, cell death by apoptosis[11, 12, 71]. Conversion of p53 to its active conformation causes this protein to become resistant to degradation by Mdm2 and, thereby, to reach higher expression levels[31, 36, 57] (Fig. 1B).

Since p53 is a pivotal tumor suppressor protein, it

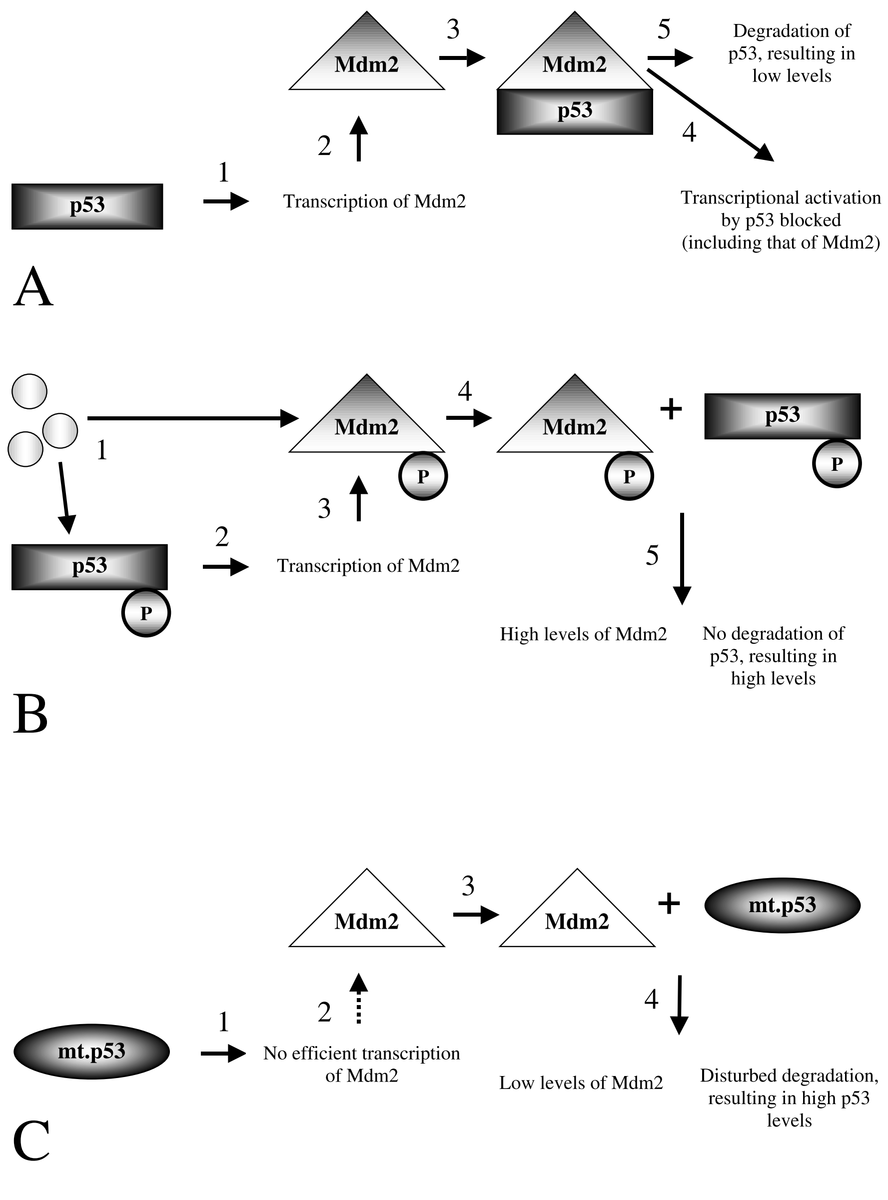

Fig. 1. Regulation of inactive p53 protein (**A**). Because p53 can induce arrest of cell growth and apoptosis, a tight regulation of its protein levels is required. Particularly the Mdm2 protein is known to be an important regulator of p53. The Mdm2 gene is a transcriptional target of p53 (1), resulting in the upregulation of the encoded Mdm2 protein (2). Subsequently, the Mdm2 targets the p53 (3), thereby not only inhibiting its function as a transcriptional activator (4), but also targeting the p53 protein for proteasome-mediated degradation (5). This autoregulatory feed-back loop can efficiently control p53 levels. Regulation of active p53 protein (**B**). DNA damage is detected and reported to p53 and Mdm2 by a cascade of sensory molecules, including DNA-PK (1). As a result, both p53 and Mdm2 are phosphorylated, preventing the binding of the two proteins (4). Because Mdm2 is still transcribed (2, 3), this results in coexisting high levels of p53 and Mdm2 (4, 5). When the DNA damage is repaired, the p53 and Mdm2 are dephosphorylated. Under these conditions the high levels of Mdm2 assure a rapid break--down of the high amount of p53. Disturbed regulation of mutated p53 protein (**C**). The enhanced stability of mutated p53 is not an intrinsic feature of the aberrant protein, but merely the result of failure of the autoregulatory feed-back loop by Mdm2. Mutant p53 cannot efficiently transcribe Mdm2, leading to low amounts of this protein (1, 2). Moreover, the binding of Mdm2 to mutant p53 is disturbed (3). Consequently, accumulation of the non-functional mutant p53 can be detected (3, 4). mt.p53 stands for mutant p53

is not surprising that mutation of the p53 gene is one of the most frequently occurring events in human oncogenesis[23]. Another consequence of p53 gene mutation is disruption of the normal homeostatic pattern of the protein. Mdm2 cannot degrade mutated p53 and, therefore, the non-functional protein can accumulate to high levels (Fig. 1C). Overexpression of p53 is frequently found in many types of human cancers, including colorectal carcinomas (Fig. 2), prompting research concerning the possibility to use p53 as a target antigen for immunotherapeutic intervention against cancer.

p53-Specific Immunity

The detection of antibodies against p53 in mice bearing mutant p53-overexpressing tumors provided the first clue that the immune system can respond against the p53 protein[10, 34]. Likewise, antibodies against p53 have frequently been detected in the sera of cancer patients, including patients with colorectal carcinoma[1, 9]. The presence of these antibodies proved to be an indicator of poor prognosis[26] and most likely reflects an abundant release of tumor--derived p53 in patients with large disseminated tumors and, thereby, an increased availability of this antigen for the activation of B cells. However, an anti-tumor efficacy of these antibodies is precluded by the fact that p53 is an intracellular antigen that is not expressed at the cell surface. Importantly, peptides derived from p53 through proteolytic degradation can be presented on the cell surface in the context of MHC class I or II, thereby serving as potential targets for T cells.

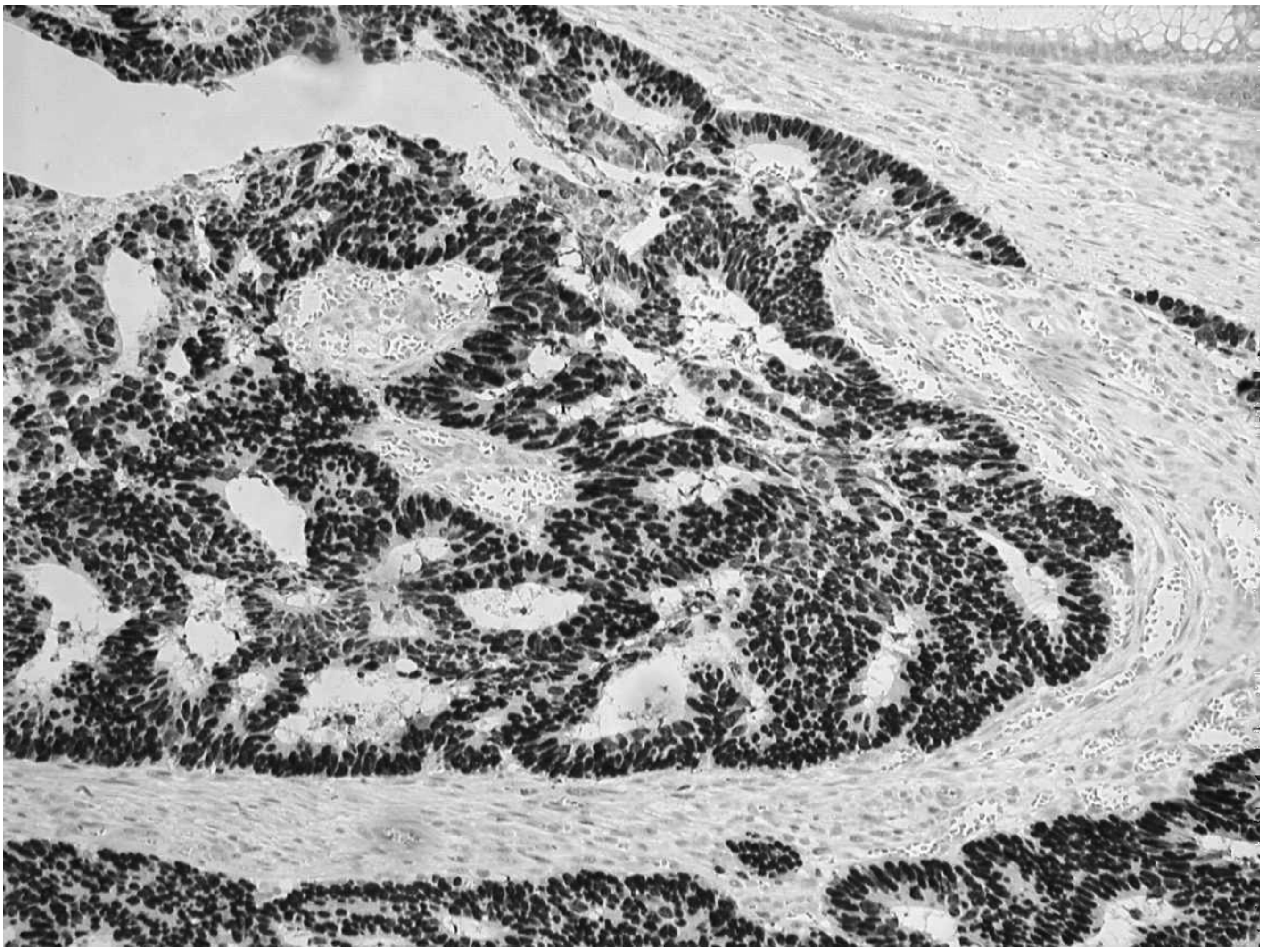

Fig. 2. Overexpression of p53 in colon carcinoma. The p53 protein is often overexpressed in many tumor types, such as colon carcinoma. In the depicted section of a colorectal cancer, the p53 protein is stained with a p53-specific antibody. Clearly, the tumor cells overexpress p53, while the stroma cells do not

Immunological Tolerance of p53

Immunological tolerance creates a potential hurdle for the use of auto-antigens such as p53 as a target for T cell-mediated immunotherapy against cancer. The p53 protein is ubiquitously expressed in all body cells and, therefore, also in the thymus[47]. Consequently, high affinity T cells reacting against this self-antigen may be deleted from the T cell repertoire. Because tumors often express mutated p53, tolerance could be circumvented by targeting of the mutated areas of the protein, which can be considered as "non-self". However, the mutations occurring in the p53 protein are very heterogeneous. Identification of the mutations would be required for every individual patient prior to immunotherapy. Moreover, the mutations are rarely contained within the processed p53-derived peptides that are presented in MHC molecules[72], rendering this approach far from generally applicable. Tumor immunological research has therefore focused on the use of wild-type p53 as a target for T cell-mediated immunotherapy. The potential of this strategy was illustrated by the finding of wild-type p53-specific T helper (Th) cells in patients with colorectal cancer[68]. In addition, cytotoxic T lymphocytes (CTL) and Th cells against wild-type p53 could be isolated from peripheral blood mononuclear cell (PBMC) cultures of healthy subjects[3, 7, 17, 25, 49]. Together with the outcomes of studies in mouse models, in which p53-specific vaccination raised wild-type p53-specific T cell immunity[29, 37, 51], these data indicate that tolerance against p53 is not complete and that exploitation of the p53-specific T cell repertoire for the immunotherapy of cancer may be feasible.

Split-Tolerance for p53

Although protective immunity against p53-overexpressing tumors could be achieved in 4 studies in mice following p53-specific vaccination[28, 37, 51, 65, 66], only one of these studies provided direct evidence that this was the result of p53-specific CTL activity[37]. In several other studies involving the analysis of p53-specific CTL, the anti-tumor efficacy of p53-specific CTL was only demonstrated *in vitro*, not *in vivo*[13, 14, 18, 21, 26, 40, 49, 61]. Furthermore, it was shown that in the periphery of p53$^{+/+}$ mice only low avidity CTL against p53 could be found[20, 21, 62]. With regard to the tremendous effort put into the induction of p53-specific CTL, these results are rather meager, suggesting that tolerance for p53 at the CTL level may be hard to break. There is convincing evidence that tolerance is far less pronounced in the case of p53-specific Th responses. The fact that antibody responses of the p53-specific IgG-type are found spontaneously in the sera of tumor-bearing mice and cancer patients points to a Th cell component, because class switching from IgM to IgG antibodies requires the involvement of CD4^{+} T cells[5, 8]. Moreover, various studies clearly indicate the existence of p53-specific Th immunity in humans[17, 63, 68]. Recently, we (Zwaveling et al., Cancer Res., in press) and others[15] demonstrated in a preclinical model in mice the lack of tolerance for p53 at the Th cell level. Furthermore, we showed that a p53-specific Th line can assist in clearing p53-overexpressing tumors *in vivo*. The intriguing difference found for p53-specific CTL and Th tolerance can be explained by taking into account the metabolism of wild-type p53. Under normal circumstances, the p53 protein has a very high turnover, as it becomes rapidly degraded via the ubiquitin- and proteasome-dependent pathway[2]. It is highly conceivable that this degradation pathway efficiently feeds p53-derived peptides into the MHC class I presentation pathway, enabling normal somatic cells, including thymic antigen-presenting cell (APC), to present these peptides at their surface (Fig. 3A). In contrast, the dominance of the proteasome-dependent degradation will not leave a considerable amount of p53 available for direct or cross-presentation through the MHC class II-processing pathway. These circumstances make it very unlikely that, in a healthy subject, p53-derived peptides are presented in the context of MHC class II, either in the thymus or the periphery, thereby explaining the lack of tolerance at the Th level[41] (Fig. 3B).

Anti-Tumor Efficacy vs Autoimmune Pathology

Apart from tolerance, another potential complication that must be considered with the use of wild-type p53 as a target for T cell-mediated immunity is the danger of inducing autoimmunity. The p53 protein is expressed, albeit at low levels, throughout the body and the consequences of an uncontrolled immune response against p53 could be disastrous. Importantly, we have demonstrated that high-affinity wild-type p53-specific CTL, isolated from p53 knock-out mice, can eradicate p53-overexpressing tumors in p53$^{+/+}$ mice in the absence of immune pathological damage to normal tissue cells[70]. Similarly, the *in vivo* action of p53-specific Th cells against p53-overexpressing tumors did not coincide with autoimmune pathology (Zwaveling et al., Cancer Res., in press). Therefore, we can conclude that the difference in p53 expression between tumors and

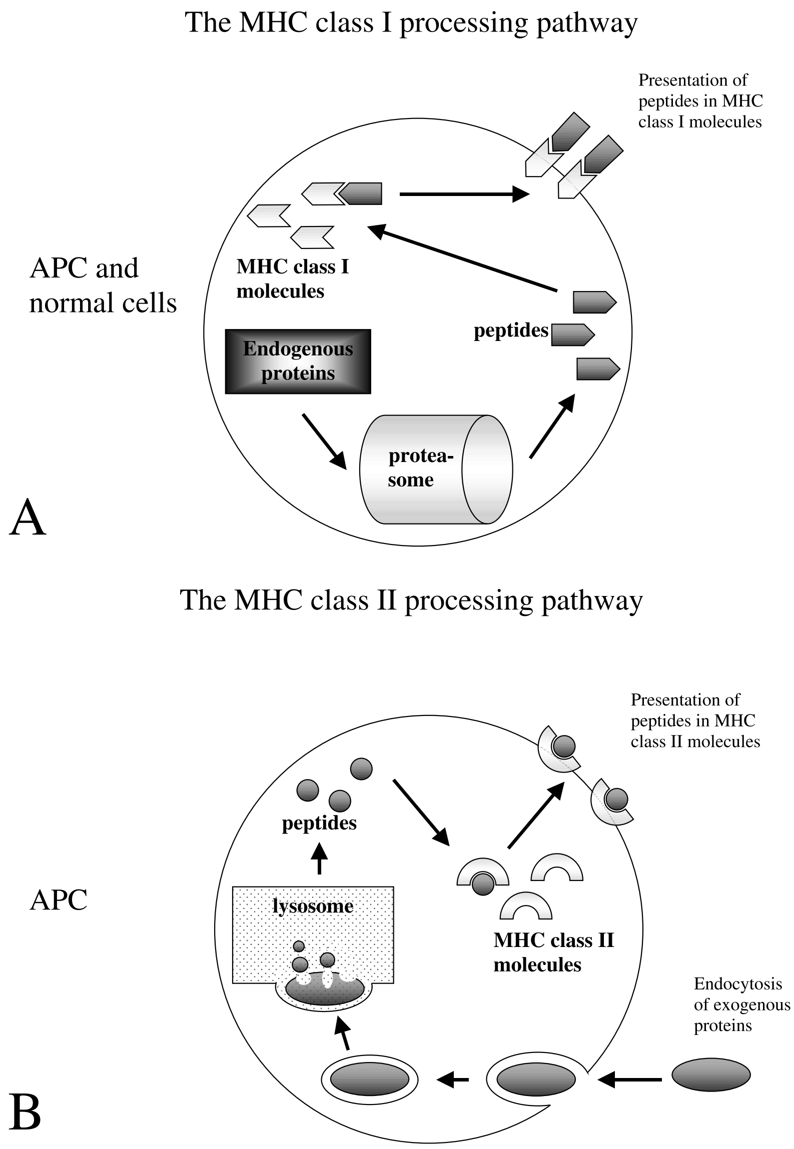

Fig. 3. Antigen presentation via the MHC class I pathway (**A**). All normal tissue cells endogenously express self antigens such as p53. Following degradation by the proteasome, antigen-derived peptides can be presented by MHC class I molecules on the cell surface. The presence of antigen-specific CD8$^+$ T cells, recognizing these peptides, would consequently lead to autoimmunity. Therefore, maturing CD8$^+$ cells that recognize these self antigens have to be deleted from the T cell repertoire in the thymus prior to their systemic release. APC in the thymus are responsible for this negative selection process. Like other cells, the APC can express self antigens, including p53, in a MHC class I context. Upon recognition of these antigen-derived peptides, maturing CD8$^+$ cells are mainly deleted from the T cell repertoire via a process that is not yet fully identified. Antigen presentation via the MHC class II pathway (**B**). APC do not only express MHC class I molecules, but also MHC class II molecules. Proteins obtained from the exterior environment (e.g. proteins derived from dead cells) can be taken up by APC via endocytosis. The proteins are degraded by various proteases in the lysosome and subsequently presented in a MHC class II context on the cell surface of the APC. Due to the highly efficient degradation of p53, this protein will usually not be present in the debris obtained from dead cells. Therefore, the pathway leading to presentation of p53-derived peptides in MHC class II will not take place and, as a result, no p53-derived peptides will be presented by MHC class II molecules on APC to CD4$^+$ cells. Consequently, maturing p53-specific CD4$^+$ T cells will not be deleted from the repertoire in the thymus. When a p53--overexpressing tumor develops, high amounts of stable p53 will become available, due to necrosis and apoptosis of tumor cells, for processing and presentation in the MHC class II pathway of APC in the vicinity of the tumor. Since p53-specific CD4$^+$ T cells have not been deleted from the T cell repertoire, this will lead to recognition of the p53 protein by these T helper cells

normal tissue creates a therapeutic window. In accordance with this notion, a recent phase I/II clinical trial performed at our institute showed that patients vaccinated with recombinant canarypox virus (ALVAC) encoding wild-type human p53 elicited p53-specific T cell immunity in the absence of detectable autoimmune pathology[69]. Previous immunization studies with ALVAC-p53 in mice and primates had shown similar results[50, 51]. Although these data demonstrate that autoimmunity can be avoided while T cell responses against p53 are successfully raised, application in humans of vaccines aimed at eliciting p53--specific T cell responses should still be conducted with great care.

A Role for p53-Specific T Helper Cells

So far, the efforts of raising p53-specific immune responses have primarily focused on CTL. Because tolerance against p53-specific Th cells appears to be less restricted than tolerance against p53-specific CTL, it is of interest to further explore the anti-tumor efficacy of the p53-specific Th component. Although most solid tumors do not express MHC class II, thereby excluding cognate interactions between Th cells and tumor cells, it is by now evident that Th cells contribute essentially to the eradication of such tumors[38, 42, 48, 55, 64]. This help can act through the recruitment of tumoricidal macrophages and eosinophils[19, 27], but also through CTL-dependent mechanisms involving the delivery of essential growth stimuli to CTL[30] and the triggering of APC through CD40-CD40 ligand interactions[4, 46, 56]. The latter mechanism induces antigen-presenting dendritic cells (DCs) to fully display their antigen-presenting and costimulatory functions, providing naïve CTL with a "license to kill" signal, and requires the DC to present relevant epitopes in the context of both MHC class I and class II. Several studies by others[6, 22, 33, 43, 58] and us[75] have indeed demonstrated the superiority of vaccines comprising tumor-specific CTL and Th epitopes over comparable vaccines containing CTL epitopes only. Importantly, these epitopes do not have to be derived from the same antigen[42], implying that p53-specific Th cells can also enable the activation of CTL directed against tumor-associated antigens other than p53. The strategy of evoking p53-specific Th cells in combination with CTL directed against other tumor-specific antigens therefore deserves further exploration.

Targeting of p53 in Colorectal Cancer

Colorectal cancer is the second cause of cancer-related death in the developed part of the world, following lung cancer in males and breast cancer in females. Because the perspectives for patients with metastases, for whom chemotherapy is at present basically the only option, are rather bleak, T cell-mediated immunotherapy against targets such as p53 could provide an important alternative. As mentioned before, the option of raising p53-specific T cells in combination with T cell immunity against other tumor-associated antigens may be feasible. Carcinoembryonic antigen (CEA) or epithelial cell adhesion molecule (Ep-CAM, KSA, 17-1-A), antigens often overexpressed in colorectal carcinoma, are being studied extensively in this respect[44]. In view of the above, a clinical trial involving ALVAC-based vaccination against p53, CEA and Ep-CAM will be performed in colorectal patients in the near future. More recently, several additional wild-type and modified antigens have been proposed as targets for therapeutic T cell immunity against colorectal tumors. These include β catenin, which is frequently overexpressed in colorectal cancers[16], and frameshift mutation-derived peptides from several cellular proteins, for example the transforming growth factor receptor II[54, 73].

Directions for Further Research

In order for anti-cancer vaccines to have therapeutic impact in patients, newly designed powerful adjuvants need to be co-administered. In a recent peptide-vaccination study we tested the effect of several of these adjuvants on the induction of CTL responses[75]. Adjuvants constituting a strong trigger for DC activation, such as CpG oligodeoxynucleotides and MPL, proved capable of raising more vigorous CTL responses than incomplete Freund's adjuvants (IFA, Montanide ISA45). Most importantly, whereas the peptide vaccines in IFA were only capable of inducing protective immunity against a subsequent challenge with tumor cells, the use of such stronger adjuvants upgraded the peptide vaccine to a therapeutic formulation capable of inducing rejection of pre-existing tumors. Some of these DC-activating adjuvants are, or will soon be, available for clinical use. Although more powerful adjuvants can improve the expansion of the desired T cell repertoire, such formulations cannot overcome the lack of certain T cell specificities due to clonal deletion. Nevertheless, several strategies may still allow the exploitation of p53-specific CTL immunity, for instance through the use of allogeneic T cell repertoires that have not been subjected to self MHC-restricted tolerance induction[52, 53, 60]. CTL against p53 in an allogeneic MHC class I context could be generated from donor-lymphocytes. Subsequent adoptive transfer to cancer patients, who receive bone marrow transplants from this donor beforehand to create a surrounding in which the transferred CTL can survive, may lead to a reduction of the tumor burden. Alternatively, transfer of p53--specific T cell receptor (TCR)-raised in HLA-transgenic p53 knock-out mice, to human CTL precursors should be considered[32, 59]. Especially TCRs that recognize human wild-type p53-derived peptide epitopes in the context of HLA molecules that are highly prevalent in the human population, such as HLA-A*0201, can constitute highly versatile tools for this approach. Last but not least, several groups have found evidence for the presence of at least a limited p53-specific CTL repertoire in humans[3, 14, 18, 49, 61]. Together, these findings prompt research concerning the "rules" that allow escape of certain p53-specific CTL from thymic deletion. Van den Eynde and Morel[67] have recently shown that auto-reactive CTL can escape deletion in the thymus if they are directed against epitopes that are exclusively, or at least primarily, formed by constitutive proteasomes and not, or to a much lesser extent, by immunoproteasomes. Because the thymic APC predominantly express immunoproteasomes, the degradation pattern imposed by this enzymatic configuration dominates the peptide repertoires presented to thymocytes during negative selection. In view of these findings, it is of interest to examine whether digestion of human p53-derived peptides by constitutive versus immunoproteasomes would reveal similar differences that could point to an escape of a p53-specific CTL subset from thymic deletion.

In spite of the fact that new techniques and recent insights have tremendously boosted the development of immunotherapeutic strategies against tumors, the clinical impact of these strategies has so far been very modest. This is partly due to the long process of extensive testing in pre-clinical systems and subsequent clinical trials that is required before approval for clinical use is granted, but could also relate to the difficulties in translating pre-clinical data to a clinical situation. Therefore, at this moment chemotherapy and irradiation are still the main options for the adjuvant therapy of cancer.

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