Immunotherapy of melanoma : toward clinical application

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Chapter 7

Discussion

Discussion: what defines the success of immunotherapy?

This thesis describes different immunotherapeutic strategies, ranging from activation of the endogenous T cell repertoire to infusion of genetically modified T cells. Until now, cell-based immunotherapy of cancer unfortunately has met with little success. Here, I will describe the factors that contribute to the efficacy of immunotherapy and that might enhance the success rate of immunotherapeutic strategies.

Target selection

One of the main issues in cancer immunotherapy is selection of the right target antigen. As discussed in the introduction, melanoma antigens can be divided into four different categories. Unique antigens, that arise from somatic point mutations, enable strictly selective targeting of tumor cells. They are however less suitable for immunotherapy since they are only expressed on individual tumors. Antigens belonging to the other categories, i.e. cancer/testis, melanocyte-differentiation and overexpressed antigens, are expressed in tumors from different patients. These shared antigens are therefore usually favored targets, but T-cells directed against these antigens often lack tumor specificity and can also target normal cells. In the case of melanoma differentiation antigens, destruction of healthy melanocytes can result in vitiligo and uveitis. However, since uveitis can be treated via the administration of corticosteroids, and vitiligo is considered a relatively mild side effect, the toxicity associated with targeting these antigens seems acceptable. In contrast, when target antigens are expressed on vital tissues, like the ubiquitously expressed p53 antigen, autoimmune destruction of normal cells could cause severe toxicities. Although the higher expression level of p53 on tumor cells is thought to provide tumor selectivity[1], recent experiments have indicated that targeting of this antigen can result in destruction of host peripheral blood and stem cells, leading to lethal cytopenia (M. Lauwen et al, manuscript submitted). Tissue distribution of target antigens is therefore a very important issue in the selection of targets for immunotherapy.

Another factor in target selection, is the tendency of tumors to escape immune attack by loss of antigen expression. The induction of T cell responses against specific antigens can lead to the selection of tumor cell variants that have lost expression of these target antigens, an event that has been observed for melanoma in mice [2] (chapter 5), as well as in humans[3-5]. To circumvent this, one could select target antigens that are essential for cellular transformation, such as PRAME, which is overexpressed in melanoma and is associated with an advantage in cell growth and survival[6]. Alternatively, multiple antigens could be targeted simultaneously to reduce the chance of outgrowth of antigen loss variants. Obviously, these strategies to circumvent antigen loss can only be successful if MHC-expression by tumor cells is maintained, whereas loss of MHC class I expression, caused for example by mutations in the β2-microglobulin gene or defects in antigen processing and transport pathways, will prevent tumor elimination by cytotoxic T cells.

T cell avidity

Since most tumor antigens are self antigens, naive T cells circulating in the periphery will generally only recognize these antigens with low affinity TCR-peptide/MHC interactions, due to the process of negative selection in the thymus. Activation of this endogenous repertoire

via vaccination will therefore be much more difficult than vaccination against pathogens. Also in the case of adoptive transfer of T cell clones or cell lines that have been obtained from patients, the clinical use of T cells specific for self antigens is restricted to the endogenous repertoire, although in vitro selection and expansion enable the infusion of high numbers of activated T cells. TCR gene transfer on the other hand, enables the introduction of high affinity receptors into T cells, thereby circumventing the limitations of the endogenous repertoire.

One strategy to acquire high affinity TCRs is to isolate them from T cells that managed to escape negative selection. This strategy is based on the assumption that TCRs present in some melanoma patients, e.g. patients that showed strong T cell responses or even tumor regression upon immunotherapy, are of an above average quality and would therefore be of value for broader groups of patients. This strategy is only feasible for antigens for which tolerance is not absolute, such as the melanoma differentiation antigens[7-9] (chapter 6).

To circumvent the restrictions of the endogenous repertoire, different strategies can be used that enable isolation of TCRs from a non-tolerant environment. First, high affinity TCRs can be isolated from a setting where the relevant MHC molecule is absent. In such an allogeneic setting, tolerance against self peptides bound by this molecule will not occur, and T cells (TCRs) with a high avidity for the relevant MHC/self peptide complexes will still be present within the endogenous repertoire[10]. Some of these TCRs might display cross-reactivity towards other self antigens, and receptors obtained in this manner should therefore carefully be evaluated before using them for clinical application.

A second strategy is to make use of a setting where negative selection is absent. In an in vitro display of libraries of TCRs, T cells do not undergo selection in the thymus and this will enable the screening of a large number of TCRs for increased affinity towards a specific antigen. Also here, cross-reactivity is an important issue of concern since negative selection is completely absent. Indeed, loss of specificity is known to occur after in vitro receptor selection[11].

Finally, high affinity TCRs can be isolated from a setting where the thymus does not present the relevant peptide. One example of such a setting is the isolation of TCRs specific for minor histocompatibility antigens (mHags). These antigens are derived from polymorphic genes, and are thought to be responsible for the graft-versus-leukemia effect upon MHC-matched stem cell transplantation. TCRs with a high affinity for a certain mHag can be isolated from an individual that does not express this antigen and subsequently used to target $mHag^+$ tumors[12]. Also in mice transgenic for human HLA genes, human epitopes that are not conserved between mice and men will be absent during thymic selection, and tolerance towards these antigens will therefore not occur[13,14]. TCRs obtained by this approach will be of murine origin, which could result in anti-receptor immune reactions. In current TCR gene transfer protocols, lymphodepleting pre-conditioning of the host will presumably prevent reactivity towards TCR modified cells and preliminary data from the Rosenberg lab indeed confirm this (R. Morgan, personal communication). However, in settings without lymphodepletion, the immunogenicity of infused TCR modified cells might be partially circumvented by replacing the murine constant domains with their human counterparts[15]. The generation of mice transgenic for human TCR loci, analogous to mice carrying human Ig loci[16], would completely solve this problem and markedly simplify the isolation of high affinity TCRs.

Although TCR gene transfer offers the possibility to employ receptors with an increased affinity compared to the endogenous repertoire, the level of expression of introduced TCRs is generally low due to the formation of mixed dimers (i.e. heterodimers of exogenous and endogenous TCR chains) and competition for components of the CD3 complex. Since the number of TCRs on the surface of a T cell is thought to (partially) correlate with T cell functionality, the low level of expression of introduced TCRs could potentially prevent optimal effector function of transduced cells[17,18]. One strategy to enhance the level of cell surface expression is the optimization of TCR genes. Although the sensitivity of gene-optimized TCR transduced cells as determined in in vitro assays seems unaffected, the in vivo functionality of these cells is markedly improved (chapter 5&6). Other approaches to enhance TCR expression can also reduce, or perhaps even prevent, the formation of mixed dimers via the introduction of an additional disulphide bond or via the use of murine constant domains[19-21]. A combination of these different strategies would presumably enhance TCR expression even further. We are currently evaluating these approaches for both human and murine TCRs (see section on safety issues).

Instead of introducing full length alpha and beta TCR chains, gene transfer can also be performed using chimeric receptors, which generally consist of an antibody-based external receptor structure linked to the TCR signal transduction domain[22]. These receptors offer the advantage of MHC-unrestricted antigen recognition, and TCR expression of these receptors is not hampered by the formation of mixed dimers. However, the in vivo functionality of cells transduced with chimeric receptors has never been directly compared to full length TCRs, and issues such as the potential immunogenicity of these receptors have not been addressed in in vivo studies.

Recently, it has been shown that microRNA 181a (miRNA181a) is involved in the posttranscriptional regulation of T cell sensitivity during T cell development. High levels of this microRNA are present in immature thymocytes, associated with increased T cell sensitivity that allows interaction with self antigens during thymic selection. On the other hand, miRNA181a is downregulated in mature T cells and targeted expression of miRNA181a in tumor-specific T cells might therefore enhance tumor antigen sensitivity and improve the efficacy of immunotherapy[23]. Careful evaluation of this strategy is needed however, since lowering the activation threshold of T cells could also induce crossreactivity towards other self antigens.

Conditioning regimen

Tumor-specific cells, either transferred or present in the endogenous repertoire, can be activated via active immunization, which will result in a proliferative burst (expansion phase), promptly followed by contraction of the effector T cell pool with only a low number of memory cells remaining in the circulation. Although T cell responses against tumor antigens are detectable upon vaccination, they are often of limited magnitude and persistence of these cells is only short-term[24,25,26].

In contrast, host conditioning via irradiation or chemotherapy can create an environment in which tumor-reactive T cells are activated and induced to undergo homeostatic proliferation[27,28]. T cells that are transferred into an “empty” host have unlimited access to proliferative cytokines and antigen presenting cells, and are less susceptible to suppression by regulatory elements. In melanoma patients, adoptive transfer of high numbers

of tumor infiltrating lymphocytes following lymphodepleting chemotherapy, resulted in long term persistence of adoptively transferred cells and marked tumor regression[29,30].

Chapter 3 describes a setting in which vaccination and irradiation are combined and where only a low number of antigen-experienced cells is transferred to the recipients. In this setting, transferred cells undergo vaccine-induced activation, followed by contraction of the antigen-specific T cell pool. This results in a marked but transient skewing of the T cell repertoire towards tumor recognition, suggesting that the transferred cells are not able to use the empty environment. Different T cell kinetics are described in chapters 5 and 6, where a relatively high number of TCR-transduced cells is transferred into irradiated hosts. In this setting, transferred cells repopulate the host's immune system and persist over a significant period of time. Additional vaccination of recipients has no effect on the persistence or functionality of transferred cells. Although these models differ in several aspects, the data suggest that the composition of the graft, i.e. the percentage and/or number of antigen-specific cells, influences T cell kinetics upon adoptive transfer into irradiated hosts.

Increased intensity conditioning or even complete myeloablation followed by hematopoietic stem cell transfer might further enhance treatment efficacy. Full ablation could lead to more complete removal of regulatory T cells and depletion of cytokine sinks. In addition, high-dose total body irradiation can induce diffuse tissue injury and a generalized inflammatory reaction, which could further drive the T cell response. Recent data showed that lymphodepletion to a level that required hematopoietic stem cell transplantation, combined with adoptive transfer of tumor reactive T cells, resulted in an enhanced anti-tumor effect compared to pretreatment with non-myeloablative conditioning[31]. However, increasing the intensity of conditioning will also increase lymphodepletion-associated toxicities (i.e. prolonged neutropenia, risk of infections, pulmonary complications), and therefore selection of an optimal conditioning regimen and careful monitoring of patients will be crucial[32].

Alternatively, selective depletion of competing cellular subsets using monoclonal antibodies against antigens expressed exclusively on T lymphocytes could provide a milder and therefore safer means of induction of lymphopenia. However, T cell-specific antibodies will not deplete NK cells, so that these cells remain present in the recipients and compete with transferred T cells for cytokines such as IL-7 and IL-15. Furthermore, depleting antibodies will not induce tumor tissue damage and will therefore lack the potential beneficial effect of improved antigen presentation or tumor accessibility.

T cell differentiation state

Prolonged antigenic stimulation during in vitro culture, such as often is required for the adoptive transfer of high numbers of tumor reactive lymphocytes, can cause the generation of exhausted T cells[33-35]. These cells might have an optimal antitumor activity in vitro, their proliferative capacity is however often decreased and these cells show poor survival in vivo. In melanoma patients, adoptive transfer upon extensive proliferation of T cell clones or vaccine-induced tumor specific cells did not result in long term persistence of transferred cells and clinical responses could not be observed[36,37].

Several studies suggest that in vivo persistence can be increased when adoptive transfer is performed with T cells that still express costimulatory and lymphoid homing receptors. These cells are thought to have an improved capacity to home to secondary lymphoid tissue, mediated via the expression of CD62L and CCR7. Interaction with peptide-MHC complexes

on APCs and simultaneous costimulation, e.g. via CD27/CD70, within these lymphoid tissues subsequently induces T cell activation and proliferation, and enables long-term persistence[38-40]. Studies in mice have shown that adoptive transfer of cells in a less differentiated state yields superior antitumor immunity as compared to adoptive transfer of fully differentiated cells[41-44]. Furthermore, although the tumor infiltrating lymphocytes responsible for tumor regression in the successful TIL trial generally had a late stage effector phenotype, the T cells that persisted in these patients for a long period expressed the costimulatory molecules CD27 and CD28, characteristic of a less differentiated state[45]. These data suggest that selection of less differentiated subpopulations before adoptive transfer can result in more effective antitumor immunity, and is a more preferred strategy than the selection of tumor reactive T cells based on in vitro IFNγ production and tumor cell lysis. Besides the expression of costimulatory and homing molecules, telomere length is also correlated with the long-term persistence of transferred cells[46]. To counteract the defective proliferative capacity of in vitro expanded cells, the hTERT gene can be introduced in order to prevent telomere erosion[47] (chapter 4). Although this approach can greatly enhance the number of population doublings, it has also been associated with genomic instability, which may limit its clinical application[48-50].

TCR gene transfer circumvents the need for extensive in vitro culture to generate large numbers of tumor specific T cells, although retroviral transduction does require strong activation of cells in order to induce cell-cycling. Selection of less differentiated subpopulations has not been tested for TCR transduced cells, but this approach seems worthwhile to pursue. As an alternative, infection with lentiviral vectors could be used, since this does not require cell division for integration of the transgene. However, activation of T cells with cytokines is still required for lentiviral transduction because infection of totally quiescent cells is blocked[51,52].

A different approach to prolong the persistence of transferred cells, is to optimize the in vitro stimulation procedure so that fit, rather than exhausted, T cells are generated. Murine TCR transgenic cells cultured in IL-15 maintained expression of CD62L and CCR7, whereas cells cultured in IL-2 had reduced expression of these molecules. Furthermore, subsequent adoptive transfer of IL-15 cultured cells into sublethally irradiated hosts resulted in a more pronounced anti-tumor effect as compared to cells cultured in IL-2[53]. Human T cells modified with a chimeric TCR and cultured in the presence of IL-15 were capable of long-term persistence in SCID-Beige mice and could eradicate established tumors[54]. Also, cell culture in the presence of artificial APCs, which can be engineered to express different co-stimulatory molecules, can generate high numbers of T cells that retain a substantial replicative capacity[55,56].

The presence of exogenous cytokines is not only required during in vitro culture, also in vivo CTLs often depend on the administration of cytokines. Administration of IL-2 can improve CTL persistence and expansion, but high doses of IL-2 are associated with serious toxicities, such as the vascular leak syndrome. These toxicities can be avoided by using lower doses of IL-2, which still support the growth of transferred cells[57]. IL-2 not only activates T cells, but is also known to cause activation induced cell death in vivo, and is involved in the induction of CD4+ CD25+ regulatory T cells[58]. Other γ-chain cytokines, such as IL-15 and IL-7, which only induce the activation of T cells, might be more suitable for exogenous

administration[53,59]. Alternatively, transfer of the IL-2 gene can be used to specifically deliver the cytokine to tumor-specific T cells[60]. This strategy has shown to allow maintenance of human melanoma-reactive T cells in vitro without the need of exogenous IL-2 administration. However, data from a first clinical trial with IL-2 modified TILs have been disappointing (B. Heemskerk, personal communication). Furthermore, constitutive expression of IL-2 might enable autonomous T cell growth and therefore require additional safety measures[61].

Finally, the in vitro production of tumor-specific T cells by TCR gene transfer into hematopoietic stem cells (HSCs) offers the possibility to generate T cells with long telomeres and presumably a much greater proliferative capacity than the memory cells currently used[62,63]. Furthermore, allelic exclusion of endogenous alpha and beta chains in these progenitor cells would also reduce the problem of mixed dimer formation, although allelic exclusion is far from complete for TCR alpha chains[63]. However, retroviral transduction of HSCs may increase the chance of transformation due to insertional mutagenesis, since genes involved in growth and development are active in progenitors, and insertions into these "high risk" genes are therefore more likely to occur. In addition, progenitor cells have a high proliferative potential and transformed cells will thus be more prone to uncontrolled growth. In a gene therapy trial of SCID-X1, leukemia was observed in several patients upon infusion of modified HSCs[64] (see also paragraphs on safety issues). Although there are indications that additional factors were involved in the development of leukemia in these patients[65,66], the potential risks of TCR gene transfer into hematopoietic precursors should be carefully evaluated in mouse models that mimic gene therapy of SCID-X1 and other diseases.

Modification of tumor environment

Progressing tumors often develop strategies to evade tumor recognition, and these mechanisms can lead to T cell dysfunction or anergy, and may thereby prevent effective anti-tumor immunity. The combination of immunotherapy with strategies that counteract tumor immune evasion and other inhibitory mechanisms could therefore improve the anti-tumor activity.

One strategy that tumors use to hamper immune responses is the production of inhibitory molecules such as IL-10, TGFβ, and IDO. The best characterized immunosuppressive cytokine is TGFβ, which is frequently found to be present in high concentrations in cancer patients, and is associated with disease progression and poor responses to immunotherapy. TGFβ supports tumor growth through the promotion of angiogenesis, the inhibition of T cell proliferation and activation, and the induction of regulatory T cells[67]. The immunosuppressive actions of TGFβ can be inhibited via large and small molecule inhibitors, but global blockade of TGF may result in side effects[68]. Alternatively, T cells can be provided with a dominant-negative TGFβ receptor, a strategy that has shown to induce preferential tumor infiltration and elimination with minimal side effects[69-71]. The infusion of TCR-transduced cells that were also modified with the TGFβDN receptor, led to an increase in T cell responses, but also to an increase in mortality that requires more attention before the possible merits of TGFβ blockade can be studied further (M. de Witte, unpublished observations, see also discussion of safety issues).

Besides secretion of immunosuppressive molecules, tumors can also express inhibitory ligands, such as FasL, PD-L1 (B7-H1) and B7-H4, on their cell surface and in this way hamper immune responses via the inhibition of T cell activation and proliferation and the

induction of T cell apoptosis. To circumvent Fas-mediated tumor immune evasion, T cells can be rendered resistant to FasL by gene transfer of small interfering RNA (siRNA). Human EBV-specific CTLs modified via this strategy were no longer sensitive to Fas-induced apoptosis, maintained their polyclonality upon prolonged cultured and remained dependent on antigen-specific stimulation for their proliferation and survival[72]. The inhibitory effects of programmed death receptor ligand -1, PD-L1, can be counteracted via the administration of blocking antibodies, and has shown to enhance tumor eradication in in vivo mouse studies[73,74]. However, PD-L1 is also expressed on normal tissue and the PD-L1/PD-1 pathway is involved in the regulation of peripheral tolerance, so extensive testing is warranted before clinical application.

T-cell mediated kill of tumors can also be prevented by the lack of expression of costimulatory molecules. The presence of costimulatory signals is thought to be required for both the activation of tumor-specific cells, as well as for efficient tumor cells lysis by transferred cells[75,76]. In the absence of costimulatory molecules such as CD28, costimulation to infused cells can be provided by modification of these cells with CD28-derived chimeric receptors. These single chain receptors contain an antigen-specific domain fused to the signal transduction domain of CD28, which can supply T cells with a costimulatory signal in the absence of B7-positive tumor cells[77]. The design of these chimeric receptors can be further optimized so that they provide both activation and costimulatory signals (CD28/CD3ζ)[78]. As for the regular chimeric receptors (see above), the functionality of T cells transduced with these receptors needs more extensive in vivo evaluation.

The transfer of TCR genes into T cells specific for viruses that have a latent persistence in vivo, might enhance the survival of modified cells by providing both antigen-dependent activation and costimulation via targets expressing viral antigens. Heemskerk et al. showed that modification of CMV-specific cells with a TCR specific for the minor antigen HA-2 generated cells that could recognize both HA-2^+ and CMV^+ targets[79]. An additive advantage of this strategy is that the formation of mixed dimers will be limited due to gene transfer into a T cell population with a restricted TCR repertoire. Furthermore, using virus-specific cells as recipients also limits the chance of activating ignorant, self-reactive cells via the introduction of an exogenous TCR (see safety issues). This approach has not been tested in vivo but clinical application should be feasible based on the presence of CMV or EBV specific cells in the majority of individuals, and the possibility to isolate these cells using MHC-tetramers[80-82]. A potential problem in this approach could be the in vivo selection of T cells that have a high expression of the viral-specific TCR and therefore a low expression of the introduced TCR.

Lastly, tumors can suppress immune responses via the induction of regulatory T cells. These cells are responsible for the induction and maintenance of peripheral tolerance towards self antigens and in that way they can also prevent effective anti-tumor immunity. For many different cancer types, including melanoma, an increased frequency of regulatory T cells has been observed in the peripheral blood of patients[83,84]. Furthermore, accumulation of regulatory T cells within ovarian tumors has been associated with a decreased survival[85]. As discussed previously, regulatory T cells can be eliminated by lymphodepleting chemotherapy or total body irradiation. However, this effect will only be transient when regulatory cells are reinfused with the graft or induced via the administration of IL-2[86]. Several mouse models have demonstrated that systemic depletion of regulatory T cells by targeting CD25, CTLA-4, or GITR results in an enhanced anti-tumor response[87-89]. Although administration of

antibodies against CTLA4 resulted in tumor regression in melanoma patients, it was also occasionally associated with severe autoimmunity[90,91].

Safety issues

An increasing number of immunotherapy trials is being performed in recent years, and some of these have resulted in clinical success. Before embarking on such studies, several factors concerning safety should be considered. A first safety issue in cancer immunotherapy is the choice of target antigens. The development and severity of on-target toxicity (i.e. reactivity towards the target antigen expressed on healthy tissue), will depend on the expression pattern of these antigens. As discussed previously, expression on tissues such as the skin is not likely to result in serious toxicity, whereas expression on vital tissues could potentially lead to more severe problems.

In TCR gene therapy, there are several additional safety issues, associated with either the process of T cell transduction via retroviral integration, or with the introduction of an exogenous TCR into mature T cells. Retroviral transductions could potentially lead to malignant transformation when the expression profile of oncogenes is altered by integration of the therapeutic gene. This was considered to be mainly a hypothetical risk, but became reality in a gene therapy trial of X-SCID where 3 out of 11 children developed leukemia upon retroviral integration in the LMO2 oncogene[64]. However, the risk of transforming events upon infusion of TCR-modified cells is likely to be much smaller than in the X-SCID trial, since there are several factors specific for this trial that might have contributed to the occurrence of leukemia. First, transduction of hematopoietic stem cells is more likely to result in integration in genes involved in self-renewal and growth as compared to transduction of mature T cells. In fact, there are no reports of side effects due to insertional mutagenesis in preclinical or clinical studies with mature T cells[92,93]. Second, γc-transduced cells have a strong growth advantage due to the severe immunodeficiency in X-SCID patients. Finally, the γc transgene itself may be tumorigenic, as suggested by a recent study in mice where overexpression of this gene caused the induction of T cell lymphomas[65].

Nevertheless, several approaches could be followed to minimize the risk of transforming events upon TCR gene transfer. For example, lentiviral or self-inactivating vectors might be considered instead of the retroviral vectors currently used in most clinical trials. Lentiviruses preferentially integrate downstream of transcriptional start sites, and in self-inactivating vectors transgene expression is driven from an internal promoter instead of the strong viral LTR[94,95]. In our hands, however, T cell transduction using different self-inactivating vectors did not result in substantial TCR expression (R. Gomez, unpublished observations). Possibly, with the development of engineering approaches that yield more robust TCR expression (see below), the use of lentiviruses may have more potential.

Another approach that is likely to enhance safety is to induce efficient TCR transgene expression while minimizing the number of viral integrations. The use of stronger promoter/enhancer elements may enhance transgene expression, but could also result in an enhanced effect on neighboring genes. Alternatively, transgene expression can be increased at the post-transcriptional level. As shown in chapter 5, gene-optimization enhances TCR expression without influencing viral titers, suggesting that the number of retroviral integrations is also unaffected. In addition, usage of the Woodchuck Hepatitis post-transcriptional regulatory element (WPRE) has been reported to strongly enhance transgene

expression, although this effect presumably also depends on an increase in viral titers[96]. However, in our own experience, introduction of WPRE into different retroviral vectors abolished expression of the DMF4 Mart-1-specific TCR (A. Jorritsma, unpublished observations).

Finally, inclusion of a suicide switch in retroviral vectors would allow the selective elimination of transformed cells. Introduction of the herpes simplex virus thymidine kinase (HSV-TK) gene in combination with administration of ganciclovir has shown to control graft versus host disease upon donor lymphocyte infusions[97,98]. However, immunogenicity of the HSV-TK gene product limits application of this safety switch[99,100]. A non-immunogenic alternative consists of the pro-apoptotic caspase 9 molecule fused to a FK506 binding domain, in combination with a chemical dimerizer[101,102]. In the RIP-OVA mouse model, autoimmune diabetes caused by OT-1 transgenic cells expressing the suicide switch could be blocked upon infusion of the dimerizer, although this approach has not yet been tested for TCR-transduced cells[103].

Besides the risk associated with retroviral integrations, the introduction of a new TCR into mature T cells could in itself lead to side-effects. Theoretically, three different mechanisms could result in the induction of autoimmunity[104]. First, introduction of exogenous TCR chains can lead to the formation of heterodimers with endogenous alpha and beta chains. This can generate TCRs with new specificities that might be reactive towards self-peptides. Second, if ignorant self-reactive T cells are transduced, triggering of these cells via the introduced TCR can result in an expanded population of autoreactive cells. Finally, MHC-mismatches between the TCR donor and recipients may result in recognition of allogeneic MHC molecules complexed to self antigens. For both human (chapter 6) and murine[105] transduced T cells, settings with an MHC-mismatch between TCR donor and recipient did not demonstrate alloreactivity, although these observations cannot guarantee safety of TCR-peptide/MHC combinations other than the ones tested.

Until now, both preclinical and clinical studies with TCR modified T cells have not shown any signs of off-target reactivity. However, recent experiments within our lab demonstrated that in a setting that strongly promotes the proliferation and activation of transferred T cells, i.e. in a lymphodepleted environment in combination with additional adjuvants such as IL-2 or TGFβ-blockade, severe pathology was induced upon infusion of TCR-modified cells, characterized by bone marrow failure and other signs of graft versus host disease. These side effects occurred in the absence of target antigen and ongoing experiments suggest that this pathology may also arise when a different TCR is used, indicating that the effect may be TCR-independent and not caused by cross-reactivity of the introduced TCR (G. Bendle, unpublished observations). Since introduction of exogenous TCRs is known to result in the formation of mixed dimers[106], these new TCRs could be responsible for the observed off-target autoimmunity. In this scenario, mixed dimers with self-reactive specificities would induce bone marrow failure analogous to settings of MHC-mismatched lymphocyte infusions[107-108].

Currently, experiments are being performed to assess whether mixed dimers are indeed the cause of the autoimmune pathology observed in our models. If so, several strategies could be employed to avoid damage by these heterodimers. First, pairing of exogenous and endogenous chains may be reduced or prevented by remodeling of the TCRαβ interface via

introduction of an extra disulphide bond or usage of murine constant domains[19-21]. Preliminary experiments with the Mart-1 –specific 1D3 TCR showed that these modifications could enhance the level of expression of the intended 1D3 heterodimer, but the effect on mixed dimer formation has not been assessed yet (R. Gomez, unpublished observations). Alternatively, cells expressing the correct αβ heterodimer could be selected using specific MHC-tetramers[82], thereby presumably minimizing the presence of autoreactive cells that express mixed dimers within the graft. Finally, the formation of mixed dimers can be avoided by the usage of single chain chimeric receptors[109], the transduction of γδ T cells[110], or by –at least to some extent- the transduction of hematopoietic stem cells (see section on T cell differentiation state). However, these approaches have not yet been evaluated in in vivo models.

Concluding remarks

Immunotherapy provides new possibilities for the treatment of tumors that do not, or no longer, respond to conventional therapies. Active immunization, which acts by stimulating the naive T cell repertoire, can be a simple, "off the shelf" method for the induction of T cell responses against tumors expressing viral antigens. The development of strategies such as DNA tattoo vaccination resulted in a more rapid and potent induction of immune responses, and combination of this strategy with host conditioning is likely to improve the clinical efficacy of vaccination.

However, when the endogeous T cell repertoire is affected by tolerance, such as is the case for melanoma and many other tumors, passive immunization via the transfer of TCR genes seems to be a more preferable approach. The selection of high affinity TCRs and the development of TCR formats that are well expressed without competition with endogenous receptors, are likely to improve the anti-tumor efficacy of TCR modified cells, while minimizing chances of autoimmunity. In addition, optimization of the host conditioning regimen, the selection of "fit" T cell subpopulations, and modification of the tumor environment can lead to further improvements. These factors should be taken into consideration when designing new clinical TCR gene transfer protocols.

If current limitations can be solved and future clinical trials are successful, TCR gene therapy has the potential of becoming a potent addition to conventional cancer therapies such as surgery, chemotherapy and radiation. The modification of patient's T cells with TCR genes has to be performed in specialized laboratories, but since this procedure is relatively simple, "off the shelf" application will become possible.

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