

Chapter 9

Therapeutic Impact of Immune Responses in Cancer

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Abstract Therapeutic targeted oncoimmunology has a long history reaching back to the nineteenth century and represents the basis of modern tumor immunology. Cell biological and molecular genetic techniques have uncovered crucial cellular and molecular mechanisms underlying effective cancer immunotherapies used in the clinic. To illustrate the scientific way that led to actual insights into the molecular and cellular approaches realized in recent cancer therapies, this chapter introduces into the history of oncoimmunology. Experimental findings of adoptive cell transfer-based cancer therapy are summarized under functional, immunological aspects. An actual overview of the antitumor prosperity of all genetically engineered tumor cells expressing recombinant cytokines which were characterized by animal experiments is given. The application of antigen-presenting cells which are triple transgenic for immune stimulatory cytokines, tumor specific antigens, and the correlated major histocompatibility complex class I necessary for tumor antigen presentation is explained exemplarily. A recent experimental animal model characterizing critical parameters for preconditioning the host prior to ACT of transgenic T cells and essential therapeutic conditions is described.

Keywords Adoptive cell transfer · Animal model · Cytokine · Effector cells · Immune evasion · Immune surveillance · Transgene · Tumor-specific antigen

Abbreviations

ACT	Adoptive cell transfer
DCs	Dendritic cells
GM-CSF	Granulocyte macrophage colony stimulating factor
HSCT	Hematological stem cell transplantation
IFN- γ	Interferon-gamma
IL	Interleukin
LAK cells	Lymphokine activated killer cells

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MCA	Methylcholanthrene
NK cells	Natural killer cells
MHC	Major histocompatibility complex
M-MuLV	Moloney murine leukemia virus
MMTV	Mouse mammary tumor virus
RAG-2	Recombination-activating gene-2
SCID	Severe combined immunodeficiency
TILs	Tumor-infiltrating leukocytes
TAAAs	Tumor-associated antigens
T _{cyt}	Cytotoxic T cells
T _H cells	T Helper cells
T _{H2} cells	T _H type 2
T _{SCM} cells	T memory cells
T _{CM} cells	T central memory cells
T _{EM} cells	T effector memory cells
TSAs	Tumor-specific antigens

9.1 Introduction: Milestones in Cancer Research

Cancer immunology is a field of immunology that comprises all aspects of interactions between the immune system and cancer cells that are functionally involved in the generation, course and control of tumors. The cornerstone for cancer immunology was laid in 1891, when Coley described a successful therapeutic approach for treatment of cancer by generating an inflammatory immune response [1]. Encouraged by observations from Martha Tracy and S. P. Beebe of the Huntington Cancer Research Fund, who demonstrated that large multiple sarcomas in dogs rapidly disappeared under local or systemic injections of bacterial toxins, Coley applied a mixture of bacterial toxins derived from the *Streptococcus erysipelas* and the *Bacillus prodigiosus* in cancer patients. The thus generated erysipelas infection resulted in tumor clearance in ~30% of patients with lymphoma or sarcoma. Based on the success of this cancer therapy, Coley concluded that infections "...may have an important bearing upon the whole cancer problem, since, if by the administration of certain bacterial toxins we can cause the degeneration, death, and absorption of living tumor cells of one variety of cancer—sarcoma—it is not unreasonable to suppose that by the use of some other forms of bacterial toxins we may succeed in destroying or inhibiting the growth of the other and more common variety—carcinoma." [2] Based on his clinical observations he formulated the theory that post-surgical infections can help patients to recover better from their cancer by provoking an inflammatory response. But while Coley assumed that in most cases the etiology of cancer is associated with an acute injury [3, 4], Paul Ehrlich proposed the hypothesis that nascent transformed cells arise continuously in our bodies and he further suggested that the immune system continuously scans for transformed cells and can suppress and finally eradicate such cells [5]. As a possible mechanism to combat transplanted neoplastic cells, Paul Ehrlich assumed an antibody-mediated athrepsy

of nutritive substances essentially required for the development of the neoplastic cells but was unable to identify any tumor-specific nutrition factor, due to technical limitations at that time.

The first experimental proof demonstrating the generation of a tumor-specific immune response was provided by Richmond Prehn and Joan Main in 1957 who showed that tumors induced by chemical carcinogens in mice could stimulate tumoricidal responses leading to rejection of this kind of experimentally induced tumor [6]. Surprisingly, spontaneously arising tumors were not rejected when tested in the same experimental manner. From this and subsequent studies it was a deductive option that naturally originating tumors were not immunogenic due to a given immune tolerance against the body's own antigens rendering the immune system powerless in the control of spontaneously developing neoplasms.

However, this assumption was challenged by Burnet and Thomas. Burnet implied that neo-antigens specific for degenerated cells can arise in tumors and might provoke an effective immune response that would eliminate developing cancers [7–9]. Thomas incorporated the age of an organism and the thus increasing probability for cancer development due to somatic mutations. The existence of somatic mutations rendering normal cells to become tumorigenic was shown by Sanford and colleagues in 1954 [10]. They verified that a normal fibroblast cell develops spontaneous mutations by *in vitro* culturing over several passages and acquires the propensity to generate sarcomata when injected into mice of the homologous strain.

Based on the experimental verification of spontaneous mutations as a natural source of cancer, Thomas suggested that complex long-lived organisms must possess mechanisms to protect against neoplastic disease similar to those mediating homograft rejection [11]. Describing the existence of mouse tumor-specific antigens and their functional role for immune system based tumor defense [12], represented a further crucial brick in the composition of the cancer immunosurveillance hypothesis, first conceptualized by Burnet in 1957. Citation: “*The failure in cancer is due not to any weakness of the organism but to a change in the character of the cells rendering them in one way or another insusceptible to the normal control.*” Interestingly, this prediction includes also phenomena in oncoimmunology not known in former times but today termed as “immune evasion”.

In 1984, Snyder and Bishop observed that a single mutation in the oncogenic tyrosine kinase *v-src* of the *Rous-Sarcom-Virus* resulted in the loss of tumorigenicity of the virus in immunocompetent mice [13]. They explained this result with the presumption that due to the mutation the virus evasion of the host immune response was no longer possible and concluded that evasion of the host immune response is a necessary step in tumorigenesis by *v-src*. In 1985, Mullen and Schreiber observed, in an UV-induced fibrosarcoma mouse model, that tumor cells can actively suppress the specific tumoricidal immune response without suppressing the immune system in general [14] and termed this effect a “tumor-induced evasion from the immune system” [15]. At the same time, Rooney and colleagues demonstrated that Epstein-Barr virus-positive Burkitt's lymphoma can evade from the cytotoxic T cell response [16]. Based on these observations the hypothesis of an immune evasion strategy of neoplastic cells was introduced to the field of tumor immunology.

So far, the immune system was regarded as a tumor defense system only. But in the late 1990s, it was reported that tissue sites with inflammation were more frequently associated with tumor formation, leading to the assumption that leukocytes might also contribute to tumor growth. In 1986, Dvorak and colleagues indicated tumor sites as “wounds that never heal” [17] and in the last 20 years numerous immune cell derived factors with pro-tumorigenic properties have been identified. But to cope with the history of tumor science, it has to be pointed out, that this “novel” insight in tumor development was previously recognized by Virchow in 1863, who postulated during one of his lecture at the University of Berlin [18], that a chronic irritation and previous injuries are preconditions for tumorigenesis. In addition, multiple resident stromal cell types which are part of the tumor microenvironment can collectively contribute to tumor progression. The interplay of neoplastic cells, stromal cells and invading leukocytes leads to a miscellaneous activity pattern of the various cell types and a complex cocktail of soluble mediators, which altogether sculpture the developing tumor. By unraveling the tumor sculpting effects of the immune system on developing tumors, the term cancer immunosurveillance used as a host-protecting mechanism was no longer appropriate in its original form. To describe more accurately the dual host-protecting and tumor-sculpting interplay between immune and tumor cells, Dunn, Bruce, Ikeda, Old and, Schreiber proposed the use of the broader term “cancer immunoediting” [19]. They envisaged the model of cancer immunoediting, which includes three phases of cancer development termed as “Elimination, Equilibrium and Escape” [20, 21]. The outcome of the cancer immunoediting process is uncertain and depends on whether the immune system is able to completely eliminate all neoplastic cells during the “Elimination phase,” or tumor sculpting processes successfully interfere with leukocyte functions by induction of immunologic anergy, tolerance or indifference against the neoplastic cells.

The “Elimination phase” starts at the point when cells of the innate immune system recognize the presence of a growing tumor, due to the tumor dependent local remodeling and damage of stromal tissue. As a consequence, inflammatory signals are generated leading to the attraction and activation of cells of the innate immune system to the local tumor site. These tumor-infiltrating leukocytes (TILs) produce various cytokines and chemokines, which further promote infiltration and activation of more TILs, in a self-enhancing circuit. Some mediators of this complex cytokine/chemokine storm carry inhibitory effects on the formation of new blood vessels. Other cytokines/chemokines function as activators of cytotoxic cells or promote tumor death via apoptosis. The resulting tumor cell debris become ingested by dendritic cells (DCs) which afterwards migrate to the draining lymph nodes, where they function as antigen-presenting cells (APCs). In the case of the presence of tumor-specific antigens (TSAs) these dendritic cells are able to recruit cells of the adaptive immune system. In the final phase of elimination, tumor-specific CD4⁺ and CD8⁺ T cells home to the tumor site and the TSA-specific killer T cells then destroy the antigen-bearing tumor cells. The anti-tumorigenic immune response runs out and terminates the “Elimination phase”.

However, in rare cases some neoplastic cells survive the “Elimination phase” and enter the “Equilibrium phase” which is clinically characterized by a dormancy of the tumor cells. This immune-mediated tumor dormancy is sustained by a fragile balance between the presence of tumoricidal and tumor promoting cytokines and might persist over several years. During this time, tumor editing occurs and it gradually becomes likely, that tumor cell variants develop, which are able to avoid immune surveillance due to their loss of antigenicity. Additionally, tumor cell variants might actually become more and more capable to use leukocytes or stromal cells of the tumor microenvironment to support tumor development. At this stage the tumor cells pass into the “Escape phase”, in which the balance of the tumor microenvironment is skewed towards tumor progression by generating a complex immune suppressive milieu and enhancing the vascularization of the growing solid tumor. In fact, the “Elimination phase” and the “Escape phase” share many similarities, except that the novel tumor cell variants have acquired an enhanced malignancy during their functional dormancy at the “Equilibrium phase”. Interestingly, the period of dormancy of tumor cells can last over many years, as observed for human melanoma. In 2003, two cases of malignant melanoma have been reported in kidney allograft recipients, that received the organs from a donor which had been cured from melanoma over a period of 16 years and was classified as melanoma free [22]. In both cases the donor kidney originated from the same donor.

9.2 Immunotherapies at the Beginning of the Twenty-First Century

The most important message of tumor medicine, starting with early observations by Coley, Ehrlich and, Virchow in the nineteenth century up to actual insights into the molecular and the cellular mechanisms underlying the hallmarks of cancer is: “Cancer can be overcome by the body’s own immune system at all phases of tumor editing.” Enormous effort has been made in the past 30 years to identify suitable cytokines, TSAs and tumor-associated antigens (TAAs) to develop biochemical or molecular biological tools for targeting tumor cells for inhibiting tumor growth supporting factors and/or for triggering antitumorogenic immune responses. To date, three main groups of immunotherapies are accepted for cancer treatment and used in the clinic: (i) antibody therapies (ii) cytokine/chemokine therapies, and (iii) cell-based therapies.

The following sections will discuss current cancer immunotherapeutic approaches and their underlying cellular mechanisms. An overview about the therapeutic potential and challenges of up to current antibody-based strategies used in human cancer therapies is given in another topic of this book.

Cytokine-based therapies use specific cytokines or cytokine cocktails to manipulate and direct immune responses to generate tumoricidal effector cells able to eradicate existing tumors. The rationale for this approach is based on the observation that during the phase of immunoediting tumor cells can exploit cytokines to

reduce immune effector functions, increase resistance against apoptosis, or even support tumor growth and dissemination. The therapeutic application of cytokines might counteract the tumor controlled editing process mediated by a sophisticated cytokine milieu and reverse the tumor microenvironment from tumor permissive to tumoricidal. The first clinical results of cytokine-based cancer therapies, which used partially purified cytokines, started in the late 1970s by the application of leukocytes derived interferons [23, 24]. In the following years, further trials demonstrated the therapeutic benefits of leukocyte derived interferons on non-Hodgkin's lymphomas, myeloma, malignant melanoma, and other malignancies even if only with moderate success. Additionally, cytokines from the group of interleukins known to exhibit immune stimulatory properties were found to be effective in cancer treatment. The major advantage of using cytokines in cancer treatment is the unlimited availability of recombinant cytokines. The huge disadvantage of using systemically applied cytokines or cytokine cocktails are the associated severe side effects [25–31]. A novel approach to deliver cytokines to cancer patients is the usage of genetically modified cells which express and release one or more different cytokines. Transfer of such cells into the tumor microenvironment seems to be more effective for tumor treatment with simultaneously reduced adverse effects observed in association with systemic application of high dosages of purified cytokines. This approach combines classical approaches of cytokine/chemokine therapies, but also represents a cell-based cancer therapy.

9.3 Cell-Based Cancer Immunotherapies

The method of cell-based cancer therapies consists of adoptive cell transfer (ACT) of isolated viable autogenic, allogenic or syngenic cells into a cancer patient. Application of ACT for tumor eradication might be effective during all phases of cancer immune-editing but typically, treatment starts after a tumor disease has been diagnosed. This mostly implies, that neoplastic cells have successfully undergone dysplasia probably followed by anaplasia and have reached the phase of "Escape". At this stage genetically/ functionally modified cells become transferred with the purpose to sensitize, reactivate and, support remaining tumoricidal immune cells from the equilibrium phase. Figure 9.1 summarizes current ACT strategies in cancer medicine based on the transferred cell type. The application of hematological stem cell transplantation (HSCT) can be included to the method of ACT but, in fact, it consists of a therapeutic option for post tumor treatment of hematopoietic and lymphoid malignancies after elimination of the tumor cells by various lymphoablating schedules (Fig. 9.1). The main goal of the non-tumor targeting strategy of HSC transfer is to re-establish a functional immune system rather than to eradicate existing tumor cells. All other ACT strategies are tumor targeted and are directed to transfer the immunologic capability to generate an effective tumoricidal response against

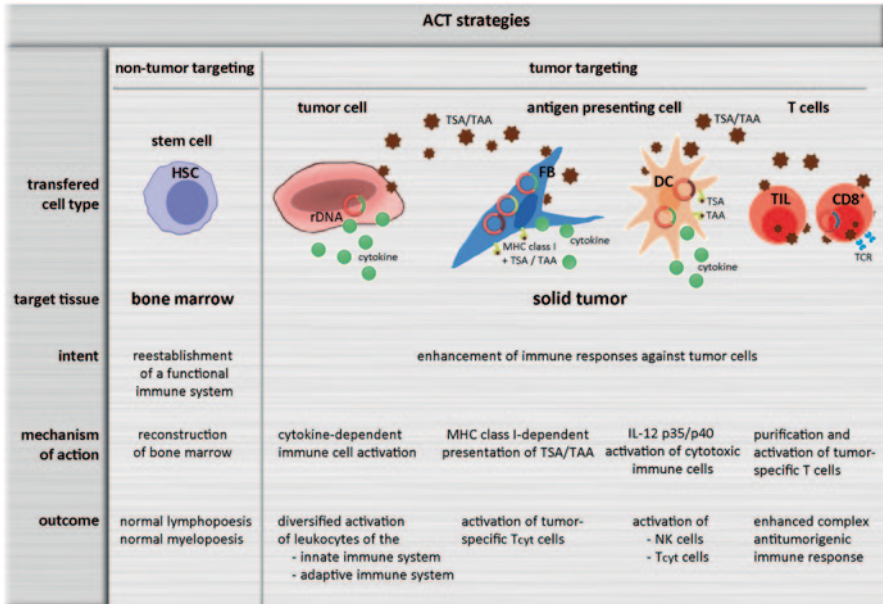


Fig. 9.1 Illustration of Adoptive Cell Transfer strategies. ACT strategies are arranged on the basis of the different immune cell sub-populations used for non-tumor targeting approaches such as hematopoietic stem cells (*HSC*) and targeted tumor therapies such as tumor cells fibroblasts (*FB*), dendritic cells (*DC*) or T cells either purified directly from the tumor stroma (tumor-infiltrating leukocyte, *TIL*) or from blood (*CD8⁺*). To summarize the various strategies for which the specified cell types are used only the most representative manipulations are depicted. To show, when one of the specified cell types has been used genetically modified, is illustrated as follows: a transgenic vector is depicted by a circle; the transfected genes in the vector are color coded with green: representing a gene encoding for a cytokine; yellow: for molecules relevant in antigen presentation such as the MHC class I molecule; blue: for a T cell receptor (*TCR*); and brown: for tumor cell specific/associated antigens (*TSA/TAA*). When cells were loaded with antigens prior to adoptive transfer, brown stars are depicted. The target tissue, the intent, the most representative and proven mechanism of action observed in different tumor entities and the underlying mode of action are listed below each cell type

present neoplastic cells back into the recipient. In most cases, the stimulation of tumoricidal responses became realized by genetical manipulation of the transfected cells with various expression vectors for recombinant cytokines, TSAs or TAAs, or major histocompatibility complex (MHC) class I antigens. Eligible cells for delivering the different recombinant tools into the tumor stroma are resident cells of the tumor stroma such as fibroblasts, professional APCs of the immune systems such as DCs, and even tumor cells by themselves (Fig. 9.1). Recently, the autologous cell transfer of *ex vivo* isolated and *in vitro* TILs has been established as an additional ACT-based tumor therapy (Fig. 9.1) and actually the usage of recombinant TILs is under investigation. Altogether, each cell type used for ACT fulfills a specific point of action within the tumor-immune-microenvironment network.

9.4 Tumor Cells Transgenic for Cytokines Used in ACT

One of the first ACT tumor strategies used genetically modified tumor cells to deliver immune stimulatory cytokines into the tumor microenvironment in a way of a Trojan horse. Based on the assumption that an immune suppressive milieu within the tumor microenvironment is responsible for tumor persistence, the rationale of this approach is to break open the immune suppressive milieu and to elicit or increase a cell-mediated antitumoral response. Based on the experience of some tumor therapeutic effects of purified cytokines systemically applied at high doses, the first recombinant genes delivered into the tumor via transgenic cells are cytokines. For this, the coding sequence of the cytokine of interest became cloned, ligated into an expression vector, which is then transferred into cells of a defined cell line or primary cells isolated from the host. The transgenic cells are then applied to the host either systemically or directly into the solid tumor. Once these cells have reached the tumor, they start to express and release the recombinant cytokine which leads to an enhanced immune stimulatory milieu within the tumor microenvironment and, hopefully, to an induction of an effective anti-tumorigenic immune response. The functional characterization of a still increasing number of cytokines, chemokines and other immune response modulating, soluble mediators, has helped to identify appropriate candidates for recombinant cell-based ACT strategies. The advantage of using tumor cells as supplier of recombinant cytokines is that the necessary cytokine dose is much lower in comparison to systemic application and, thus, adverse side effects might be reduced. Additionally, a long lasting, local expression of immune mediators is assured, which will not cease before all tumor cells, *de novo* arisen as well as transgenic tumor cells become eradicated in equal measure. Hence, various tumor cell lines were genetically modified to express recombinant cytokines known to exert tumoricidal effects directly upon tumor cells or to induce or enhance host-mediated mechanisms (for overview see Table 9.1).

One of the first cytokines used for proving the novel concept, that delivery of a recombinant expressed cytokine via genetically transfected tumor cells can be an effective therapeutic approach in tumor treatment, was the T Helper (T_H) cells-derived interleukin (IL)-4. This T_H type 2 (T_{H2}) synthesized-derived lymphokine was previously characterized as a multifunctional cytokine which exhibits a broad range of activities on B- and T cells and on hematopoietic cell lineages *in vitro* [32]. For construction of IL-4 expressing tumor cell lines, a genomic DNA fragment that contained the entire murine IL-4 coding region and about 3.5 kb of its 3' flanking sequences was cloned from an embryonic BALB/c library, placed under the control of the promoter/enhancer from the LTR of either the Moloney murine leukemia virus (M-MuLV) or the mouse mammary tumor virus (MMTV) [33]. Transfection of these plasmids into mammary adenocarcinoma cells (K485) resulted in the generation of several transgenic mammary adenocarcinoma cell lines, which differ in their amount of released IL-4. The IL-4-transgenic mammary adenocarcinoma cell line K485/D2B-1, which was found to express the highest levels of IL-4 when compared to other cell lines, was used for tumor transplantation into *nu/nu* mice [33]. The results showed that IL-4 expression substantially reduced the tumor growth,

Table 9.1 Overview of antitumor effects of ACT approaches using tumor cell lines transgenic for cytokines in various animal models

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for	Outcome ^a	References		
IL-1 α	Bladder carcinoma	MBT2	Irradiated	Development	Therapy	Delayed	[41]	
	Bladder carcinoma	MBT2	Irradiated	Development	Therapy	Delayed	[41]	
IL-1 β	Fibrosarcoma	CMS-5	Living	Development	Vaccination	Rejected	[42-44]	
	Melanoma	B16	Living	Development	Vaccination	Rejected	[45, 46]	
IL-2	Melanoma	B16	Irradiated	-	Vaccination	-	[45]	
	Melanoma	Cloudman S91	Irradiated	-	Vaccination	-	[47, 48]	
	Myeloma	X63-Ag8.653	Living	Development	-	Therapy	Eliminated	[49]
	Mammary tumor	4T07	Irradiated	Development	Vaccination	Rejected	Rejected	[50]
	Mammary adenocarcinoma	TS/a	Living	Development	Vaccination	Rejected	Rejected	[51]
	Lewis lung carcinoma	D122	Irradiated	Development	Vaccination	Therapy	Delayed	[52]
	Lewis lung carcinoma	LLC	Living	Development	Vaccination	-	Rejected	[53]
	Colon carcinoma	CT-26	Living	Development	Prevention	-	Rejected	[46]
	Bladder carcinoma	MBT2	Irradiated	Development	Vaccination	Therapy	Rejected	[41, 54]
	Renal cancer	SK-RC-29	Living	Development	-	-	Rejected	[55]
	Prostate cancer	R3327-Mat-LyLucell	Irradiated	-	Vaccination	Therapy	-	[56]
	IL-4	Melanoma	B16	Living	Development	-	Delayed	[45]
Melanoma		B16	Irradiated	-	Vaccination	-	[45]	
Mammary adeno-carcinoma		K485	Living	Development	Prevention	-	Rejected	[33]
Plasma cytoma		J558L	Living	Development	Prevention	-	Rejected	[33, 35]
Lewis lung carcinoma		LLC	Living	Development	Vaccination	-	Rejected	[53]
Renal cell carcinoma		Renca	Living	Development	Vaccination	Therapy	Rejected	[40]

Table 9.1 (continued)

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for	Outcome ^a	References
IL-5	Melanoma	B16	Irradiated	–	–	[45]
IL-6	Melanoma	B16	Living	Development	Delayed	[45]
	Melanoma	B16	Irradiated	–	–	[45]
	Lewis lung carcinoma	D122	Irradiated	Development	Delayed	[57]
IL-7	Lewis lung carcinoma	LLC	Living	Development	Shortened	[58]
	Ependymo-blastoma	203-glioma	Living	Development	Rejected	[59]
	Plasmacytoma	J558L	Living	Development	Rejected	[60]
	Leukemia	WEHI-3	Irradiated	–	–	[61]
	Fibrosarcoma	MCA 205	Living	Development	Rejected	[62]
IL-12 p35/p40	Colon carcinoma	CT-26	Living	Development	Rejected	[63]
IL-23	Colon carcinoma	CT-26	Living	Development	Rejected	[63]
	Fibrosarcoma	CMS-5	living	Development	–	[44, 64]
	Melanoma	B16	Living	Ddevelopment	Delayed	[45]
IFN- γ	Melanoma	B16	Irradiated	–	–	[45]
	Bladder carcinoma	MBT2	Irradiated	Development	Delayed	[41, 54]
	Neuroblastoma	C1300	Living	Development	Rejected	[65]
	Renal cancer	SK-RC-29	Living	Development	Not effective	[55]
	Prostatecancer	R3327-Mat-LyLucell	Irradiated	–	–	[56]

Table 9.1 (continued)

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for	Outcome ^a	References	
TNF α	Melanoma	B16	Living	Development	–	–	
	Melanoma	B16	Irradiated	–	Vaccination	–	
	Skin tumor	159I-RE	Living	Development	–	Protected	
	Bladder carcinoma	MBT2	Living	Development	Prevention vaccination	–	
G-CSF	Plasma cytoma	J558L	Living	Development	–	Rejected	
	Fibrosarcoma	MCA 205	Living	Development	–	Rejected	
	Colon adenocarcinoma	C-26	Living	Development	Prevention	–	
	Melanoma	B16	Living	Development	–	Rejected	
GM-CSF	Melanoma	B16	Irradiated	–	Vaccination	–	
	Bladder carcinoma	MBT2	Irradiated	–	–	Therapy	
	Renal cell carcinoma	Renca	Irradiated	–	Prevention	–	
	Colon carcinoma	CT-26	Irradiated	–	Prevention	–	
	Leukemia	WEHI-3	Irradiated	–	Vaccination	–	
	Prostatecancer	R3327-Mat-LyLucell	Irradiated	–	Vaccination	–	
	Fibrosarcoma	CMS-5	Living	Development	–	–	
							–
							–
							–

Table 9.1 (continued)

Transfected cytokine	Type of tumor	Tumor cell line	Status of transfected tumor cells	Used for	Outcome ^a	References
IL-2 + IFN- γ	Melanoma	B16	Living	–	Vaccination –	Protected – [45]
	Fibrosarcoma	CMS-5	Living	Development	Vaccination –	Rejected – [44]
GM-CSF + IL-2	Melanoma	B16	Living	–	Vaccination –	Protected – [45]
JE/MCP-1	Chinese hamster ovary	CHO	Living	Development	Prevention –	Rejected – [71]

Definition of terms used:

“irradiated”: mitotic activity of cells was inactivated by X-irradiation; “living”: cells were vital; “development”: experimental approach, in which exclusively transgenic cytokine-expressing tumor cells were injected into the animal; “vaccination”: transgenic tumor cells were injected prior to wild-type tumor cells; “prevention”: transgenic tumor cells were injected in parallel with wild-type tumor cells; “therapy”: transgenic tumor cells were injected after a wild-type tumor had been formed in an animal; “rejected” (term is used in context of prevention): transgenic tumor cells become completely eradicated; “delay”: expansion rate of tumor cells was reduced compared to wild type tumor cells; “protected” (term is used in context of vaccination): transgenic tumor cells become completely eradicated; “eliminated” (term is used in context of vaccination): existing tumor cells are killed by the transgenic tumor cells

^a Note, that the tabulated rating in most cases does not reflect 100% efficacy but the successful therapeutic outcome

when compared to non-transfected K485 cells, which in fact implies a tumor suppressive effect rather than a tumor therapeutic effect. However, a more pronounced tumor therapeutic effect was found, when murine plasmacytoma cells (J558L) [34] were transfected with an expression vector containing the *IL-4* gene under the control of a promoter/enhancer derived from the mouse immunoglobulin heavy chain genes (transgenic plasmacytoma cell lines: J558L-I3L6 [33]; J558L-XEPIL4 [35]). While subcutaneous injection of non-transfected plasmacytoma cells in *nu/nu* mice as well as in BALB/c mice led to the formation of large tumors, the injection of the IL-4 recombinant plasmacytoma cell lines J558L-I3L6 or J558L-XEPIL4 never led to any tumor formation [33, 35].

Furthermore, a paracrine antitumorogenic effect of IL-4 was found, as a mixture of non-transfected plasmacytoma cells with IL-4 expressing plasmacytoma cells prevented *nu/nu* mice and also BALB/c mice from tumor formation. This paracrine-mediated tumor suppressive property of IL-4 was not limited to syngenic plasmacytoma cells but also transferable to a variety of other murine tumor cell types. Mixed inoculation of IL-4 transgenic plasmacytoma cells with SMF cells, a mammary adenocarcinoma line [36] or with A.6R.1 cells, an Abelson virus-transformed fibroblast cell line [37], prevented *nu/nu* mice from tumor formation, and mixture with B16-FO, a C57BL/6-derived melanoma [38] or the sarcoma 180 [39] markedly inhibited growth of the melanoma tumors or the sarcoma tumor, respectively [33]. These experiments suggest a general tumor suppressive effect of IL-4 on diverse tumor cells of epithelial and mesenchymal origin, which is mediated in a paracrine fashion.

In vivo experiments using an IL-4 transgenic cell line derived from a spontaneously arising renal cell carcinoma of BALB/c mice (Renca-IL-4C) exhibited similar local tumor suppressive effects on the parental Renca cells in BALB/c mice and *nu/nu* mice [40] as observed with the mammary adenocarcinoma and the plasmacytoma cell lines [33]. In all of these tumor models histological analyses revealed an influx of primarily macrophages and granulocytes and only few T cells at the site of mixed (non-transfected- and IL-4 expressing) tumor cell injection. This might reflect a mainly innate immune system mediated tumoricidal response. Interestingly, when non-transfected- and IL-4 expressing Renca-cells were inoculated at distant sites, a mainly CD8⁺ T cell-dependent systemic immune response was generated and was responsible for eradication of the renal tumor [40]. This systemic tumoricidal T cell response was blocked when CD8⁺ T cells were eliminated prior to injection of the parental Renca cells. Additionally, when the cured mice were injected with parental Renca cells, about 50% rejected the challenge [40], indicating some level of immune memory had been generated. In summary, the animal experiments of IL-4 expressing tumor cells show that the location at which IL-4 becomes synthesized determines the kind of tumoricidal immune response. If IL-4 is locally expressed within the tumor microenvironment, macrophages and granulocytes of the adaptive immune system mainly contribute to the tumor eradication whereas systemically synthesized IL-4 outside the tumor microenvironment mainly leads to an activation of CD8⁺ cytotoxic T (T_{cyt}) cells, which belong to the adaptive arm of the immune system. A further important finding of these data is that different immune cell types

including macrophages, granulocytes and/or T_{cyt} cells can mediate the cytokine-induced tumoricidal response. Notably, activation of cytotoxic cells of the adaptive immune system seems to generate a significant and, most important, a long lasting antitumor response.

One cytokine having the propensity to activate cytotoxic cells is the T cell-derived lymphokine IL-2. This lymphokine has been found to stimulate the proliferation of T_{cyt} cells [72], T_{H} cells [73] and natural killer (NK) cells [74] and is able to transform resting lymphocytes into lymphokine activated killer (LAK) cells [75, 76]. All these cell types are known to participate in the antitumor response which is the reason why IL-2 is the most frequently used cytokine in tumor cell-based ACT studies. Tumor cells transgenic for IL-2 were successfully applied in tumor models of fibrosarcoma, melanomas, myeloma, mammary carcinomas, carcinomas of the lung, colon and bladder, as well as renal and prostate cancers. The therapeutic benefit was mostly associated with high levels of cytotoxic cells. Immunohistochemical analyses by using antibodies directed against various leukocyte differentiation antigens (e.g. anti-CD4 detecting T_{H} cells, anti-CD8 for T_{cyt} cells, CD11b/CD18 for NK cells) revealed an enhanced presence of T cells [41], NK cells [41, 42], monocytes/macrophages and granulocytes [47] at the site of IL-2 transgenic tumor cell injection. The local presence of these cells suggests their effector role in antitumor immunity, but the distinct functional proof of the tumoricidal potency of distinct leukocyte subpopulations was provided by either depletion of defined immune cell populations *in vivo* or by the usage of animal strains, which genetically lack functional T cells (e.g. SCID mice, RAG-2 mice). For depletion experiments, antibodies able to kill a defined cell subpopulation were injected into the animal before application of IL-2 transgenic tumor cells. Depletion of $CD4^{+}$ —and $CD8^{+}$ T cells in the IL-2 CMS-5 fibrosarcoma mouse model did not bypass the IL-2 mediated rejection of the transgenic tumor cells suggesting that the protective immune response is not mediated by T cells [42]. The rejection of IL-2 transgenic CMS-5 fibrosarcoma cells in T cell deficient BALB/c *nu/nu* mice further supports the T cell independent antitumor immunity [42]. Additionally, the growth reduction of IL2 transgenic MBT2 bladder tumor cells in Swiss *nu/nu* mice also supports a T cell independent antitumorigenic effect of IL-2, at least when expressed at higher concentrations [52]. Nevertheless, IL-2-activated T cells exhibit a pivotal role in the establishment and long-lasting protection against tumor reimplantation. In various tumor models it was found, that once animals were cured from the tumor, an immunological protection in the sense of a vaccination developed in most cases, which protected animals of the reimplantation of the tumor [45, 48, 50, 54]. This protection was only possible, when the animals possessed functional T cells and the protection was associated with the generation of lytic cells such as LAK cells [41–43, 46, 47, 49, 52, 56].

The most promising observation of the IL-2-based ACT using transgenic tumor cells is that this approach was also successful in the therapeutic treatment of wild-type tumors [41, 47, 48, 52, 54, 56] which reflects the clinical situation in human cancer treatment. Other cytokines were cloned and transfected in tumor cells, from which IL-4, IL-6, IL-7 and granulocyte macrophage colony stimulating

factor (GM-CSF) showed some and interferon (IFN)- γ highly effective therapeutic properties (Table 9.1). In addition, the combined transfer of two recombinant cytokines into the same tumor cell may enhance the antitumor immunity [44, 54].

In summary, the findings raised by the various animal tumor models of ACT of cytokine-transfected tumor cell lines revealed that, depending on the cytokine or cytokine-mix expressed, an effective tumoricidal response can be induced by activation of nonspecific cytotoxic effector cells and/or tumor-specific T cells. Furthermore, for the acquisition of a long-lasting antitumor immunity, the activation of CD8⁺ T cells is essential. The central role of tumor-specific lymphocytes of the adaptive immune system in the tumoricidal immune response further supports the existence of a specialized APC system which enables/enhances the presentation of TAAs or TSAs after the ACT of living or irradiated transgenic tumor cells. This led to the concept to transfer MHC class I genes or genetic information of TAAs and TSAs into transferable cells. For the gene transfer of such factors, cells of the tumor stroma such as fibroblasts or DCs are most applicable, because both cell types are known to function as efficient APCs and long lasting presentation of translated TAAs via the MHC class I complex seems most likely. Furthermore, the danger that living auto- or allogenic transferred tumor cells could spread and metastasize within the patient can be avoided.

9.5 Antigen Presenting Cells Transgenic for Tumor Antigens Used in Adoptive Cell Transfer

Based on genetic alterations neoplastic cells express specific antigens which are either present only on tumor cells—in case of TSAs—or on both tumor cells and also some normal cells—in case of TAAs—within the tumor microenvironment. The presentation of these antigens together with MHC class I molecules to effector T cells has been found to be a critical step in the generation of an efficient T_{cyt} cell-dependent response against the tumor. Insufficient MHC class I-dependent presentation of TSAs/TAAs by either tumor cells or host professional APCs can be a basic cause for a failure of the immune response in the tumor bearing host, even when TSAs or TAAs are expressed [77]. However, within the scope of immune evasion most neoplastic cells down regulate their expression of MHC class I molecules and, thereby, reduce the level of MHC class I-mediated activation of T_{cyt} cells [78–80]. For example, human papilloma viruses (HPVs) express the oncoprotein E5 which is implicated in MHC-class I downregulation [81, 82] and was shown to affect MHC class II maturation in IFN- γ -treated keratinocytes [83]. The therapeutic stimulation of MHC class I expression within the tumor entity via application of recombinant cytokines known to enhance the MHC expression such as IFN- γ [84] is one possibility to overcome this status. In fact, the use of IFN- γ transgenic tumor cells in ACT strategies, as listed above, is a proven possibility to elicit an efficient antitumor response mediated by TILs.

Recent strategies to induce or augment the host antitumor immune response included the transfer of genes encoding MHC class I, costimulatory molecules or cytokines, and TAAs into tumor cells and APCs. A functional proof of the correlation between MHC class I expression, generation of cytotoxic cells within the tumor microenvironment and the clearance of existing metastases by adoptive immunotherapy was given by Restifo and coworker [85]. They showed that transfection of methylcholanthrene (MCA)-induced sarcoma cells with recombinant IFN- γ led to enhanced MHC class I expression on the surface of the tumor cell line which converted it from poor presenter of antigen to high antigen presenter cells. Tumors derived from high presenter cells made it possible to isolate and clone CD8⁺ TILs, which, when transferred *in vivo*, revealed a tumoricidal response against present metastases from the wild-type MCA-induced sarcoma [85]. These data prove that the presence of a sufficient level of MHC class I and, thus, it is appropriate to assume that an enhanced presentation of TSAs/TAAs is required to achieve therapeutic effects.

Many other animal experimental models used the strategy of enhancing the presentation of TSAs/TAAs by ACT strategies. Because tumor cells are genetically instable [86] and thus cannot be stably transfected with viral vectors, the usage of cells which are known to be efficient in antigen presentation came into the focus of cancer immunotherapies. However, this requires the identification of therapeutic relevant TSAs/TAAs for a given type of tumor, which is indeed, one of the major challenges in tumor medicine. Various methods, such as differential gene analysis, exome sequencing and proteomics are actually used methods to identify genes, peptides or proteins specifically expressed in neoplastic cells or solid tumors and, therefore, possibly they are usable as prognostic or therapeutic factors. Promising experimental results have been observed when fibroblasts and DC were used to transfer TSAs/TAAs into the tumor. To deliver or express TSAs/TAAs into APCs to this day cells were pulsed with unfractionated tumor-derived peptides [87], tumor cell lysates [88], apoptotic cell bodies [89, 90] and mRNA [91, 92] or cDNA libraries [93, 94] derived from tumor cells. Fibroblasts are readily available to be cultured, transfected and selected, and were found to produce physiologically relevant levels of cytokines after the introduction of cytokine genes [95–97]. Furthermore, these cell types can provide a useful manipulation of key aspects of antigen presentation, such as epitope choice, antigen density, and selection of immune-stimulating molecules, to promote the induction of potent cytotoxic T lymphocyte responses.

One sophisticated strategy of using fibroblasts as APCs of TSAs/TAAs is to generate double or triple transgenic fibroblast cell lines which possess transgenic vectors expressing MHC class I molecules, a stimulatory cytokine and either one or more TSAs or TAAs previously identified to be specific for the corresponding tumor entity. Using the murine model of highly malignant SB5b breast carcinoma, Cohen and coworker genetically engineered a fibroblast cell line (LM cells) of C3H/He (H-2^k) mouse origin which expressed an allogenic (H-2^d) MHC class I determinant, the immune stimulatory cytokine IL-2, and a cDNA library derived from a small pool of SB5b breast cancer cells [93, 94]. To enrich the pool of tumor DNA-transgenic cells with those cells, which synthesize tumor relevant antigens in association with

MHC class I, the whole transfected LM cell pool was subdivided in several sub-pools, which were tested on their potency to induce a cytotoxic response against SB5b cells *in vitro*. Only pools of high responders were used for a second round of this immunoselection and the most effective sub-pool of these triple transfected cells was used for ACT against the breast cancer cells. As shown, by further *in vitro* and *in vivo* testing, the tumoricidal response of triple transgenic high responder fibroblasts was mediated by the activation of CD8⁺ T_{cyt} cells [94]. The immunization of breast cancer bearing mice with these cells had a therapeutic effect and led to eradication of the SB5b tumors in some animals and prolonged survival in others [98]. Furthermore, an immunological long-term immunity against the tumor cells had developed in the cured mice, protecting the animals from re-transplantation of tumor cells [93]. In the related model of intracerebral metastatic breast cancer such triple transgenic cells were also effective in eradication of the intracerebral SB5b metastases [99] suggesting that the tumoricidal immune protection was independent from the organic location of the tumor cells.

9.6 T Cells Used in Adoptive Cell Transfer

The most recent transgenic cell-based ACT strategy is the usage of genetically modified TILs. TILs have been found to serve as a good prognostic marker for many human tumor entities [100–107]. Isolation of TILs from tumor tissues with subsequent *in vitro* stimulation, expansion of tumor specific T cells and transfer back into the patient is hence a promising therapeutic approach. Until now, TIL populations that become therapeutically effective after *in vitro* stimulation were primarily isolated from melanomas thereby limiting the therapeutic usage of TILs to this tumor type. Nevertheless, beside TILs, *in vitro* modified T cells genetically redirected to recognize TSAs/TAAs on the surfaces of tumor cells are powerful therapeutic tools that can be used against virtually all types of tumors [108–113]. For the generation of T cells, expressing TCRs with a high affinity and specificity for TAAs, various techniques are currently available that are reviewed in detail by Restifo and colleagues [114]. A novel approach, termed “Chimeric Antigen Receptor” (CAR) therapy, genetically engrafts the gene sequence encoding the variable region of a target cell-specific antibody onto the TCR intracellular domain that is capable of activating T cells. The resulting transgenic T cells then become activated *in vivo* when the CAR binds to the tumor target antigen, which can take place independent from MHC class I or II. It is also possible to isolate TCR RNA from humanized mice, which bear T cells transgenic for human MHC class I. The immunization of such mice with human tumor antigens results in the generation of T cells specific for human MHC class I-restricted tumor antigens. As a third method, T cells isolated from a patient, found to be a high responder against a defined tumor type, are used as a genetic source to transfect autologous T cells of low responder patients. Numerous clinical trials using T cell based ACTs are on their way and the plethora of individual treatment schemes makes it nearly impossible to identify

critical parameters such as preconditioning treatment and therapeutic parameters like cell dose and differentiation phenotypes of the T cell. Similarly, the impact of other factors such as vaccination against the tumor or cytokine delivery in parallel to the T cell transfer remains unclear. For systematical characterization of such critical therapeutic conditions, animal studies are indispensable.

One animal model that has been extensively used to define some of the above mentioned critical key determinants for successful ACT immunotherapy is the Pmel-1 CD8⁺ T cell receptor transgenic mouse model [115]. One animal model that has been extensively used to define some of the above mentioned critical key determinants for successful ACT immunotherapy is the Pmel-1 CD8⁺ T cell receptor transgenic mouse model. This animal model uses the mouse melanoma B16 cell line to induce solid melanomas, which are in concordance with human melanomas, since they share the melanocyte/melanoma (self/tumor) -antigen gp100 [116]. The shared self/tumor-antigen gp100 also known as Pmel can be used in both, human (Pmel-17) and mice (Pmel-1) as a target for T cell-based tumor treatment. To generate murine Pmel1-TCR-transgenic T cells for evaluation in the B16 C57BL/6 mouse model, splenocytes were isolated from Pmel1 transgenic mice and incubated *in vitro* in the presence of gp100 (human gp100₂₅₋₃₃) and recombinant IL-2 [117]. This priming led to the expansion of mainly Pmel1-TCR-transgenic T cells. For testing T cells which have reached a defined differentiation status, CD8⁺ T cells or CD8⁺ T memory (T_{SCM}) cells, CD8⁺ T central memory (T_{CM}) cells, or CD8⁺ T effector memory (T_{EM}) cells were isolated [118]. The rationale for testing different developmental stages of CD8⁺ T memory populations for ACT treatment is based on clinical and preclinical observations showing that the success of ACT-based approaches depends on the differentiation state of the transferred T cell population. Less differentiated T_{SCM} and T_{CM} were found to be more effective in tumor patients than more differentiated T_{EM} cells [119, 120].

Studies using the Pmel-1 mouse model observed a superior tumoricidal effect of T_{SCM} cells on the elimination of existing primary tumors [117]. However, Pmel1-TCR-transgenic CD8⁺ T cells were able to eliminate the tumor and enhance the survival rate of the animals (up to 100%), large numbers (1×10^7) of these cells were necessary to reach this therapeutic effect. When using low numbers of Pmel1-TCR-transgenic CD8⁺ T cells tumor eradication was incomplete. In contrast, the number of Pmel1-TCR-transgenic T_{SCM} cells necessary to reveal comparable tumor protection was only 1×10^4 . The antitumor efficacy of the more differentiated T memory cells decreased in correlation to a more differentiated status in the order T_{SCM} > T_{CM} > T_{EM}. These results show that next to the absolute number of adoptively transferred cells, the T cell differentiation status significantly contributes to the efficacy of the tumor therapy. The aspect of T cell differentiation has to be taken into account particularly within the context of the cell expansion time during the priming and expansion of T cells *in vitro* prior to ACT. Longer culturing time leads to a higher number of transferable T cells but the cellular differentiation status increases too, resulting in a less effective tumor-directed T cells [121]. A second critical parameter is the amount of *in vivo* applied antigen used for re-stimulation of the transferred cells. The Pmel-1 mouse model revealed a strong correlation between

the amount of vaccine and the therapeutic outcome [117]. This observation underlines the importance of identifying effective TSAs/TAAs to enhance clinical outcomes. Thirdly, recombinant cytokines were used to support the success of ACT. For example, in the Pmel-1 mouse escalating dosages of cytokines known to activate, expand, or promote the survival of T cells such as IL-2, IL-7, IL-15, and IL-21 were used in parallel to ACT of Pmel1-TCR-transgenic CD8⁺ T cells, demonstrating only moderate effects on the therapeutic outcome [117]. These results are challenging the requirement of cytokines in ACT treatment.

Targeting antibody therapies, unspecific cytokine/chemokine therapies as well as cell-based therapies are the three central pillars of modern oncoimmunology. The variety of possible treatment schemes makes it often difficult to recognize the central mode of action. On the other hand, the large number of paths that can be used to artificially influence immune responses gives us hope to develop highly effective immune-based anti-tumor strategies in the future. ACT therapies will help us to better understand basic immunological processes, including the role of various immune cell types in the antitumor inflammatory response.

No Conflict Statement No potential conflicts of interest were disclosed.

Acknowledgement Many thanks for helpful discussions to A. Bette and Prof. R. Mandic.

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