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Targeting of peripheral blood T Lymphocytes

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Introduction

New prospects for an immunologically based strategy of the treatment of cancer and infectious diseases are regularly highlighted in the preclinical and clinical literature. The hallmark of the immunologically based therapeutic strategies is the specificity for the cancer or virally infected tissues by virtue of the presence of specific receptors on populations of immune cells, e.g., T lymphocytes. Hence immune attack leaves normal or non-infected tissues intact. This idea goes back to the beginning of the twentieth century; in Germany, Paul Ehrlich put forward his hypothesis of "magic bullets" [30] and Von Leyden and Blumenthal initiated their studies aimed at active immunization of cancer patients against their own cancerous tissue. During the subsequent 6 decades, cancer patients were non-specifically immunostimulated using relatively crude leukocyte extracts such as transfer factor, immune RNA, or from bacterial extracts, e.g., bacillus Calmette-Guérin (BCG), levamisole, and Coley's toxin. In those days very little was known about the various components of the immune system that could react against cancer, and even less was known about tumor-associated antigens.

In the 1960s Burnett formulated the immuno-surveillance theory, highlighting that cell-mediated immunity played a critical role in the control of cancer and infectious diseases [18]. In order to discriminate between malignant cells or infected cells and normal tissues it was postulated that T cells during ontogeny learned to distinguish "self" (which should be left intact) from "non-self" (which should be eliminated). Hence, one of the major tasks of the host immune T cells was to continuously monitor whether in the host "self" became "altered self" due to, e.g., viral infection of cells or neoplastic transformation.

Recently, Ridge et al. [84] have proposed that discrimination between "self/non-self" is a life-long during process in which foreign molecules are also tolerant to the

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T cells, if the co-stimulatory signal (second signal) is not accompanied by a "danger" signal. Tumors, when they arise, do not represent imminent danger to their host, and hence the immune system may become tolerant to the tumor-associated antigen or, in other words, anergic. By the time tumors are life threatening, the tolerant immune system ignores the tumor, unless immune activation occurs by artificially targeting T cells to the tumor cells with the ultimate aim of breaking tolerance and possibly inducing systemic immunity with immunological memory. The important task of the immune system therefore is to guarantee the integrity of the internal environment of the host by detecting and eliminating potentially harmful, undesired alterations or infiltrations: immune surveillance.

Research during the past 30 years has led to the identification and characterization of a number of immune effector cells, such as natural killer (NK) cells, macrophages, and immune T and B cells, the latter producing antibodies following their maturation into plasma cells. The effector mechanisms range from cytolysis, which is the process of target cell destruction, to cytostasis, the latter being the growth arrest of target cells. The advent of new biotechnologies, such as the production of monoclonal antibodies (mAbs), in vitro expansion of immune T cells, gene cloning, and viral vector-mediated gene transfer to recipient cells, has allowed the identification of the individual molecules on immune effector cells and their target cells involved in immune reactions. The concerted, scaled-up use of these technologies allows us to specifically target genes of choice to either immune cells or target cells, aiming to repair genetic defects in cells and to cause their destruction or to redirect specificity of immune cells.

In this review we will first describe the T cell and target cell structures that are well known to play major roles in effector lymphocyte/target cell interactions and subsequently will focus our attention on the following targeting strategies: (1) production of bispecific mAbs (bs-mAbs) and single chain Fv(scFv)-mAb for targeting of T cells; (2) targeting of T cells with bs-mAb; (3) targeting of T cells by genetically engineering single chain Ig/γ or ζ receptors into T cells.

Cytotoxic T lymphocyte/target cell interactions

The physical interaction between effector cells, i.e., cytotoxic T lymphocytes (CTL) and target cells involves the establishment of multiple bonds between effector and target cells mediated by T cell receptors (TCRs) and adhesion molecules, which are both present on all CTLs.

TCRs and immunoglobulins

Antigen-specific T cells and CTL express TCRs on their membrane which specifically bind target cell antigen (Ag). Most T cells, including CTL, recognize and bind to "non-self" Ag. These Ags are bound to and presented by major histocompatibility complex (MHC) class I or II molecules on the surface of Ag-presenting cells (e.g., dendritic cells) or target cells by the effector cells. The TCR initiates the recognition and binding of target cells. The TCR is a multi-component complex consisting of an immunoglobulin (Ig)-like Ag binding portion, the polymorphic $\alpha\beta$ or $\gamma\delta$ TCR, which is non-covalently linked to the non-polymorphic signal-transducing proteins, CD3/$\delta\epsilon$,

CD3/$\gamma\epsilon$, ζ and η. TCR recognize Ag in the context of MHC on the target cells, whilst Ab molecules, i.e., Igs, recognize soluble Ag. In the B cell receptor complex, CD79 functions as the analogue of CD3 for TCR. Nevertheless TCR and Ig share many structural characteristics, e.g., both molecules are disulfate-linked heterodimers or tetradimers, TCR and Ig genes are assembled in an identical way, and both contain an invariant constant (C) region and an Ag-binding variable (V) region [21, 25]. The Ag diversity of the TCR and Ig, the hallmark of the immune system, is created by similar processes of genetic rearrangements involving the variable (V), diversity (D, and for Ig heavy chain, H), and joining (J) gene segments. Only in the case of Ig do somatic mutations add to Ag diversity. In short the V,D, and J segments are combined to form the Ag-binding domains. For Ig-mediated effector functions, the Ig C region interacts with complement and Fc receptors on monocytes, NK cells, and some T cells. In contrast, the C region of the TCR contains a transmembrane structure anchored to the T cell surface, which takes part in the signalling process via the CD3 complex following Ag recognition by the TCR. The fashion and form in which proteins fold are critical for their biological function, and for Ig and TCR, both members of the Ig supergene family, the foldings are similar.

Adhesion and co-activation molecules on T cells

TCR and CD28 each have counter-regulatory molecules which are expressed on the T cell membrane, for TCR the NK receptor gene family [81] and for CD28 the CTLA-4 [55, 69, 92]. These counter-regulatory molecules, which only become expressed on activated T cells, deliver a negative signal to T cells, and the immune response returns to the baseline state: self-limiting immune response. TCRs which recognize antigens without a co-stimulatory signal will become anergic or tolerant. The best-characterized second signalling molecule thus far is CD28 [69, 92]. The intrinsic binding affinities of TCRs for Ag are low to moderate in comparison to Ab, and the number of MHC peptides per target cell that form complexes with the TCR is also low [109]. Nevertheless, even the low numbers of TCR/Ag complexes formed following Ag recognition by the TCR induce a rapid increase in affinity of the adhesion molecules, e.g., lymphocyte function-associated antigen-1 (LFA-1) for its target cell ligand intercellular adhesion molecule-1 (ICAM-1). The increase in affinity does not require an increase in LFA-1 cell surface density on the T cells [11]. CD2 on T cells and ICAM-3 on the target cells are also involved in the adhesion processes [14, 38]. Consequently, where no foreign Ag is expressed on normal cells, turning dangerous "self" into "non-self", T cells with dangerous "non-self" recognizing TCRs will not bind to any "self" and continue their immunosurveillance.

A second group of accessory molecules are the very late Ag (VLA), a sub-family of integrins (VLA 3, 4, 5, and 6]. These molecules recognize collagen, fibronectin, and laminin, which are present in the extracellular matrix of target cells [102, 114]. Thirdly, CD44, which also recognizes extracellular matrix proteins such as glycosaminoglycans (e.g., hyaluronate and chondroitin sulfate), does not belong to the integrin family [24, 67]. The extracellular matrix proteins are critical in guiding the trafficking of immune cells to particular organs and most likely influence the maturation process of T cells as they interact with the receptors on the T cells [114].

Several of the adhesion molecules, such as CD2, can also serve as signal-transducing receptors themselves, provided they become crosslinked [10, 37]. Com-

binations of mAbs against distinct signalling molecules (e.g., CD2 and CD3 or CD16 etc.) critically enhance the T lymphocyte activation process [9, 13, 110, 111]. Other molecules that can serve as signal-transducing receptors are CD44 [34, 97], present on interleukin-2-activated NK cells but also on a small amount of $CD56^+$ T cells [93], and CD69, an early activation marker on T cells which serves as a cytotoxic triggering molecule on NK clones and $\gamma\delta$ T lymphocyte clones [76]. The CD8 and CD4 molecules on T cells which specifically associate with the same MHC class I or II molecule that present Ag also secure stabilization of the T cell/target cell interaction and enhance the signalling process.

In summary, crosslinking of multiple TCRs on individual effector cells with multiple Ags on the individual target cells, the occurrence of additional bonds between CD4 or CD8 cytotoxic T cells and MHC/Ag, and crosslinking between T lymphocyte adhesion molecules and target cell ligands, which all move towards the contact region between T cells and target cells, result in conjugate formation between T cells and target cells, stabilization of the cell conjugates, increase in overall TCR/Ag affinity, signal transduction through the CD3/$\epsilon\delta$ [15, 23, 26] and ϵ portions [8, 62, 66, 80, 90, 91, 98] (patch formation in the contact region), lymphokine production by T helper/inducer cells and CTL, and cytolysis of target cells by CTLs.

The CTL lytic mechanisms

CTL-induced target cell lysis is an apoptotic process. During CTL/target cell interaction aggregation of TCR, co-stimulatory molecules, and adhesion molecules takes place. The CTL microtubule organizing center arrives at the CTL-target cell contact region followed by an accumulation of cytoskeleton elements at the interaction site [36, 61]. The result is selective exocytosis of specialized cytolysins into the narrow gap between the interacting cells [45].

Perforins. The exocytosed granules contain perforin and a plethora of other enzymes including granzymes [108]. Perforin shows homology with complement proteins, in particular the pore-forming protein C9, and produces the lysis-inducing pores in the target cells [29, 51, 68, 99] (in the presence of calcium perforins form aggregates resulting in cylindrical structures that are inserted into the lipid bilayer of the target cell membrane). The result is apoptosis: the target cell chromatin condenses, its membrane blebs, its DNA breaks up into oligonucleosome-length DNA fragments, consequently the cells shrink, its endoplasmic reticulum dilates, and finally the target cells become fragmented: sealed membrane fragments (apoptotic bodies) are produced [4]. The susceptibility of the target cell to this apoptotic death is dependent on the stage of the mitotic cycle of the target cell: quiescent (G0) cells are refractory whereas G1 stage target cells are susceptible to CTL-induced DNA fragmentation [78, 89]. Consequently the physical association and aggregation at the contact region between CTL and target cells does not only stabilize the conjugate formation between the effector and target cells following initial TCR recognition, but also channels the exocytosed perforins and granzymes, thereby avoiding death of innocent healthy "bystander" cells which are adjacent to the aberrant target cells. The chance of innocent bystander target cell lysis is further decreased because of the short half life of cytolysins in serum.

In summary, the lytic protein perforin is a critical cytolysin in this cytocidal mechanism. Besides these natural serine proteases (perforins, granzymes), both of which are stored in unique secretory CTL granules [45, 82, 100], calcium is involved

in the degranulation process of the effector cell [29, 51]. The DNA breakdown, however, is caused by the granule proteases [45, 101].

Fas–Fas-ligand interactions. Recently it has been shown that CTL devoid of perforin could nevertheless lyze their target cells. These CTL showed a specific alloreactive CTL response in mixed lymphocyte cultures and protected mice in vivo against intraperitoneally administered tumor allografts [56, 57, 70, 116]. This mechanism is now known to involve Fas and Fas ligand (FasL), two cell surface proteins that are homologous to tumor necrosis factor (TNF) and TNFα receptor, respectively [52, 72]. When the FasL on the target cells is crosslinked by Fas on the effector T cells [72] or by Abs [107], the target cell is triggered into an apoptotic suicide mechanism.

In summary, potent and specific CTL-mediated lysis of target cells occurs by at least two distinct mechanisms: (1) the secretion of cytolysins and the perforin-mediated pathway cause direct pore-forming channels in target cells through which the lysis-inducing enzymes can enter the target cells, resulting in granule protease-induced DNA fragmentation, the process of target cell apoptosis; (2) the CTL Fas/target cell FasL-dependent pathway involves the triggering of a programmed disintegration of the CTL-bound target cell.

Anti-TCR mAb mimic TCR/Ag interactions

For T cell activation, Ag can be substituted by mAbs, provided they are specific for and hence bind to: the Ag-binding part of the polymorphic TCR$\alpha\beta$ (e.g., anti-TCR idiotypic Abs) or any of the non-polymorphic signalling structures of the TCR/CD3 complex, because otherwise only cell/cell conjugate formation will occur upon interaction with irrelevant target cells. Following crosslinking of the TCR complexes by these mAbs, T cells then become activated as if the TCRs were interacting with their natural target cell ligands, as described earlier [15, 23, 26]. Again, all these cell surface molecules co-aggregate in the contact region between T cell and target cell as if normal TCR/Ag interactions were taking place. This also explains why triggering through any of these signalling molecules have the same result: T lymphocyte activation and/or triggering of T cell-mediated target cell lysis.

Targeting of CTL by bs-mAbs or chimeric Ig-receptor genes

Cellular based therapies utilizing tumor-reactive T cells have been developed for patients with metastatic melanoma, resulting in significant tumor reduction in some patients [87, 88]. However, the generation or isolation of tumor-specific CTLs has proved extremely difficult, laborious, and time consuming, and hence has, as yet, not been exploited for immunotherapy. In contrast, a plethora of mAbs have been generated that recognize tumor-associated antigens common to certain cancers. To combine the ability of mAbs to selectively recognize tumor-associated antigens with the potent anti-tumor effector functions of T cells, and specifically CTLs, we and others designed bs-mAbs and chimeric Ig-receptor genes to retarget the specificity of T cells for tumor-associated antigens.

Production of bs-mAb and single chain Fv (scFv) Ab

The bs-mAbs can be produced by chemically coupling two types of mAbs with defined specificities [44]. This method is inefficient and unreliable because it yields unstable material. The hybrid-hybridoma technology has already proved to be a more sophisticated and reliable method [112], although this method also produces bs-mAbs with undesirable chain combinations, requiring the isolation of the relevant bs-mAbs.

The central concept of the bs-mAb approach for the retargeting of T lymphocyte specificity is that the bs-mAb with specificities for a particular tumor-associated antigen on target cells and for effector T cells crosslink the effector and target cells thereby triggering immune attack [112]. The availability of large-scale culture technologies for the production of human activated T cells combined with an array of mAbs specific for T lymphocyte activation molecules has allowed the application of the general concept of redirection of T lymphocyte specificity by means of adoptive transfer of bs-mAb redirected T cells to clinical treatment of cancer. It has now been clearly demonstrated that bs-mAbs specifically redirect CTLs of all specificities towards human and animal cancer cells inducing tumor cell lysis [94–96].

With the recent advances in antibody gene engineering [121], bispecific molecules can be made even more easily by the production of molecularly engineered versions, including scFv fragments provided with multimerization domains or tags, scFv2s, or dimerized and crosspaired scFv's, so called diabodies [46, 47, 58, 79]. In particular, the relative ease of producing diabodies [123], combined with their demonstrated efficacy in retargeting T cells to tumor cells [123], suggests that this format will provide a significant stimulus to the further development of therapeutic bispecific molecules. A second advantage of the molecular route is the speed with which many different bispecific molecules can be made and screened, allowing the tailoring of both affinity and specificity of the antibodies.

Immune responses to the bispecific antibody or the retargeted T cell can be reduced dramatically and sometimes avoided entirely by using genes encoding antibody fragments of human origin. These types of Abs are now readily available, mainly due to the advances made with the phage display technique. Phage display involves the expression of proteins on the surface of phage as fusion proteins attached to normal phage coat proteins. The physical link between the protein on the surface of the phage and the gene which encodes it within the phage allows a rapid evolution of protein properties on the basis of binding, by a series of repetitive cycles of phage binding, elution, and regrowth to amplify the selected phage population. One of the most successful applications of phage display has been the derivation of mAbs using large phage antibody libraries, and the subsequent improvement of the affinity of selected antibodies by their mutation and further selection [122]. Antigen-specific antibodies are selected from libraries of randomly paired combinations of variable domains of heavy and light chains (formatted as scFv [74], Fab [48], or diabody fragments), which are displayed on the surface of filamentous bacteriophage via fusion to the minor coat protein pIII. To generate human antibodies, the libraries are made from "naive" B cells from human donors [73], or from cloned human V gene segments [49]. A number of very large collections of human antibodies have been made, with over 10 billion different antibodies, allowing the isolation of antibodies to any chosen antigen and with a moderate to high affinity (10 nM–0.3 nM) [40, 115]. Human Fab or scFv fragments with tumor antigen binding characteristics can thus be isolated from

such libraries and their genes directly transfected into CTLs for retargeting to tumor cells.

bs-mAb-mediated T cell targeting: redirection of CTL specificity

We and others have exploited the phenomenon that mAbs, by crosslinking the relevant antigens, induce lymphocyte activation and trigger the lytic machinery of T cells by generation and use of bs-mAbs [112]. bs-mAbs recognize a T cell triggering molecule, e.g., CD3ζ, and a tumor-associated antigen cell surface molecule. Crosslinking of the signalling/adhesion molecules occurs when bs-mAb crosslink them to the tumor-associated antigens on tumor cells by virtue of their dual specificity: bs-mAb recognize, e.g. CD3-ζ on T cells on the one hand and tumor-associated antigens on the target cells on the other. The same CD3-ζ is expressed on all T cells, irrespective of their antigen specificity, all T cells become activated and all activated CTL are triggered to lyze the tumor-associated antigen-expressing target cell.

Lymphokine production by bs-mAb-retargeted T cells

From in vivo studies aimed at tumor neutralization in nude mice it appeared that much lower numbers of effector cells were required to inhibit tumor growth in vivo than required to induce lysis in vitro [106], and that supernatants obtained from peripheral blood lymphocytes inhibited growth of tumor cells which themselves were not recognized by the Ab-targeted T cells. Hence, the inhibition of "bystander" tumor cell growth did not require effector cell/target cell contact. That lymphokine production induced by lymphocyte triggering molecules was indeed responsible for the inhibition of tumor growth was demonstrated by the addition of cytokine-specific mAbs, particularly anti-TNFα and anti-interferon-γ mAbs, which abrogated the tumor growth inhibitory activity [28]. A number of other lymphokines are also triggered by crosslinking of activation molecules, in particular inflammatory lymphokines that can recruit and act on other cellular components of the immune system and induce homing of immune cells to target cells.

This site-specific induction of the immune reactions has great advantage over the systemic administration of cytokines such as interferon-α, TNF-α or interleukin-2, and others, because these highly active biomolecules have pleiotropic effects on many different cell types, and hence may cause unwanted toxic effects in the patient. Moreover, generally these lymphokines have a short half-life, function at a short range, and interact with specific cell surface receptors. These interactions trigger the delivery of signals to target cells which affect their gene expression, proliferation and differentiation, and ultimately cause their death (apoptosis); the effect(s) occur depending on their degree of differentiation and maturation. Site-specific immune reactivity avoids systemic toxic effects of cytokines by virtue of specific trafficking and homing of immune T cells to the target site by retargeting T cell specificity with bs-mAb or scFv chimeric receptors.

In summary, redirection of T cell specificity results in cytokine-triggered inflammatory reactions at the target site, mobilizing the different effector arms of the immune system. The immune activation may result in systemic, specific humoral and cellular immune responses against the tumor and tumor regression by CTLs.

Immunotherapy

Animal models

Now that in vitro studies have proved that bs-mAbs efficiently redirect T lymphocyte specificity towards tumor cells (or virus-infected cells) and subsequently trigger cytokine production and cytolysis of the target cells, suitable animal models have been developed to study their in vivo functions. Two models have been developed (1) a xenograft model in which human tumors were transplanted into immunodeficient nude mice, followed by treatment of the tumor xenograft by bs-mAb-targeted human T cells; (2) a syngeneic model in which murine bs-mAbs, tumors, and T cells are used.

Early studies involved the treatment of syngeneic lymphoma with activated host-derived T cells and bs-mAbs directed against Vβ8 TCR and Thy1.1. on the lymphoma cells, and showed tumor protection [15, 16, 26–28, 117–119]. bs-mAb-mediated therapy was also successful in syngeneic solid tumor animal models [5, 118]. In the xenograft studies CTLs, bs-mAbs, and tumor cells were mixed and injected subcutaneously into nude mice: a Winn-type assay [106]. The anti-cancer effect involved direct lyzing of the tumor cells [106], as well as tumor growth inhibition by cytokines released by these activated T cells [83]. Even established ovarian carcinoma xenografts growing intraperitoneally in nude mice could be successfully treated with bs-mAbs (OCTR) targeted to CD3 on the T cells on the one hand and to a human folate binding protein (MOV-18), which is overexpressed in ovarian carcinoma cells on the other hand [19, 35]. Also, human T cells prolonged survival in this model system [75, 113]. Subsequently, syngeneic models [1, 3] were developed to avoid irrelevant activation conditions obviously occurring in a xenogeneic system due to incompatibilities between donor and host adhesion molecules and their ligands. Such incompatibilities can result in abnormal trafficking of the human T cells in animals. These studies served as preclinical models for the design of patient treatment protocols.

Clinical studies

The first international multicenter study involved locoregional treatment of ovarian carcinoma cells in patients with advanced disease [12, 20]. The high frequency of relapse of advanced ovarian carcinoma after induction chemotherapy called for a new therapeutic strategy. Prior to entry into the study, laparotomy was performed to reduce tumor load and to indicate remaining macroscopical lesions. We treated 28 patients with two 5-day cycles of treatment, consisting of daily intraperitoneal injections of autologous activated T cells, which had been expanded in vitro following activation with phytohemagglutinin and addition of exogenous interleukin-2 for each of the ten infusions and retargeted with the bs-mAb OC/TR specific for CD3 and for the folate receptor overexpressed by ovarian carcinoma cells. T cells [10^9] were incubated with 1 mg bs-mAb. The patients received additional infusions of interleukin-2 and bs-mAb after each administration of targeted T cells to maintain the lytic activity of the retargeted T lymphocytes; 6–10 weeks following treatment those patients without signs of tumor progression underwent explorative laparotomy for objective assessment of tumor response by comparing remaining tumor with that documented by pretreatment

laparotomy. The overall intraperitoneal response rate was 27%. Complete responses were seen in 3 patients, and lasted 26 months, 23 months, and 18 months, respectively. Toxicity of the treatment was mild, WHO grade II.

Of 24 patients evaluated, 23 developed a human anti-mouse antibody (HAMA) response [20]. In vitro studies showed that although during treatment cycles the levels of HAMA increased with time, HAMA did not block the bs-mAb-mediated triggering of cytolysis and cytokine production by the patient's T cells until day 40 following the first administration of bs-mAb, i.e., after the 2 cycles of treatment were completed [64]. Other pilot studies that followed reported the treatment of patients with malignant ascites or pleural effusions resulting from colon, mammary, ovarian, lung, and gastric carcinomas. These patients were treated locally with ex vivo activated autologous cells and an anti-CD3-based bs-mAb. Again, the bs-mAb-targeted T cells exerted strong local biological effects within hours: conjugates between tumor cells and T cells were identified with a decrease in tumor cell number and in serum levels of carcino-embryonic antigen. Elevated levels of cytokines and an increase in granulocyte numbers were also observed [59].

The anti-cancer effects obtained so far with bs-mAb-retargeted T lymphocytes have been locoregional. Systemic administration of bs-mAb-retargeted activated T cells is expected to be more effective for systemic anti-cancer effects, as demonstrated by mouse studies. There is only limited experience of systemic treatment with bs-mAb. One ovarian cancer patient treated with intravenous infusion of 1 mg of the bs-mAb used for the locoregional treatment suffered severe toxicity starting 30 min after administration [105], and other patients showed similar toxicity (Bolis, Milan; personal communication). The patients developed chills, headache, fever in combination with hypertension, and fatigue, although all symptoms resolved after treatment was discontinued. The toxicity was most likely due to an increase in serum TNFα levels. Other studies revealed extravasation of T cells following intravenous injection of bs-mAb and a subsequent rise in TNF-α [54, 60], probably due to crosslinking of the bs-mAb-targeted TCR, following binding to tumor cell antigens or crossreactive normal tissue.

In conclusion, the clinical studies involving bs-mAb-targeted T cells have shown "proof of principle," and have yielded encouraging results, even in the locoregional therapy of advanced stages of disease with tumors already resistant to standard treatment regimens [20]. Therefore this approach deserves further testing, focusing on how toxicity following systemic administration can be avoided. The most appropriate setting for this bs-mAb-targeted T lymphocyte approach is ultimately expected to be the treatment of minimal residual disease following standard therapy. In such an adjuvant setting the bs-mAb-targeted T cells are expected to effectively attack and destroy circulating tumor cells and small metastases.

Immuno-gene-therapy

Retargeting of T lymphocyte specificity with chimeric receptor genes encoding Ab-type specificity

The use of bs-mAb for therapy may be hampered by the inaccessibility of solid tumors to antibody penetration [53]. Moreover, bs-mAb-redirected CTL retain the bispecific antibody for only limited periods (i.e., 48–96 h) due to dissociation from the CTL

surface [7, 65]. In addition, bs-mAb-redirected CTL lose signal transduction and lytic capacity following target cell recognition, lysis, and TCR/CD3 complex clustering [7].

Because systemic eradication of tumors requires long-lasting, systemic immune responses, molecular engineering of chimeric receptors with Ab-dictated specificity into T cells or other immune cells may result in such a sustained immune response. Because each immune cell population shows specific patterns of cytokine production, e.g., T helper 1 versus T helper 2 cells, NK cells, antigen-presenting dendritic cells, etc., each population can trigger different immunological responses and therapeutic outcomes [86, 104]. The future gene retargeting of the specificity of these individual populations will allow further fine-tuning of immuno-gene-therapy strategies.

To this end we and others have adopted an approach in which T cells are "gene grafted" with a permanent antibody-dictated specificity [31, 103]. This molecular approach to graft T cells with a predetermined antibody specificity exploits the already described structural similarity between Ab and TCR molecules. It is possible to construct chimeric genes in which the Ab recognition unit has been used in the form of a single-chain Fv (scFv)-encoding gene, to create hybrid molecules in which the signalling subunits are molecules such as the ζ-chain derived from the CD3 complex or the γ-chain from the high-affinity receptor for IgE Fcϵ-RI. In another approach the TCR variable region is replaced by an Ab variable region in a chimeric TCR gene. The variable domains of mAb fused by a flexible linker sequence have been shown to display similar binding affinities and specificities compared with those of the natural mAb [6, 22, 120].

To transfect genes to primary T cells, retroviral vectors have been adapted for the delivery of foreign genes to primary human cells [2, 16, 32, 33, 39, 41–43, 63, 71, 85]. Chimeric receptors are then cloned into these retroviral vectors, which transduce the chimeric genes into, e.g., T cells where they become stably integrated in the cellular DNA. The integration of the chimeric receptor encoding gene in the cellular DNA results in synthesis of the chimeric proteins and expression on the lymphocyte surface. The original studies used the two-chain receptor approach, with V_H and V_L linked to the α and β chains of the TCR [2, 41, 42, 63], followed by the single chain receptor (scFv) approaches, which allowed the expression of full Ab specificity on a single receptor molecule. Various anti-tumor mAbs have been engineered into a single-chain format and expressed on the surface of the CTL [50]. Single-chain antibodies (scFv) juxtaposed to a signal-transducing molecule, like the Fc(ϵ)RI γ or TCR ζ chain, have now been functionally expressed in mouse T cell hybridomas or CTL [17, 31, 77, 103], tumor-infiltrating lymphocytes, and human $CD8^+$ T cells [50, 85].

Conclusions

In summary, human single-chain gene transduced T cells were shown: to express the scFv on their surface, to recognize their relevant ligand (tumor-associated antigen) on tumor target cells, to produce cytokines and, to lyze tumor cells. In our earlier review on retargeting T lymphocyte specificity [11], we concluded that a number of questions needed to be answered: (1) Is triggering of cytolysis by CTL or lymphokine production most important to generate anti-cancer effects? Both are important, especially to eliminate bystander cells which do not express tumor-associated antigen [35, 75, 83, 106]. (2) Can targeted CTL traffick and home to the tumor site? Yes, they can.

(3) Does "humanization" of mouse mAbs reduce HAMA responses? Our preliminary experiences suggest that this is the case (unpublished data).

Significant progress has therefore been made in the laboratory and in clinical tests, and will continue to be made. We are now preparing for the clinical phase I/II testing of in vivo anti-tumor activity of T lymphocytes retargeted by transfer of chimeric receptor genes encoding Ab-type specificity.

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