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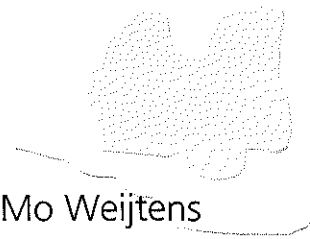
chimeric receptor-mediated lysis of tumor cells



Mo Weijtens

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Immuno-gentherapie voor nierkanker

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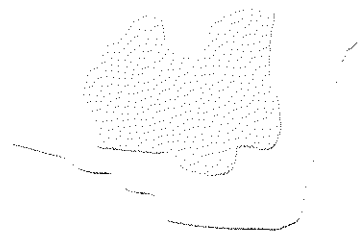
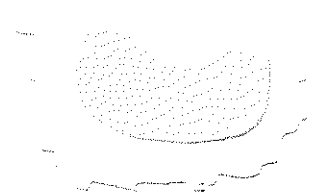


Introduction



INTRODUCTION

The immune system serves as a protective system against infectious agents such as bacteria, viruses and parasites. Foreign molecules (antigens) can be recognized by the immune system and induce an immune response resulting in destruction and elimination of the pathogens. In addition to infectious agents, also tumor cells can distinguish themselves from normal cells either by quantitative and/or qualitative different expression of genetic information. This idea forms the basis of cancer immunotherapy, which aims at the elimination of tumor cells using the patient's natural immune system. The concept of tumor-specific immune reactivity goes back to the postulation of immunesurveillance as formulated by Ehrlich in 1909 and adapted by Burnet in 1970. They proposed that the immune system might be capable of eliminating tumor cells in the same way that protects us from foreign agents, i.e. immunosurveillance. Although there might be immunesurveillance, this mechanism is not dominant, as tumor cells may escape the immune system resulting in uncontrolled cell proliferation and tumor formation. Therefore, effective anti-cancer immunotherapy should be directed at the manipulation and enhancement of the immune capacity to fight cancer cells. This can be accomplished either in an active way, by activating and amplifying the destructive potential of the individual's endogenous immune cells able to recognize and destroy the tumor, or in a passive way by providing ex vivo activated immune cells with anti-tumor capacity to a tumor-bearing individual.

The first known immunologically based treatment of cancer patients was performed at the end of the 19th century by Coley, who tried to induce an immune reaction against the tumor with bacterial extracts. This treatment resulted in tumor regression but with inconsistent results. Year's later, bacterial vaccines such as *Bacillus* Calmette-Guerin (BCG) or *Corynebacterium parvum* (C. parvum) were again used as immuno-modulating agents to stimulate cellular and humoral immune reactivity. Other than in melanoma and bladder cancer, treatment with bacterial products was not very successful (1). This non-specific approach was followed by the use of cytokines to enhance the immune system. The use of cytokines in cancer treatment has been promising in experimental animal models but clinical results were disappointing mostly due to toxicity (2-4).

When developments in the field of immunology and molecular biology allowed the production of large amounts of biological molecules (cytokines, monoclonal antibodies (mAb)) necessary to culture immune cells *in vitro*, the isolation, cloning, expansion and adoptive transfer of immune effector cells became possible (5-8). Together with the understanding of the nature of antigen recognition and the identification of tumor antigens it became possible to develop specific anti-tumor therapies.

This introduction addresses basis principles of tumor immunology with focus on the current developments in immunotherapeutical approaches for cancer treatment.

Effector mechanisms of the immune system

The immune system can be divided into an innate and adaptive arm that together provides an effective defense system. Mechanisms of non-antigen specific innate immunity serve as the first line of defense. The innate immune system reacts directly to a pathogen by the activation of complement and the destruction of pathogens by neurophils and macrophages. An important role for the innate immune system is to recruit more phagocytic cells and effector molecules on the site of infection by the release of cytokines. When pathogens escape the innate immune system an adaptive immune response will follow with the formation of antigen-specific effector cells and immunological memory. Cells of the adaptive immune system bear specific receptors for recognition of antigens. The adaptive immune system can be further subdivided into the humoral immune response involving B lymphocytes that produces soluble antibodies (Ab) and the cellular immune response comprising cytotoxic effector cells and helper T cells.

Antibodies are the secreted form of the B cell receptor for antigen and circulate in the blood. A single clone of B lymphocytes produces monoclonal antibodies (mAb). Each Ab is composed of a variable (V) and a constant (C) region. The variable region is unique for each antibody and primarily responsible for antigen recognition and contains subregions (complementarity determining regions: CDR) that are in contact with the antigen. The constant region encodes the isotype of the antibody and thus their biological function and location (9). Ab molecules can specifically bind to pathogens and thereby block their access to cells or trigger the complement cascade by activating proteins of the complement system (10). This may eventually result in lysis of the target cell by forming pores in their membrane. Another mechanism by which antibodies mediate target cell lysis is antibody-dependent cell-mediated cytotoxicity (ADCC) by monocytes, natural killer (NK) cells and mast cells (11-13). Via binding of the Ab to the Fc receptor the effector cells are activated and target cells can be killed by phagocytosis and through the release of toxic granule components.

The cellular immune response comprises different types of effector cells. NK cells and γδ T cells provide early protection without the generation of lasting immunity. NK cells are a population of lymphocytes that was first identified by their capacity to kill cells without prior immunization or activation (14, 15). They secrete cytokines and mediate ADCC after binding of an antibody-antigen complex to the CD16 receptor indicating that this structure is involved in triggering of the cytolytic machinery (16, 17). In addition, they demonstrate major histocompatibility complex (MHC)-unrestricted killing of tumor and virally infected cells (18, 19). Their target cell selectivity is mediated by the use of varying combinations of different receptors such as CD2 and NKR-P1 for recognition and cell activation and NK receptors (NKR) that mediate an inhibitory function (20-22). T cells bearing γδ T cell receptors (TCR) represent 3-10% of the total T cell population (23, 24). These γδ T cells are mostly found in surface epithelia and mediate non-MHC-restricted cytolysis (25-27). Like NK cells, γδ T cells express CD16 that serves as an activation

site for triggering cytolytic activity (28) and natural killer inhibitory receptors suggesting that they play a role in tumor defense similar to that suggested for NK cells (29).

The major components of the cellular immune system are the αβ T lymphocytes. Via the αβ TCR these T lymphocytes recognize specific antigens that are processed and presented by MHC molecules. T lymphocytes can be divided into 2 groups, $CD4^+$ T helper and $CD8^+$ cytotoxic T lymphocytes (CTL) recognizing Ag in the context of MHC class II and I respectively. The αβ TCR is expressed on the T lymphocytes in association with the signaltransducing CD3 complex: CD3/δε, CD3/γε, ζ and η-chains (30). The α and β polypeptides contain a variable, highly specific antigen binding region (V) and a constant region (C) that functions in transferring signals to the CD3 complex proteins that have cytoplasmic extensions which allow them to interact with signal-transducing molecules. Other molecules on the αβ T lymphocytes serve as important interaction structures. For activation of primary T lymphocytes is, besides the specific signal delivered through the TCR, a second signal required. This signal can be provided by interaction of the CD28 on the T lymphocytes with CD80 on professional antigen-presenting cells (APC) (31, 32). Furthermore, interaction of adhesion and accessory molecules such as CD4, CD8, CD2 and CD11a/CD18 on the T lymphocytes with their ligands on the target cells, i.e., MHC Class II and I, CD58 and CD54, respectively contribute to T lymphocyte activation (33). In addition to an adhesion function, the CD8, CD2 and CD11a/CD18 receptors play an important role in T lymphocyte activation as costimulatory molecules. T lymphocytes can be activated by cross-linking of CD2, CD3 or CD11a/CD18 molecules without requiring specific TCR/CD3 interaction with MHC-peptide complex (16, 20, 34-38). Adhesion of T lymphocytes to target cells, specific TCR recognition of antigen in the context of MHC and costimulation via accessory molecules together results in CD3 signaling, activation of T lymphocytes and subsequent lysis of target cells by CTL and cytokine production by $CD4^+$ T helper cells.

Immunogenicity of tumors

Essential in the application of immunotherapy of cancer is that tumors must be recognized by the immune system. This can only be achieved when a human tumor is immunogenic. Tumor immunogenicity is based on the fundamental observations from animal studies that tumor cells express antigens that are qualitative and/or quantitative different from normal cells. Specific immune recognition and rejection was first demonstrated in mouse experiments in which T cells recognize and reject secondary tumors after surgical removal of the primary tumor. These T cells acquired tumor-specific memory which was demonstrated by the transfer of protection from one animal to another by T lymphocytes (39-41). Later, specific CTLs against tumor antigens have been isolated and adoptive transfer of anti-tumor CTL clones was found to eradicate tumor cells in animals bearing large tumors (42).

These findings led to the idea that individual tumor cells express tumor antigens. The identification of tumor antigens was made possible both by mAbs to identify tumor specific antigens and by the isolation and generation of T lymphocyte lines and clones that recognized MHC restricted tumor antigens. Already a large number of tumor antigens which elicit humoral and cellular reactions in tumor bearing hosts have been cloned and characterized (43, 44).

Tumor associated antigens (TAA) can be classified into different groups by their expression pattern, which determine their suitability for immunotherapy:

Tumor antigen with tumor-specific expression from genes that are expressed on tumor cells but are silent in normal adult tissues. These genes include the MAGE family (45) which antigenic peptides are expressed on a large number of different tumor types (46). As the antigenic peptides are shared by tumors expressing the appropriate MHC type, it makes them promising targets for immunotherapy. The same holds true for a group of specific tumor antigens that evolved from oncogenic viruses. Viral proteins have been associated with human tumors and tumor-specific CTL can be generated in vitro (47).

Tumor antigens from ubiquitous expressed genes that contain point mutations. Expression of these antigens is restricted to a unique mutation event and therefore specific for the individual tumor. Fast methods for the identification of the genes encoding the tumor antigens and cloning of tumor-specific T cells allow the development of immunotherapeutical strategies based on these individual mutations.

Tumor associated antigens expressed from differentiation genes or genes that are overexpressed in tumors. These antigens should be used in cell-mediated immunotherapy with great care and expression in normal tissue needs to be evaluated.

Escape mechanism of tumor cells

Although tumor associated antigens are expressed on a variety of tumors and various effector mechanisms against tumor cells are present in immune system, tumors can escape immune recognition or fail to elicit an adequate anti-tumor immune response.

Tumor cells can escape immune recognition by various mechanisms involving antigen processing and presentation, co-stimulatory signals and immuno-modulation.

- Antigen processing and presentation in the context of MHC class I and class II molecules is required for recognition by T lymphocytes. It has been observed that during tumor-specific immune response selection of antigen-loss variants could occur (48, 49). Antigenic peptides can disappear as a result of loss of expression of the protein or mutation of the epitopes so they can no longer be recognized (50). In addition, loss or reduction of MHC molecule expression and/or their peptide processing machinery as a result of mutations in β2-microglobulin, transporters associated with antigen processing (TAP) proteins

or proteosomal LMP proteins (51, 52) may enable the tumor cells to escape the host immune response.

- Even when TAA are expressed on the tumor cells there can be lack of response. Lack of expression of the co-stimulatory molecules CD80 on tumor cells results in absence of the 'second signal' during the specific binding of the TCR with Ag in the context of MHC and results in diminished activation of T cells and even induction of T cell anergy (53).
- In addition to the effect on antigen presentation and T cell activation, tumor cells can manipulate their environment and thereby inhibit or eliminate cells from the immune system. A decrease in lymphocyte signal-transduction molecules, described in cancer patients and patients with chronic infectious diseases, has been proposed as a possible mechanism leading to an impaired immune response in cancer patients (54-56). However, these defects have observed to be reversed after cytokine immunotherapy or resection of the tumor mass (57-59).

Immunotherapy of cancer

The fact that tumor-specific or selective T cells and antibodies can be isolated from patients with a variety of different tumors has demonstrated that humoral and cellular immune reactions against established malignancies exist in humans. However, antibody treatment and adoptive transfer of these non-specific or tumor-specific immune cells does not result in complete tumor regression in all patients with all kinds of tumors (60, 61). Improvement of therapy might require modulation of the immune response to cancer cells. Enhancement of the immune response against cancer can be accomplished by the modification of either the tumor cells or the effector cells of the immune system. Tumor cells or tumor antigens should be manipulated in such a way that they will become immunogenic and give rise to an immune response. On the other hand, manipulation of the effector cells and antibodies could provide effector mechanisms that circumvent escape mechanisms and therefore can eradicate tumor cells.

Enhancing immunogenicity of tumors

Vaccination of patients with tumor antigens can be a strategy to enhance the immune response against cancer cells. Transfection of tumor cells with genes that encode cytokines, co-stimulatory molecules or MHC molecules can enhance the immunogenecity of tumor cells. In experimental animal models vaccination with tumor cells that secrete lymphokines like IL-2 and GM-CSF have shown to induce anti-tumor T cell responses (62-65) and tumor cells transfected with genes encoding the co-stimulatory molecule CD80 have shown to enhance tumor immunity *in vivo* even resulting in immunity to rechallenge with parental, untransfected tumor cells (53, 66). So far vaccination with tumor cells transfected with many different cytokine and activation molecules have shown increased anti-tumor T cell responses and protection

against a subsequent tumor challenge in experimental animal studies. However, in the treatment of cancer patients the results were disappointing. It became apparent that effective induction of an immune response requires antigen presentation in an environment that provides appropriate help and co-stimulatory signals. Such conditions are provided by professional APC like dendritic cells (DC) that express co-stimulatory molecules and are able to efficiently activate CD4 and CD8 T cells. Anti-tumor vaccines have been developed with DC that have been loaded or transfected with tumor derived peptides, proteins or whole lysates or mRNA of tumor cells resulting in anti tumor responses in animal model (67-70). An alternative strategy for inducing immunity *in vivo* against unidentified tumor antigens is the fusion of DC with tumor cells (71-73)

All the above described vaccination strategies require the ex vivo manipulation of either tumor cells or DC. Protective CTL responses can also be induced by vaccination with tumor-specific peptides (74) or DNA encoding for these peptides or proteins in plasmids or viruses (75-77). Recombinant viruses and plasmids encoding tumor antigens can elicit specific immune responses that even can be enhanced by the use of cytokines and co-stimulatory molecules (78, 79).

All together, promising results have been obtained with a variety of vaccination strategies in experimental animal studies. In patients with advanced cancer, vaccination with tumor cells resulted often in T cell response against the vaccine and only sometimes in clinical tumor regression (80-87)

Antibody in immunotherapy of cancer

Antibody-mediated immunotherapy is based on the use of mAb that are reactive with antigens on tumor cells to activate effector immune cells at the tumor site. Binding of mAb to tumor antigen can activate the complement system and can induce ADCC by Fc-receptor-expressing cells, both resulting in destruction of the tumor cell (11, 12). There is a large range of antigens associated with human tumors that are recognized by murine antibodies. These tumor antigens can be good candidates for antibody-mediated immuno-therapy. Besides a cytotoxic mechanism induced by binding of 'naked' mAb to the tumor, the specific binding to the tumor makes delivery of isotopes (88), cytostatic drugs (89), toxins (90) and other cytotoxic agents to the tumor possible by conjugation to the mAb.

A potential limitation of antibody therapy was expected to be the development of a human anti-mouse immunoglobulin immune response (HAMA), especially when multiple infusions are required to obtain therapeutic efficacy (91, 92). However, no effect of HAMA formation could be determined for the clinical response to antibody therapy for colorectal cancer (93, 94).

Although antibody therapy has shown to be beneficial in animal models for renal cell carcinoma (95) and melanoma (96, 97), no consistent pattern of response or improved survival has resulted from a large number of clinical trials (98). These failures are often ascribed to antigenic heterogeneity and insufficient accessibility of cells in advanced tumors (98-100). However, more recently, promising results have

been obtained with 17-1A mAb and anti-CD20 in the treatment of colon carcinoma, breast cancer and lymphoma (101-104).
Ongoing research will further improve the therapeutical capacity of antibodies. Increased tumor infiltration can be accomplished by the generation of smaller single chain molecules consisting of only the antibody variable regions fused together by a flexible linker (105). Humanization of antibodies might result in reduced immunogenicity since only the CDR that determine the antibody binding specificity are of murine origin and framework regions are replaced with human framework (106). Further improvements of mAb-mediated immunotherapy might revolve from the generation of antibodies using phage display (107-109) allowing the selection of human antibodies with the required specificity, affinity and stability for each patient.

Adoptive T cell-mediated immunotherapy

Since the description of T cell growth factor interleukin-2 it became feasible to culture T lymphocytes *in vitro* (110, 111). Activation of mouse splenocytes as well as human peripheral blood cells with IL-2 generates cells that demonstrate *in vitro* kill of autologous and allogeneic tumor cell lines and freshly isolated tumor cells (112-114).
Lymphokine activated killer (LAK) cells consist of a heterogeneous population of non-specific natural killer and T cells and in general display non-MHC restricted cytotoxicity. Adoptive transfer of LAK cells in combination with IL-2 was capable of mediating tumor regression in several animal models (115-117). In humans, therapeutic responses have been observed in only a fraction of patients treated and most notable in patients with melanoma and renal cell carcinoma (4, 118-122). This low efficiency of LAK cells *in vivo* might be partly due to the lack of homing of the effector cells to the tumor (123, 124). Furthermore, LAK therapy was limited by the toxic side effects of high dose IL-2 administration (122, 125).
A cell population that has the potential to home to tumors can be isolated from tumor-bearing patients (126). These tumor-infiltrating lymphocytes (TIL) can be expanded and can mediate regression of large metastatic tumors in a mouse model (127, 128). Such animal studies revealed that TIL are 50 to 100 times more effective in their therapeutic potency than LAK cells and display autologous tumor-specific cytotoxicity in contrast with the non-specific reactivity to histologically different tumors as described for LAK cells. Administration of TIL to autologous cancer patients resulted in partial responses in melanoma and renal cell carcinoma (129-132). The *in vivo* tumor response correlated both with the ability of TIL to localize to the side of the tumor and the ability of TIL to mediate specific lysis and cytokine production upon culturing with tumor cells (132, 133). At present the use of LAK cells and TIL does not provide a satisfactory immunotherapeutical approach for cancer treatment.
After the identification of tumor antigens it became possible to generate *in vitro* tumor-specific T lymphocytes (44). However, the generation of sufficient numbers of tumor-reactive T lymphocytes is still difficult and laborious due to the low

frequency of functional T lymphocytes with anti-tumor reactivity (134). Although still limited by the difficulties in the generation and *in vitro* expansion of tumor-specific CTL, already promising results have been obtained in leukemia patients where treatment with leukemia-reactive T cells resulted in complete remission (135-136)

Targeting of T cells to tumor cells

Targeting T cells to the tumor can circumvent the generation of tumor specific T cells from individual patients and extend the anti-tumor specificity of T lymphocytes. The combination of tumor-selective mAb or TCR with the anti-tumor functions of T lymphocytes can result in an effector cell with defined anti-tumor specificity. This can be accomplished either by bispecific monoclonal antibodies (bsmAb) that can bind to both tumor antigen and T lymphocytes or by permanent genetic grafting of T lymphocytes with tumor-selective chimeric receptors.

Bispecific monoclonal antibodies

BsmAb, with one binding site directed against a tumor antigen and the other recognizing an activation molecule on immune effector cells, can combine the advantage of antibody-specificity with the cytokine production and cytotoxic capacity of the effector cells (137-139). High levels of therapeutic efficacy have been described in several experimental tumor systems when bsmAb were used to redirect T lymphocytes (140-143). In a clinical study involving locoregional treatment of ovarian cancer patients, with bsmAb against CD3 on T cells and the folate receptor on the ovarian carcinoma cells, an overall anti-tumor response of 27% was observed (144, 145). In other clinical studies that involved infusions of bsmAb targeted T cells against malignant melanoma, renal cell carcinoma, colon carcinoma and B cell malignancies biological responses were demonstrated (146-149). In these patients conjugate formation between tumor cells and activated lymphocytes soon after injection of bsmAb-redirected lymphocytes, elevated cytokine levels and tumor regression and decreased serum levels of tumor cell markers were observed.
Although good results can be obtained *in vivo* using T cell retargeted bsmAb, there are some limitations to this approach. BsmAb-redirected CTL retain the bi-specific antibody for only limited periods of time (i.e., 48-96 h) due to their dissociation of the CTL surface (139, 150). Only in the presence of excess bsmAb, bsmAb-redirected CTL could recycle cytolytic activity (150). However, the excess bsmAb could evoke a human anti-mouse Ab response, eventually blocking cytolytic activity. Human anti-mouse antibody (HAMA) responses are often observed after repeated injections of bsmAb of murine origin and regarded as a negative effect resulting in the clearance of bsmAb from the circulation (91, 92, 151). However, in a clinical study involving locoregional treatment of ovarian cancer patients, with bsmAb, a significantly longer median survival probability has been observed in patients with high HAMA levels than in patients with lower HAMA levels (152).

The use of bsmAb for therapy may be hampered by the inaccessibility of solid tumors to Ab penetration and their limited activity (99, 150). To circumvent the limitations associated with bsmAb, T lymphocytes can be genetically grafted with permanent Ab-dictated specificity resulting in tumor-specific, MHC-unrestricted killer cells (153, 154). This can be accomplished by the expression on T lymphocytes of chimeric receptors consisting of a single chain mAb coupled to a signaltransducing molecule such as the Fc(ε)RI γ- or TCR ζ-chain (155). Single chain mAb comprises the variable heavy and light domains of mAb fused by a flexible linker sequence and have been shown to display binding affinities and specificities similar to those of the natural mAb (105, 156, 157). Chimeric receptors have been functionally expressed in mouse T cell hybridomas or CTL (155, 158-160), TIL (161) and human $CD8^+$ T lymphocytes (162, 163). Stimulation of the chimeric receptor with the relevant target cell results in T cell activation responses, including cytokine production (159) and lysis of the target cell (155, 158, 160-164). The adoptive transfer of genetically engineered chimeric receptor-positive CTLs has shown *in vivo* anti-tumor activity in mice (165-170) Furthermore, gene modification of heamatopoietic stemcells with chimeric receptors can protect transplanted mice from challenge with tumor cells expressing the relevant target antigen (171-173) The potential of the chimeric receptor approach in adoptive immunotherapy for cancer is currently under investigation in clinical trials. The application of mAb-based chimeric receptors is limited to MHC-unrestricted tumor antigens that can be recognized by mAb. However, many tumor antigens are processed intracellularly and presented as peptides in MHC molecules and therefore are recognized by the TCR on T lymphocytes. Tumor cells expressing these antigens would therefore escape mAb-based chimeric receptor recognition and escape destruction. For that reason, chimeric receptors have been constructed using TCR derived from tumor specific T cells (174-176). Efficient TCR-based chimeric expression and tumor cell kill and cytokine production upon tumor interaction was demonstrated (176). The construction of a T cell-based library of TCR can be used to select *in vitro* TCR with increased affinities or altered affinities. Using this method, variant influenza A-specific TCRs have been isolated (177). Recently, the construction of a chimeric receptor based on a phage display selected Fab fragment specific for the MAGE-1 peptide in HLA-A1 molecules has been described. Grafting of T lymphocytes with this chimeric receptor confers melanoma cells specificity resulting in specific tumor cell lysis and cytokine production (Willemsen, personal communication).

Aim of this study

Effective anti-cancer immunotherapy should be directed at the manipulation and enhancement of the immune capacity to fight cancer cells. In our studies we aimed to develop of efficient effector cells i.e. tumor-selective T lymphocytes for the treatment of renal cell carcinoma. By expressing antibody-binding fragments coupled to a signaltransducing moiety on T lymphocytes, they acquire the

predefined antibody specificity. In this way the specificity of tumor selective antibodies can be combined with the efficacy of CTL to destroy tumor cells.
In chapter 2 we describe the development of a mAb-based chimeric receptor with renal cell carcinoma selectivity. We constructed a chimeric single chain immunoglobulin/γ (scFv/γ) gene composed of the variable regions of the RCC selective mAb G250 joined to the Fc(ε)RI signaling receptor γ-chain of mast cells. Retroviral transduction of T lymphocytes with this chimeric receptor rendered them specific for renal cell carcinoma, as was shown by high levels of Ab-dictated lysis of renal cell carcinoma and lymphokine production upon stimulation with the relevant target cells. Since adhesion and accessory molecules play a critical role in T cell activation and effector function, we investigated the contribution of several molecules in T lymphocyte-tumor cell interactions mediated by chimeric immunoglobulin receptors in chapter 3. Our results demonstrated a co-regulatory role for CD2, CD3 and CD11a/CD18 molecules in scFv/γ-mediated cytolysis indicating that intracellular signaling pathways of the γ-chain can interact with these molecules. Furthermore it was shown that the requirement of CD11a/CD18-CD54 adhesion interaction is dependent on the level of antigen on the target cells. We concluded that this might prove to be an important advantage for immunotherapy of tumor cell variants that express no or low levels of the adhesion molecule CD54.
Genetic engineering of T lymphocytes for adoptive clinical immunotherapy calls for efficient gene transduction methods. Therefore we developed a transient retroviral vector system STITCH that allows efficient transduction of human primary T lymphocytes (chapter 4). This retroviral vector system STITCH in combination with an optimized single chain antibody chimeric receptor gene structure resulted in an increased chimeric receptor membrane expression on T lymphocytes. This high level of chimeric receptor membrane expression allowed us to investigate how the densities of tumor-specific chimeric receptors and tumor associated antigens (TAA), respectively, affect human T lymphocyte functions in relation to target cell susceptibilities to lysis. We therefore compared the level of cytolysis and cytokine production of T cells with high and low level of scFv/γ expression after interaction with tumor cells with high and low TAA expression in chapter 5. We concluded that the expression levels of the scFv/γ on the T lymphocyte and antigen on the tumor cells both are important for the activation of T lymphocytes. However, at high scFv/γ receptor expression levels the T lymphocytes can lyse tumor cells with a wide range of TAA densities.
In chapter 6 the results are summarized and the clinical application of chimeric receptor redirected T lymphocytes is discussed.
The findings that are reported in this thesis demonstrate the feasibility of the chimeric receptor approach for the generation of immune cells with anti-tumor specificity. The first clinical trial with these redirected T lymphocytes should reveal their *in vivo* anti-cancer activity.

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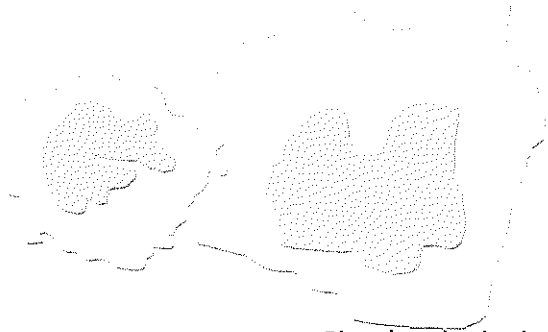
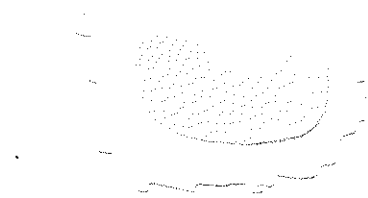


Single chain immunoglobulin/γ gene-redirected human T lymphocytes produce cytokines, specifically lyse tumor cells and recycle lytic capacity



Mo Weijtens, Ralph Willemsen, Dinko Valerio, Kees Stam, Reinder Bolhuis

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SUMMARY

To enable construction of cytotoxic T lymphocytes (CTL) with known predefined antibody (Ab) specificity for adoptive immunotherapy, we constructed a chimeric scFv/γ gene composed of the variable regions of a mAb joined to the Fc(ε)RI signaling receptor γ-chain of mast cells. Introduction of this chimeric receptor into CTL rendered these lymphocytes specific for renal cell carcinoma. This approach combines the specificity of tumor selective antibodies with the efficacy of CTL to destroy tumor cells. We not only demonstrated that the transduced CTL functionally express the scFv/γ receptor for a prolonged period of time (4.5 month of in vitro culture), but also showed high levels of Ab-dictated lysis of renal cell carcinoma similar to that of normal CTL, and importantly, we demonstrated that these CTL can recycle their lytic activity. Moreover, these scFv/γ-expressing T lymphocytes produce cytokines upon stimulation with the relevant target cell. These results together with the donor independence of our gene transduction protocol demonstrate the feasibility of redirecting T lymphocytes for cancer treatment.

INTRODUCTION

Adoptive cellular immunotherapy in cancer treatment refers to the transfer of cultured immune cells with anti-tumor reactivity into patients. Lymphokine-activated killer (LAK) cells and tumor-infiltrating lymphocytes (TIL) have shown therapeutic responses in clinical trials, although these were observed in only a fraction of patients treated (122, 132). Tumor-specific MHC-restricted cytotoxic T lymphocytes (CTL) have been isolated, primarily specific for melanoma (178), but the availability of tumor-specific lymphocytes against more common types of cancer for adoptive therapy has been limited due to difficulties in generating these tumor-specific CTL. Broadening of the range of tumor specificity has been obtained by combining biological response modifier (BRM) production, migration/homing, as well as lytic capacity of T lymphocytes with the selectivity of tumor-recognizing monoclonal antibodies (mAbs). Bispecific mAbs (bsmAb), with one binding site directed against a tumor antigen (Ag) and the other recognizing an activation molecule on the T lymphocyte, have been employed to redirect CTL in pre-clinical and clinical studies (139, 140, 142, 143, 145). However, the use of bsmAb for therapy may be hampered by the inaccessibility of solid tumors to Ab penetration (99). Moreover, bsmAb-redirected CTL retain the bispecific antibody for only limited periods of time (i.e., 48-96 h) due to their dissociation of the CTL surface (139, 150). In addition, bsmAb-redirected CTL lose signaltransducing and, hence, lytic capacity following target cell recognition, lysis and T cell receptor (TCR)/CD3 complex clustering (150).

To circumvent the limitations associated with bsmAb, we and others have adopted an approach in which T lymphocytes are grafted with a permanent Ab-dictated specificity (155, 158). Variable domains of mAb fused by a flexible linker sequence have been shown to display binding affinities and specificities similar to those of the *n*atural mAb (105, 156, 157). Such single chain antibodies (scFv) juxtaposed to a signaltransducing molecule such as the Fc(ε)RI γ- or TCR ζ-chain have been functionally expressed in mouse T cell hybridomas or CTL (155, 158-160), tumor-infiltrating lymphocytes (161) and human $CD8^+$ T lymphocytes (162). Stimulation of the chimeric receptor with the relevant target cell results in T cell activation responses, including BRM production (159) and lysis of the target cell (155, 158, 160-162).

In this study we used an scFv/γ receptor derived from a renal cell carcinoma-selective mouse mAb G250 that is directed to a membrane-bound Ag present on more than 90% of primary tumors and more than 80% of metastases (179, 180). A chimeric gene was constructed composed of the variable domains of the G250 mAb and the γ-chain from the Fc(ε)RI receptor present on mast cells (181). Retroviral gene transfer was employed to successfully transduce the scFv/γ into activated T lymphocytes. The gene-transduced T lymphocytes stably express the receptor for more than 4 months and specifically lyse renal cell carcinoma in an MHC-

unrestricted manner. Continuing and advancing the work of others (155, 161), successful transduction was demonstrated in T lymphocytes derived from all donors tested, as shown by (a) scFv/γ-redirected lysis, (b) cytokine production by the gene-transduced T lymphocytes upon relevant target cell interaction, and (c) the fact that, like normal CTL (150, 162, 182, 183), the gene-transduced CTL can recycle their scFv/γ-dictated lytic activity, i.e. one CTL enters into multiple lytic cycles with the target cells.

Materials and Methods

Cells and antibodies

PBL of healthy donors were isolated by centrifugation through Ficoll-Isopaque (density = 1,077 g/cm^3; Pharmacia Fine Chemicals, Uppsala, Sweden) and activated in culture flasks precoated for 3 h at 37 °C with 1/30 dilution of OKT3 culture supernatant at a density of 2x10^6 cells/ml for 3 days in Mix-Med culture medium (78% RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 20% AIM-V (Life Technologies, Paisley, U.K.) and 2% heat inactivated human plasma supplemented with 360 IU/ml human recombinant IL-2 (rIL-2) (Eurocetus, Amsterdam, The Netherlands), 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) (8). In addition, lymphocytes were activated in Mix-Med culture medium with 10 ng/ml OKT3 (Ortho Diagnostic System, Beerse, Belgium) at a density of 0,5x10^6 cells/ml without rIL-2. After activation, lymphocytes were washed twice and cultured in Mix-Med culture medium. The following cell lines were used as target cells in the cytotoxicity assays: renal cell carcinoma cell lines SK-RC-7 (kindly provided by S. Warnaar, Leiden, The Netherlands), SK-RC-52, SK-RC-1, SK-RC-59, SK-RC-10 (kindly provided by E. Oosterwijk, Nijmegen, The Netherlands), and A75 (generated in our laboratory); melanoma cell line G43 (kindly provided by T. Boon, Brussels, Belgium); ovarian carcinoma cell line IGROV-1 (kindly provided by J. Bénard, Villejuif, France); Burkitt lymphoma-derived cell line Daudi; and erythromyeloid-derived cell line K562. The amphotropic packaging cell line PA317 (American Type Culture Collection, Rockville, MD) was cultured in DMEM 12501 (Life Technologies) and 10% bovine calf serum (BCS; Hyclone, Logan, USA) supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The mAbs used in cytotoxicity inhibition studies were the renal tumor associated Ag (TAA)-specific G250 mAb (kindly provided by S.O. Warnaar, Centocor, Leiden, The Netherlands) and anti-HLA-A,B,C mAb (W6/32; Seralab, Sussex, U.K.).

Construction of scFv/γ genes

The genes encoding the V_H and the V_L domains of the G250 mouse mAb were isolated by anchored polymerase chain reaction (AN-PCR) (184) from cDNA prepared of G250 mAb-producing hybridoma cells, using anchored/anchored polyC primers (184) and a constant V_H (HB) or V_L (KA) primer. The V_H and V_L gene segments were cloned into the plasmid pGEM11 and the nucleotide sequence was determined using the dideoxy-mediated chain termination method (Pharmacia, Uppsala, Sweden). To construct the scFv/γ chimeric receptor, we used a pBluescript vector containing the linker sequence 212 (156). The V_H gene segment containing the leader sequence was reamplified using primers introducing EcoRI and BamHI restriction sites at the 5' and 3' ends, respectively. The V_L gene segment was reamplified without the leader sequence using primers introducing XbaI and BamHI restriction sites at the 5' and 3' ends, respectively. The V_L gene 3' primer consists of a constant light chain (C_L) gene sequence (18 nucleotides) and a extracellular ζ gene sequence (nucleotides 138-158) (185). The Fc(ε)RI γ-chain, containing the most 3' 9-basepair extracellular sequence in addition to transmembrane and intracellular sequences, was isolated from a human cDNA clone (181) using BamHI and XhoI restriction sites at the 5' and 3' ends (kindly provided by Z. Eshhar, Rehovot, Israel). To construct the chimeric scFv/γ gene, V_H, V_L, and γ gene segments were cloned into the pBluescript vector. The chimeric scFv/γ gene was subsequently subcloned into the retroviral vector LXSN (186) containing the murine Moloney leukemia virus long terminal repeat and a neomycin resistance gene under the control of an SV40 promoter. The sequences of the primers used are: HB: CTC TAA GCT TGG CTC AAA CAC AGC GAC CTC GGA TAC AGT TGG TGC AGC; KA: CTC TTC TAG AGA GTC TCT CAG CTG GTA GGA TAC AGT TGG TGC AGC; V_H5': CGC TCG AGG AAT TCG CAC TGA ACA CAG ACC; V_H3': GCG CGG ATC CTG AGG AGA CGG TGA CTG A; V_L5': CTA GTC TAG AGA CAT TGT GAT GAC CGA G; V_L3': CGC GCG GAT CCA GCA GGC CAA AGC TCT GGG ATA CAG TTG GTG CAG C.

Gene transduction and selection of gene-transduced lymphocytes

The LXSN retroviral vectors were electroporated into the amphotropic packaging cell line PA317 using a BTX electroporator (San Diego, U.S.A.) at 250 V and capacitance of 750 μF. A stable amphotropic packaging line, PA317, was obtained after G418 selection. The amphotropic virus supernatants produced had a viral titer of $1x10^4$ colony forming units (cfu)/ml, determined on the basis of neomycin resistance of infected NIH-3T3 cells. To transduce the activated PBL with the G250 scFv/γ retroviral vector (L(scFv/γG250)SN), $2x10^6$ lymphocytes were cocultivated for 72 h with a 70% confluent irradiated (25 Gy) monolayer of virus-producing cells in culture medium supplemented with 4 μg/ml polybrene (Sigma, St. Louis, MO, U.S.A.) and 360 IU/ml rIL-2. Subsequently, the gene-transduced PBL population was selected for 4 days in culture medium containing 1 mg/ml G418, followed by 5 days selection in medium containing 0.4 mg/ml G418. After selection, the gene-

transduced lymphocytes were expanded in round-bottom 96-well microtiter plates (Greiner Labor Technik, Nürtingen, Germany) at 37 °C in 5% CO_2 in the presence of feeder cells: irradiated (25 Gy) allogeneic PBL and Epstein Barr Virus transformed lymphoblastoid B cell lines (B-LCL) as described previously (7, 187). Cloning of gene-transduced T lymphocytes was accomplished through limiting dilution by seeding these T lymphocytes in round-bottom 96-well microtiter plates at 10, 3, 1 and 0,3 cells/well in the presence of feeder cells (7). The culture medium was RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM; Life Technologies), supplemented with 10% (v/v) human plasma, 150 IU/ml rIL-2, 4 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml PHA (Murex Diagnostics, Dartford, U.K.).

Polymerase chain reaction (PCR)

DNA was isolated from 10^5 PBL by incubation of the samples in DNA lysisbuffer (50 mM Tris-HCl (pH 9,0), 50 mM KCl, 7 mM $MgCl_2$, 0,5% NP-40, 0,5% Tween-20, 60 µg/ml proteinase K) at 55 °C for 60 min. Proteinase K was inactivated at 95 °C. DNA from 10^4 cells was amplified using the 5' V_K primer (CTAGTCTAGAGACAT-TGTGATGACCCAG) and 3' γ primer (GCTGCTCGAGTCTAAAGCTACTGTGG-TGG). The PCR reaction was performed in a total volume of 50 µl containing 50 mM Tris-HCl (pH 9,0), 50 mM KCl, 7 mM $MgCl_2$, 200 µM of each dNTP, 20 pmol of each primer and 0,25 U Super Taq (HT Biotechnologies, Cambridge, U.K.) and was covered with 50 µl of paraffin oil. The samples were amplified in 35 cycli (30 s at 95 °C, 30 s at 58 °C, and 1 min at 72 °C) using a Trio Thermoblock (Biometra, Göttingen, Germany). Twenty microliters of each sample was analyzed by agarose gel electrophorese. For RT-PCR, RNA was isolated from $4x10^6$ PBL using the RNeasy kit (Qiagen, Hilden, Germany). Single strand cDNA was synthesized by incubation of RNA with 500 ng oligo d(T) primer (Promega, Leiden, The Netherlands) for 10 min at 75 °C, followed by incubation at 37 °C for 60 min with 100 U of Superscript (Life Technologies), 50 mM Tris-HCl (pH 8,3), 40 mM KCl, 6 mM $MgCl_2$, 10 mM DTT (Life Technologies), 500 µM dNTP and 13 U RNAguard (Pharmacia, Uppsala, Sweden). PCR was subsequently performed on DNA from 10^5 cells as described above. As a control for cDNA synthesis, amplification with β_2-microglobulin primers was performed. Sequences of the $\beta_2$microglobulin primers are: β_2m 5': TCAGGTTTACTCACGTCATCCAG, and β_2m 3': TCACTCAATCCAA-ATGCGGC.

Measurement of cytokine production

To determine cytokine production by gene-transduced PBL upon Ag stimulation, $6x10^4$ transduced PBL were cultured for 24 h in either the presence or absence of $2x10^4$ adherent tumor cells in RPMI culture medium containing 360 IU/ml rIL-2. Plates were centrifuged for 5 minutes at 1500 rpm, supernatant was harvested, and levels of TNFα and GM-CSF were measured by ELISA (Medgenix Diagnostics, Fleuris, Belgium) according to suppliers' specifications.

Cytotoxicity assay

Cytotoxic activity was measured in a 4- to 5-h ^{51}Cr-release assay. Briefly, varying numbers of effector cells were added in triplicate to 96-well round-bottom microtiter plates (100 µl/well), followed by addition of 2500 target cells (100 µl)/well. The target cells were labeled with 100 µCi ^{51}Cr/0.5x10^6 cells for 2 h at 37 °C. At the end of the 4- to 5-h incubation period (37 °C and 5% CO_2), supernatants were collected using a Skatron harvesting system (Skatron, Lier, Norway), and radioactivity was counted in a gamma-counter. The percent specific lysis was calculated as follows: ((test counts - spontaneous counts) / (maximum counts - spontaneous counts)] x 100%. In blocking experiments, G250 mAb (10 µg/ml) or W6/32 mAb (10 µg/ml) was added to labeled target cells 15 to 30 minutes before addition of the effector cells.

CTL-target cell interactions

To examine lytic recycling capacity of scFv/γ-transduced CTL, a secondary cytotoxicity assay that we previously developed was used (150). In short, CTL (3x10^5 or 1x10^5) were incubated for 18 h in the presence or the absence of adherent target cells (3x10^5) in 1 ml of medium supplemented with 360 IU/ml rIL-2. After exposure, transduced PBL were harvested, counted, and subsequently tested for lytic activity in the ^{51}Cr release assay as described above. Target cell contamination was determined by phase contrast microscopy.

RESULTS

Cytokine secretion by G250 scFv/γ-transduced T lymphocytes upon stimulation with renal cell carcinoma

For expression of scFv/γ receptors selective for renal cell carcinoma we constructed one continuous molecule comprising gene segments of the variable region of the renal cell carcinoma-selective mouse mAb G250 and the signaltransducing human Fc(ε)RI γ-chain transmembrane and intracellular region. Retroviral gene transfer using the LXSN vector was used to generate stable integration into the genome, as we and others have previously shown to be effective in human activated T lymphocytes (188, 189). Gene transduction of activated human PBL was accomplished after cocultivation of anti-CD3-activated human PBL (frozen/thawed) with irradiated virus-producing packaging cells followed by selection in G418-containing medium and expansion in the presence of feeder cells for 2 weeks. The presence of the scFv/γ viral construct in the genomic DNA of scFv/γ-transduced

T lymphocytes and the expression of scFv/γ mRNA were demonstrated by DNA-PCR and RT-PCR using scFv/γ-specific primers (fig. 2.1 lanes 4-9).
Chimeric receptor surface expression was too low to be detected by FACS with anti-idiotype mAb (190); therefore, the percentage of scFv/γ-transduced T lymphocytes could not be determined. To examine functional expression of the receptor, the ability of scFv/γ-transduced T lymphocytes to secrete GM-CSF and TNF-α after specific target cell interaction was tested (table 2.1). ScFv/γ-transduced bulk T lymphocytes specifically secreted GM-CSF and TNF-α after interaction with A75, a G250 mAb-binding renal cell carcinoma cell line, but not after stimulation with irrelevant SK-BR-3, a breast carcinoma cell line, thereby showing functional expression of the scFv/γ receptor and specific recognition of the relevant target cells by the scFv/γ receptor.

Cytolytic activity of G250 scFv/γ-transduced bulk CTL

To further study the functional expression of the scFv/γ receptor on transduced T lymphocytes, cytolytic activity against a panel of renal and nonrenal cell carcinoma lines was evaluated in a ^{51}Cr release assay (fig. 2.2). High levels of lysis

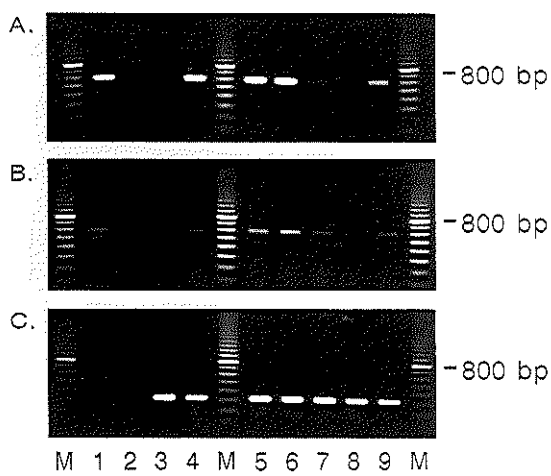

figure 2.1 PCR analysis of the detection of scFv/γ G250 DNA and RNA in transduced T lymphocytes derived from different donors. Ethidium bromide stained gel of DNA-PCR amplification products obtained using primers specific for scFv/γ G250 (A), RT-PCR amplification products using primers specific for scFv/γ G250 (B), and RT-PCR amplification products using β_2-microglobulin primers (C). Lane 1, Positive control for scFv/γ G250-specific primers: retroviral vector containing the scFv/γ G250 construct; 2, negative control (no DNA/RNA control); 3, mock-transduced PBL (PBL-LXSN); 4, scFv/γ-transduced PBL (PBL-scG250); 5, scFv/γ-transduced T lymphocytes derived from different donors: VD-2; 6, VD-3; 7, VD-4; 8, VD-5 and 9, VD-9. The marker (M) is a 100 bp ladder.

(±80% at an E/T ratio of 60) were shown against all G250 mAb-binding renal cell carcinoma cell lines. No lysis was observed of control cell lines SK-RC-59, a G250 mAb-nonbinding renal cell carcinoma cell line, and G43, an irrelevant melanoma cell line (<20% at an E/T ratio of 60). In addition, no MHC-unrestricted natural killer activity (NK) or activated kill (AK) activity was found, as shown by the absence of lysis of K562 and Daudi, respectively (fig. 2.2). That indeed the specificity of lysis was dictated by functional expression of the scFv/γ gene was further demonstrated by the inhibition of renal cell carcinoma lysis following the addition of soluble G250 mAb to the renal cell carcinoma target cells (fig. 2.2). No inhibition of lysis was seen with the control anti-HLA-A,B,C Ab (W6/32; data not shown). These combined results demonstrate (a) the specificity of the G250 mAb-mediated inhibition of lysis, and (b) the non-MHC-restricted nature of the scFv/γ receptor interaction with the target cell. Retroviral transduction has been shown to introduce foreign genes stably into lymphocytes without adversely affecting their functions over time (189). Here, we demonstrate stable functional expression over a test period of more than 4.5 months of continuous culture by serial cytotoxicity studies. During this culture period we repeatedly observed specific lysis of renal cell carcinoma by bulk-cultured, gene-transduced CTL. This scFv-redirected CTL lysis was only inhibited by renal carcinoma-specific G250 mAb and was devoid of NK and AK activity. With time, an increase in $CD4^+$ T lymphocytes in the bulk population (>25%) was observed, paralleled by a decrease in specific lytic activity. Depletion of $CD4^+$ lymphocytes with magnetic beads completely restored lytic activity up to the level observed before the preferential outgrowth of $CD4^+$ lymphocytes (fig. 2.3).

This result demonstrates that $CD4^+$ lymphocytes are not lytic and that the decline in the percentage of $CD8^+$ CTL in favor of $CD4^+$ T lymphocytes accounts for the observed decrease in scFv/γ-mediated target cell lysis. $CD4^+$ lymphocytes do produce cytokines upon scFv/γ-mediated specific target cell interaction. Compared with the $CD8^+$ scFv/γ-transduced T lymphocytes, $CD4^+$ T lymphocytes produce approximately 5.5-fold the amount of GM-CSF and about 1.6-fold the amount of TNF-α (data not shown).

table 2.1 GM-CSF and TNF-α secretion of transduced PBL upon target cell interaction[a]

	GM-CSF			TNF-α		
effector	-	A75	SK-BR-3	-	A75	SK-BR-3
PBL-scG250	103	1326	64	<12	192	13
PBL-LXSN	99	<25	38	<12	<12	<12

[a]Six times 10^4 T lymphocytes transduced with scFv/γ (PBL-scG250) or mock-transduced (PBL-LXSN) were cultured in medium or with 2 x 10^4 G250 mAb-binding or nonbinding tumor cells, for 24 h. Cytokine secretion in supernatant was measured by ELISA (Medgenix, Brussels, Belgium). Background cytokine secretion by tumor cells alone has been subtracted (GM-CSF: 78 pg/ml for A75 and 46 pg/ml for SK-BR-3; TNF-α: <12 pg/ml for A75 and SK-BR-3). Production of GM-CSF and TNF-α is in pg/ml/3 x 10^5 cells/24 h. A75 is a G250 mAb-binding human renal cell carcinoma cell line. SK-BR-3 is a human breast carcinoma cell line used as G250 mAb nonbinding control cell line. Similar results were obtained from three independent experiments. - = no stimulator cells.

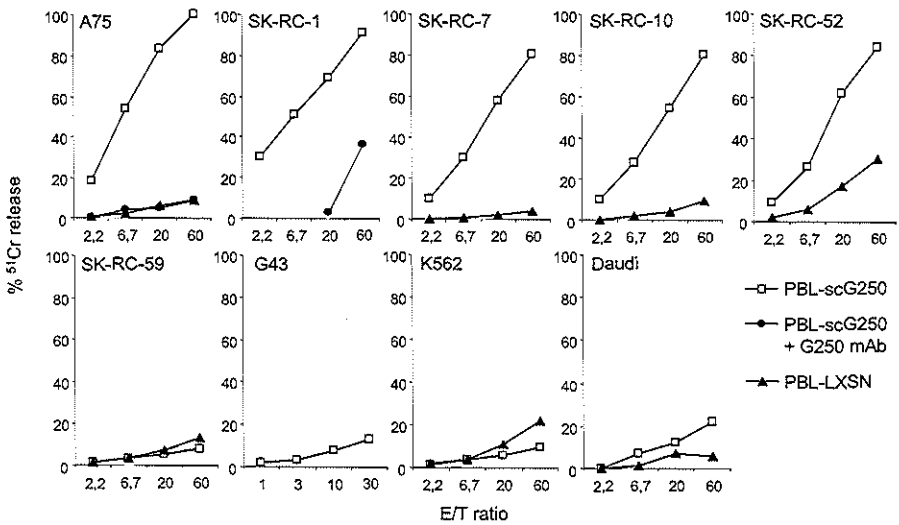

figure 2.2 Cytotoxicity of scFv/γ (PBL-scG250) and mock (PBL-LXSN)-transduced T lymphocytes against G250 mAb-binding and nonbinding cell lines. scFv/γ-transduced (PBL-scG250) or mock-transduced (PBL-LXSN) lymphocytes were incubated with the following target cells: A75, SK-RC-1, SK-RC-7, SK-RC-10, SK-RC-52 (G250 mAb-binding renal carcinoma cell lines), SK-RC-59 (G250 mAb nonbinding renal carcinoma cell line), G43 (G250 mAb nonbinding melanoma cell line), K562 and Daudi, and tested in a 5-h cytotoxicity assay. Blocking of cytolysis with G250 mAb was performed at a concentration of 10 μg/ml. The specific ^{51}Cr release is depicted at different E/T ratios. Experiments were performed in triplicate and the standard deviation did not exceed 10%. Similar results were obtained from at least two independent experiments. The percentages of spontaneous release were for A75, 22%; SK-RC-1, 23%; SK-RC-7, 16%; SK-RC-10, 40%; SK-RC-52, 12%; SK-RC-59, 8%; G43, 12%; K562, 5%; Daudi, 14%.

Cytolytic activity of G250 scFv/γ-transduced T lymphocyte clones

Limiting dilution of the CTL from the transduced bulk culture yielded CTL clones with multiple lytic activities. When cloned scFv/γ-transduced CTL were tested for (a) G250 scFv/γ-redirected lysis (target: SK-RC-7), (b) NK (target: K562), and (c) AK activity (target: Daudi), the following picture emerged: two clones with only scFv/γ-dictated lytic activity (clones 44 and 75); a clone with scFv/γ-dictated specificity and NK lytic activity (clone 27); 2 clones with scFv/γ, NK and AK activity (clones 45 and 49); and a clone without cytolytic activity against the targets tested (clone 41; fig. 2.4).

These differences in target cell specificities among CTL clones and hence CTL target recognition structures were also illustrated by the fact that only lytic activities of those CTL clones that exclusively lysed renal cell carcinoma (scFv/γ dictated) were completely inhibited by G250 mAb. The cytolytic activity of cloned scFv/γ-redirected CTL that also exerted NK and/or AK activities was partly inhibited (fig. 2.4). Clones 27, 41, 45 and 49 were phenotyped and found to be TCRαβ$^+$ and CD8$^+$. Two clones (41 and 49) were further analyzed and were negative for CD16 and ± 50% of the T lymphocytes expressed CD56. In addition, no scFv/γ G250 membrane expression could be detected by FACS using anti-idiotype mAb (190) (data not shown).

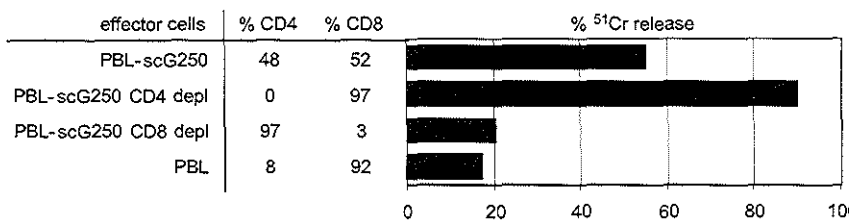

figure 2.3 Cytotoxicity of scFv/γ (PBL-scG250), CD4-depleted fraction (PBL-scG250 CD4 depl), CD8-depleted fraction (PBL-scG250 CD8 depl) and untransduced PBL against G250 mAb-binding renal cell carcinoma, A75. CD4- and CD8-depleted PBL-scG250 fractions were obtained by magnetic bead depletion of either population of the bulk culture. The percentage of CD4$^+$ or CD8$^+$ T lymphocytes was determined by flow cytometry. Specific ^{51}Cr release is depicted at an E/T ratio of 60. Experiments were performed in triplicate and the standard deviation did not exceed 10%. Similar results were obtained from at least two independent experiments. The percentage of spontaneous release was 9%.

Efficacy of functional LXSN scFv/γ gene transduction protocol

To investigate whether scFv/γ transduction and subsequent functional expression by recipient T lymphocytes is donor independent, we set out to transduce fresh T lymphocytes from 10 healthy volunteers. Activated PBL were cocultivated and selected as described. Evaluation of genomic DNA and RNA by PCR analysis showed T lymphocytes from all donors to contain and to transcribe the fusion gene (fig. 2.1 lanes 5-9). Subsequent analysis of functional expression demonstrated a complete correlation between the presence of scFv/γ transcripts and (a) GM-CSF and TNF-α

production upon Ag stimulation (table 2.2) or (b) scFv/γ-mediated target cell lysis by the $CD8^+$ scFv/γ-transduced CTL (fig. 2.5). Cytokine production was observed in all 10 gene-transduced T lymphocyte cultures. Specific lysis of renal cell carcinoma was shown, with 8 of 10 scFv/γ-transduced T lymphocyte cultures derived from 10 donors. Specificity was further demonstrated by inhibition of renal cell carcinoma lysis by soluble parental G250 mAb to the target cells before addition of the scFv/γ-expressing CTL. To exclude the possibility that lysis might involve HLA molecules on the target cells, lysis of renal cell carcinoma by these transduced T lymphocytes derived from five different donors was also tested by preincubation of the target cell with anti-HLA framework Ab W6/32. No inhibition of lysis was seen.

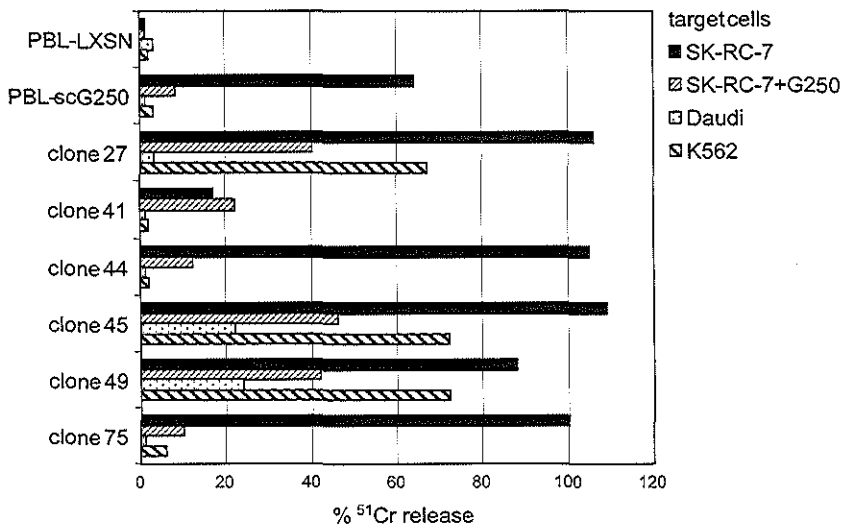

figure 2.4 Cytotoxicity of T lymphocyte clones obtained by limiting dilution of a scFv/γ-transduced PBL population. T lymphocyte clones were tested in a 5-h ^{51}Cr release assay with target cells: K562, Daudi or the renal carcinoma cell line SK-RC-7. Blocking of cytolysis was achieved with G250 mAb at a concentration of 10 μg/ml. The E/T ratio was 20. PBL-scG250 is the scFv/γ-transduced bulk population and PBL-LXSN is the mock-transduced population. Experiments were performed in triplicate and the standard deviation did not exceed 15%. Similar results were obtained from at least two independent experiments. The percentages of spontaneous release were SK-RC-7, 13%; Daudi, 17%; K562, 9%.

As cytokine production is another sensitive method to measure specific CTL/target cell interactions, we assume that the percentages scFv/γ-transduced T lymphocytes in the two nonlytic cultures are too low to monitor lysis. Differential efficacies of gene transduction and subsequent selection of gene-transduced lymphocytes will result in T lymphocyte bulk populations comprising different percentages of gene-transduced T lymphocytes. In addition, T lymphocyte composition will vary among cultures of individual donors. This may explain the different levels of cytolytic activity and the

different lytic activities (e.g. scFv/γ-dictated lysis and NK/AK activity) as well as differences in the lymphokine repertoire.

G250 scFv/γ-transduced T lymphocytes recycle chimeric receptor-dictated lytic activity

To examine the capacity of gene-transduced CTL to recycle their scFv/γ-redirected cytolysis, these CTL were allowed to enter multiple lytic interactions with relevant target cells before being tested in a secondary ^{51}Cr release assay (150). ScFv/γ-transduced CTL were added to an equal (1/1) or excess (1/3) number of unlabeled G250 mAb-binding renal cell carcinoma A75 cells and incubated for 18 h. During the incubation period more than 95% and 60% of the target cells were lysed, respectively, as determined under the phase contrast microscope. Unlabeled, irrelevant ovarian carcinoma cells (IGROV-1) were used as control target cells and were not lysed by the effector cells. ScFv/γ-transduced CTL were harvested and tested for lytic activity in a secondary cytotoxicity assay in the presence of ^{51}Cr labeled A75 cells. These preincubated cells still displayed scFv/γ-dictated cytolytic activity, which suggests that these cells, although we cannot formally exclude recruitment, retain full lytic recycling capacity (fig. 2.6).

table 2.2 GM-CSF and TNF-α secretion of transduced PBL upon target cell interaction[a]

	GM-CSF			TNF-α		
effector	-	A75	IGROV-1	-	A75	IGROV-1
VD-1	<12	716	<12	16	509	66
VD-2	<12	1500	<12	<12	1131	66
VD-3	<12	2614	<12	<12	940	52
VD-4	232	445	96	54	46	19
VD-5[b]	<12	153	<12	36	85	28
VD-6	<12	2709	<12	16	1260	56
VD-7	<12	329	<12	<12	127	16
VD-8	<12	902	<12	24	516	58
VD-9	<12	743	<12	<12	1800	<12
VD-10	<12	691	<12	<12	438	<12
PBL-LXSN	<12	31	<12	<12	<12	<12

[a]Six times 10^4 T lymphocytes transduced with scFv/γ (VD-1-10) or mock-transduced (PBL-LXSN) were cultured in medium or with 2 x 10^4 G250 mAb-binding or nonbinding tumor cells, for 24 h. Cytokine secretion in supernatant was measured by ELISA (Medgenix, Brussels, Belgium). Background cytokine secretion by tumor cells alone has been subtracted (GM-CSF: 206 pg/ml for A75, <12 pg/ml for IGROV-1; TNF-α: 22 pg/ml for A75, 47 pg/ml for IGROV-1). Production of GM-CSF and TNF-α is in pg/ml/3 x 10^5 cells/24 h. A75 is a G250 mAb-binding human renal cell carcinoma cell line. IGROV-1 is a human ovarium carcinoma cell line used as G250 mAb nonbinding control cell line. Similar results were obtained from two independent experiments. - = no stimulator cells. [b]GM-CSF and TNF-α in pg/ml/5 x 10^5 cells/24 h.

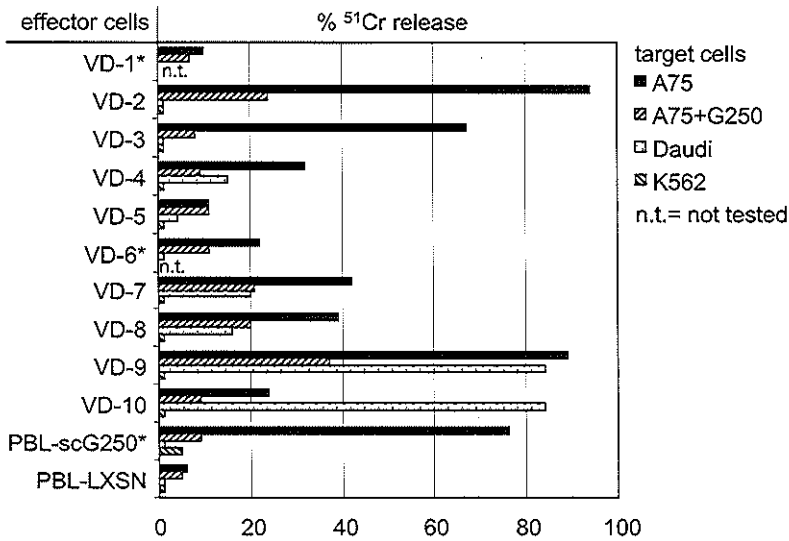

figure 2.5 Cytotoxicity of scFv/γ-transduced CTL derived from 11 different healthy donors (VD-1-10 and PBL-scG250) and mock-transduced (PBL-LXSN) PBL against G250 mAb-binding renal carcinoma cells, A75. Gene-transduced T lymphocytes were depleted for $CD4^+$ T lymphocytes and incubated with A75, K562, or Daudi as target cells in a 4- to 5-h cytotoxicity assay. Blocking of cytolysis was achieved with G250 mAb at a concentration of 10 μg/ml. The specific ^{51}Cr release is depicted at E/T ratio of 60 or 20 (*). Experiments were performed in triplicate and the standard deviation did not exceed 15%. The percentages of spontaneous release were: A75, 13%; Daudi, 6%; K562, 5%.

DISCUSSION

In this report we have demonstrated redirection of human T lymphocyte specificity by retroviral single chain immunoglobulin/γ gene transfer, obtaining CTL with a permanent, MHC-unrestricted, tumoricidal activity. Redirection of lymphocyte specificity to recognize targets not recognized by their endogenous TCR was previously achieved with bsmAb, one arm of which is directed against a tumor Ag and the other against an activation molecule on T lymphocytes (139, 140). Such bsmAb-redirected CTL have shown in vivo anti-cancer activity in mice (142, 143) and in man (144-146). Lysis of tumor cells can be attributed to bsmAb-redirected $CD8^+$ T lymphocytes, although in mice additional anti-tumor growth effects were seen due to cytokine release by $CD4^+$ T lymphocytes and subsequent activation of CTL and NK activities (191). Loco-regional immunotherapy in clinical studies has shown tumor responses that were restricted to the treated area (144, 145). This may be due to limited migration capacity of bsmAb-redirected CTL and their limited recycling of signaltransducing capacity, as we reported previously (150).

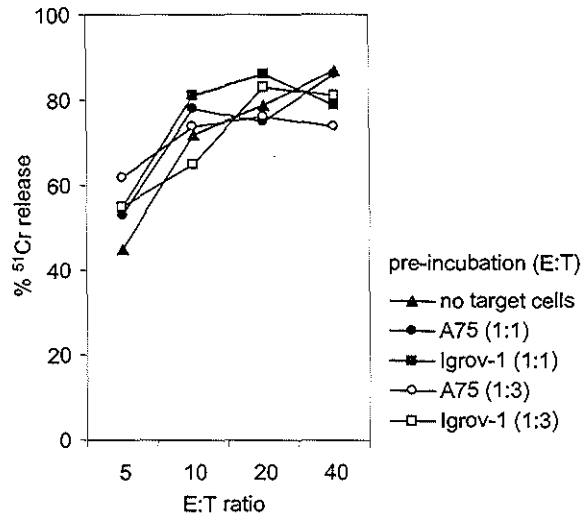

figure 2.6 Cytolytic recycling capacity of scFv/γ-transduced PBL (PBL-scG250). Gene-transduced PBL were first exposed for 18 h to (a) medium alone, (b) G250 mAb-binding renal cell carcinoma, A75, or (c) irrelevant ovarian carcinoma cells, IGROV-1. E/T ratios were 1 and 0.3. After exposure to the target cells, retention of the cytolytic capacity of the transduced PBL was measured in a 4-h ^{51}Cr release assay against renal cell carcinoma, A75. E/T ratios were 5, 10, 20 and 40. Experiments were performed in triplicate and the standard deviation did not exceed 15%. Similar results were obtained from at least two independent experiments. The percentage of spontaneous release was 9%.

Genetic engineering of T lymphocytes using an scFv/γ gene carrying LXSN retroviral vector resulted in permanently acquired, Ab-dictated target cell specificity and lysis as well as the triggering of lymphokine production and of MHC-unrestricted NK/AK lysis. Such scFv/γ-redirected CTL show normal recycling of lytic activity and may have normal migration and target cell homing capacities. Indeed, we demonstrated that G250 scFv/γ-transduced CTL show relevant renal cell carcinoma-specific kill and prolonged functional expression of the chimeric receptor for a test period of almost 5 months. Although transcription of the fusion gene could be demonstrated, levels of T lymphocyte surface expression were too low for detection by FACS analysis. Despite this low surface expression, the observed levels of lytic activities of scFv/γ-transduced CTL was high (e.g. 76% for clone 44 at an E/T ratio of 2) in a 5-h ^{51}Cr release assay. This high lytic activity may be explained by the high affinity of the scFv/γ chimeric receptor relative to that of TCR for the Ag on the target cells, although we expect the affinity of scFv/γ to be lower than the parental mAb. This low expression of scFv/γ receptors may prove advantageous from a clinical point of view, as it might lower the human anti-mouse Ab responses in patients after transfusion of scFv/γ-expressing CTL.

Because the number of tumor cells is usually in excess of lymphoid effector cell number, their elimination by CTL in vivo requires that individual CTL recycle their lytic machinery. We previously demonstrated that only in the presence of excess bsmAb, bsmAb-redirected CTL could recycle cytolytic activity (150). However, the excess bsmAb evoked a human anti-mouse Ab response, eventually blocking cytolytic activity (91, 92, 145). We, therefore, studied the lytic recycling capacity of scFv/γ-transduced CTL. Prolonged exposure (18 h) of scFv/γ-transduced CTL to their specific target cells and subsequent testing of these CTL in a secondary cytotoxicity assay demonstrated their capacity to enter multiple lytic cycles with their specific tumor targets. In normal CTL, the continued triggering of T lymphocytes and, hence, recycling of lytic capacity require continuous synthesis and subsequent surface expression of endogenous TCR (150, 192, 193). Therefore, we conclude that scFv/γ gene-transduced CTL, which functionally express the scFv/γ chimeric receptor and show recycling of lytic capacity, also continuously synthesize the transduced chimeric receptor.

Clinical application of scFv/γ-redirected PBL can be effective as it combines induction of BRM production and cytolysis by the redirected T lymphocytes. Release of lymphokines by $CD4^+$ gene-transduced T lymphocytes upon specific target cell interaction may also contribute to tumor growth inhibition as well as induction of NK/AK activities. These lytic activities may result in elimination of those tumor cells that lack or downregulate the relevant TAA expression (122).

An important feature of CTL expressing scFv/γ receptors is that they, like Ab per se, recognize Ag in an MHC-unrestricted manner. Hence, in contrast to T lymphocytes which recognize their ligand via their endogenous MHC-restricted TCR, the scFv/γ-dictated specificity and anti-tumor activity is not adversely affected by tumor cells that down-regulate their MHC complex or for other reasons do not express MHC-restricted Ag (194).

In conclusion, we have shown long term functional expression of scFv/γ chimeric receptors by transduced T lymphocytes, scFv/γ-triggered lymphokine production, and cytolysis of relevant target cells as well as recycling of lytic activity. The scFv/γ gene transduction was successful in all 11 donors and these results provided 'proof of principle' of the use of scFv/γ-expressing CTL for clinical anti-cancer treatment.

3

Chimeric scFv/γ receptor-mediated T cell lysis of tumor cells is co-regulated by adhesion and accessory molecules

Mo Weijtens, Ralph Willemsen, Brigitte van Krimpen, Reinder Bolhuis

Int. J. Cancer 77: 181-187 (1998)

Adhesion and accessory molecules play a critical role in T cell activation and effector function in general and in tumor cell recognition and lysis in particular. We investigated the contribution of CD2, CD3, CD11a/CD18, CD54 and CD58 molecules in T lymphocyte-tumor cell interactions mediated by chimeric immunoglobulin receptors. The chimeric receptor is composed of a single chain antibody binding site and a γ-chain signaltransducing molecule (scFv/γ). T lymphocytes expressing such scFv/γ receptors recognize the G250 antigen (Ag) on renal cell carcinoma (RCC) in a major histocompatibility complex (MHC)-unrestricted manner and exert RCC selective cytolysis. A coregulatory role for CD2, CD3 and CD11a/CD18 molecules in scFv/γ-mediated cytolysis was demonstrated using monoclonal antibody (mAb)-induced inhibition of scFv/γ-mediated cytolysis. The inhibition of lysis was not due to inhibition of cytotoxic T lymphocyte (CTL)-target cell conjugation but rather to a post-conjugate signaling event. Binding of CD54 and CD58 mAbs to the RCC did not inhibit cytolysis of RCC that expressed high levels of both CD54 and the G250 Ag (A75), whereas cytolysis of RCC expressing intermediate levels of CD54 and G250 Ag (SK-RC-17 cl.4) was partly inhibited by the CD54 mAb. Binding of low concentrations of G250 mAb to RCC (A75) rendered these cells sensitive to CD54 mAb inhibition, demonstrating a direct functional relation between G250 Ag expression level and adhesion molecules. Taken together, our findings indicate a coregulatory role for CD2, CD3 and CD11a/CD18 molecules in the scFv/γ-mediated cytolysis of tumor cells and show that the requirement of CD11a/CD18-CD54 interactions is dependent on the level of free Ag. This makes these gene-transduced T lymphocytes attractive tools for adoptive immuno-gene therapy of cancer.

INTRODUCTION

T lymphocytes recognize their target cells via the T cell receptor (TCR). Interaction of CTL with target cells requires specific recognition of the TCR with Ag in the context of MHC molecules resulting in CD3 signaling, activation and subsequent effector functions of T lymphocytes. This specific target cell recognition is preceded by non-specific conjugate formation, primarily mediated by non-polymorphic cell-surface receptors such as CD4, CD8, CD2 and CD11a/CD18 on the T lymphocytes with their ligands on the target cells, i.e., MHC Class II and I, CD58 and CD54, respectively (33, 195). In addition to an adhesion function, the CD8, CD2 and CD11a/CD18 receptors play an important role in T lymphocyte activation as costimulatory molecules. T lymphocytes can be activated by cross-linking of CD2, CD3 or CD11a/CD18 molecules without requiring specific TCR/CD3 interaction with MHC-peptide complex (16, 20, 34-38). A coregulatory role in anti-CD3 and anti-CD16 mAb-induced activation has also been demonstrated for CD11a/CD18 (36, 37, 196). Adhesion of T lymphocytes to target cells, specific TCR recognition of Ag and costimulation via accessory molecules together results in triggering of, e.g., the lytic machinery and subsequent lysis of target cells.

Cancer-specific CTL, particularly in melanoma, can be used for immunotherapy as tumor cell killers (132). These cancer-specific T lymphocytes for most tumors are difficult to obtain and to expand in tissue culture. To circumvent this requirement for tumor-specific CTL, $CD3^{Pos}$ T lymphocytes can be selectively activated by anti-CD3 mAb (16, 197) and their cytolytic effector function can be directed against a predefined target specificity using bispecific mAb (bsmAb) with two distinct binding sites, one for the CD3 complex and the other for a tumor-associated Ag (TAA) (138-140, 145). Binding of the bsmAb to both the CD3 complex and the TAA results in triggering of cytokine-producing capacity and of the cytolytic effector function of the CTL. In this high affinity interaction the CD11a/CD18-CD54 adhesion pathway and the CD2-CD58 pathway are also involved in both adhesion and coregulation of bsmAb-mediated cytolysis (37, 198, 199). Another approach to redirect the specificity of CTL is the permanent grafting of these cells with mAb-dictated specificity. This can be realized by genetic engineering of CTL to express antibody-based chimeric receptors (scFv/γ) in their membrane (154, 155, 161-163). These chimeric receptors comprise the Ag binding part of mAb and the signaltransducing γ-chain of the high affinity Fc(ε)RI (181) that can function independently of CD3 complex (200, 201). We previously showed that such scFv/γ-transduced CTL can (a) specifically lyse target cells via their scFv/γ chimeric receptor in a MHC-unrestricted manner, (b) produce cytokines upon specific target cell interaction, and (c) like normal CTL (150), these gene-transduced CTL recycle their scFv/γ-dictated lytic activity (163).

Interactions between TCR-antigen complex and activation or accessory molecules, as well as a role for adhesion molecules in T lymphocyte-target cell conjugate formation have been described for MHC-restricted, TCRαβ-mediated activation of T lymphocytes

(35). We also demonstrated these functional interaction to occur with the TCR-complex in situations where activation took place via bsmAb involving antigen recognition in a MHC-unrestricted way (36, 37). In both cases, coactivation/adhesion-ligand interactions result in enhanced activation of T lymphocytes. This T lymphocyte activation occurs either through interaction of the intracellular signaling pathways of coactivation receptors on the one hand and the TCR/CD3 complex on the other, involving δγε- and ζ-chains or via enhanced effector-target cell binding. The question we wanted to address is whether such interactions also occurred between the intracellular signaling pathways of coactivation molecules and a chimeric receptor consisting of a tumor-specific single chain antibody linked to the γ-chain of the Fc(ε)RI.

In this study, we show that CD3, CD2 and CD11a/CD18 on the scFv/γ-redirected CTL can act as accessory molecules in the lytic process. We also show that the contribution of CD11a/CD18-CD54 interaction in the cytolytic process depends on the expression level of TAA on the target cell. High numbers of G250 Ag on the target cell bypass the requirement of CD11a/CD18-CD54 interaction for scFv/γ-mediated T cell cytolysis. These findings pave the way for clinical anti-tumor therapy using scFv/γ gene-transduced T lymphocytes.

MATERIALS AND METHODS

Cells and antibodies

Peripheral blood mononuclear cells (PBMC) of healthy donors were isolated by centrifugation through Lymphoprep (d=1,077 g/cm^3) (Nycomed, Oslo, Norway) and activated in Mix-med culture medium (78% RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 20% AIM-V (Gibco BRL, Paisley, England), 2% heat-inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) (8) with 10 ng/ml OKT3 (Ortho Diagnostic System, Beerse, Belgium) at a density of 0.5x10^6 cells/ml. After 3 days activation, lymphocytes were washed and cultured in RPMI culture medium (RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 10% heat inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 360 IU/ml human recombinant IL-2 (rIL-2) (Chiron, Amsterdam, The Netherlands). The following cell lines were used as targets in cytotoxicity assays and conjugate assays: RCC A75 (generated in our laboratory), SK-RC-17 cl.4 (G250 Ag cDNA transfected RCC cell line)(kindly provided by E. Oosterwijk, Nijmegen, The Netherlands) and IgR39 melanoma cell line (kindly provided by L. de Ley, Groningen, The Netherlands). The antibodies used for FACS staining and cytotoxicity and conjugate inhibition studies were: renal tumor associated Ag (TAA)-specific G250 mAb (kindly provided by S. O. Warnaar, Centocor, Leiden, The Netherlands), anti-HLA-A,B,C mAb (W6/32; Seralab,

Sussex, U.K.), anti-CD3 (CLB-CD3), anti-CD2 (CLB-CD2), anti-CD58 (CLB-CD58), anti-CD11a/CD18 (CLB-CD11a), anti-CD54 (CLB-CD54; all from CLB, Amsterdam, The Netherlands), anti-CD4 (OKT4 culture supernatant), and anti-CD8 (B116.1.1).

G250 scFv/γ gene transduction

Activated T lymphocytes were transduced with the G250 scFv/γ retroviral vector (L(scFv/γG250)SN) as described (163). In short, activated T lymphocytes were cocultivated for 3 days with the irradiated L(scFv/γG250)SN-producing amphotropic packaging cell line PA317 in the presence of 360 IU/ml rIL-2 and 4 μg/ml polybrene (Sigma Chemical Co., St. Louis, MO). Cocultivation was followed by 9 days G418 selection and expansion in the presence of feeder cells as described previously (202, 203).

G250 Ag gene transfection

Melanoma cell line IgR39 was electroporated with 10 μg of G250 Ag cDNA in expression vector pCDM8 (kindly provided by E. Oosterwijk, Nijmegen, the Netherlands) by using a BTX electroporator (BTX, San Diego, CA) at 250 V and a capacitance of 2000 μF. Three days after electroporation, the cells were used as target cells in the ^{51}Cr release assay.

Flow cytometry

The expression of different surface molecules on effector and target cells was measured by direct and indirect immunofluorescence and flow cytometry on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cells were washed in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA) and resuspended in 50 μl of PBS/1%BSA. Fifty microliters of the diluted mAb was added and incubated for 30 min at 4 °C. Cells were washed once in PBS/1%BSA, and for the indirect fluorescence, the second step antibody goat-anti-mouse phycoerythrin (PE)-conjugated was added for 30 min at 4 °C. After incubations, cells were washed once in PBS/1%BSA, resuspended in 1% paraformaldehyde containing 1 μg/ml 7-AAD (Brunswig Chemie, Edison, NJ) and analyzed by flow cytometry. Incubation of cells with goat-anti-mouse PE-conjugated served as control staining.

Cytotoxicity assay

Cytotoxic activity was measured in a 4- or 5-h ^{51}Cr-release assay. Briefly, varying numbers of effector cells were added in triplicate to 96-well microtiter plates (100 μl/well), followed by the addition of 2500 target cells (100 μl)/well. The target cells were labeled with 50 μCi ^{51}Cr per $0{,}5x10^6$ cells for 2 h at 37 °C. MAbs were added to the effector cells (CLB-CD2: 10 μg/ml, CLB-CD3: 10 μg/ml, OKT4: 1:4, B116.1: 10 μg/ml, and CLB-CD11a: 10 μg/ml) or to the target cells (W6/32: 10 μg/ml, CLB-CD58: 10 μg/ml, CLB-CD54: 10 μg/ml and G250: 5 μg/ml) 15-30 minutes before the addition of a fixed amount (n=2500) of ^{51}Cr-labeled target cells. At the end of the 4-5-h incubation period (37 °C and 5% CO_2), supernatants were collected using a Skatron harvesting

system (Skatron, Lier, Norway) and counted in a gamma-counter (Wallac, Breda, The Netherlands). Percentage specific lysis was calculated as follows: ((test counts - spontaneous counts) / (maximum counts - spontaneous counts)) x 100%.

Conjugate formation assay

Effector/target cell conjugate formation was assessed by FACS analysis (37). ScFv/γ-transduced CTL ($5x10^6$) were labeled with 0,075 μM Calcein AM (green) (Calbiochem, San Diego, CA) in a volume of 5 ml at 37 °C for 60 min. Target cells ($10x10^6$) were labeled with 40 μg/ml hydroethidine (red) (Molecular Probe Inc., Eugene, OR) in a volume of 1 ml for 30 min at 37 °C. Effector and target cells were washed three times before use and diluted to $10x10^6$ cells/ml. Twenty microliters of labeled target cells were mixed with an equal or 10-fold amount of labeled effector cells with or without blocking mAb. MAbs were incubated with either effector or target cells for 30 min at 4 °C before conjugate formation. The final volume was adjusted to 220 μl. Cell mixtures were centrifuged for 3 min in a serofuge and conjugates were allowed to form at 21 °C for 15 min. Conjugate assay was stopped by the addition of 500 μl ice-cold PBS and kept on ice until analyzed on a FACScan. Cells were resuspended on a vortex for 5 sec before analysis. Ten thousand target cell events were counted and the total number of events simultaneously emitting red and green fluorescence (conjugates) was divided by the total number of events emitting red fluorescence (target cells), yielding the percentage of target cells that had formed conjugates.

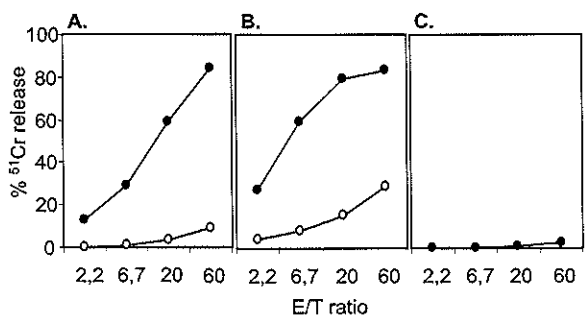

figure 3.1 Cytotoxicity of scFv/γ-transduced CTL against RCC cell lines. ScFv/γ-transduced CTL were incubated with renal carcinoma cell line A75 (A), SK-RC-17 cl.4 (B) and SK-RC-17 (C) and tested in a 4-h ^{51}Cr release assay. Blocking of cytolysis with G250 mAb was performed at a concentration of 5 μg/ml. The specific ^{51}Cr release is depicted at different E/T ratios. (•) scG250-CTL; (o) scG250-CTL + G250 mAb. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from at least two independent experiments.

RESULTS

Cytolysis of RCC by scFv/γ-redirected CTL

To demonstrate redirection of human CTL specificity by molecular grafting with scFv/γ receptor specific for RCC, cytolysis of different RCC cell lines was investigated. RCC A75 expressing G250 Ag was efficiently lysed by scFv/γ-redirected CTL, and binding of parental G250 mAb to the target cells inhibited lysis (fig. 3.1A). No specific lysis was demonstrated against RCC target cell SK-RC-17 not expressing G250 Ag (Fig. 3.1C). Transfection of these G250 Ag^{neg} cell SK-RC-17 with the cDNA encoding G250 Ag resulted in membrane expression of G250 Ag and, consequently, these cells were specifically lysed by the scFv/γ-redirected CTL (fig. 3.1B). This lysis was also inhibited by G250 parental mAb.

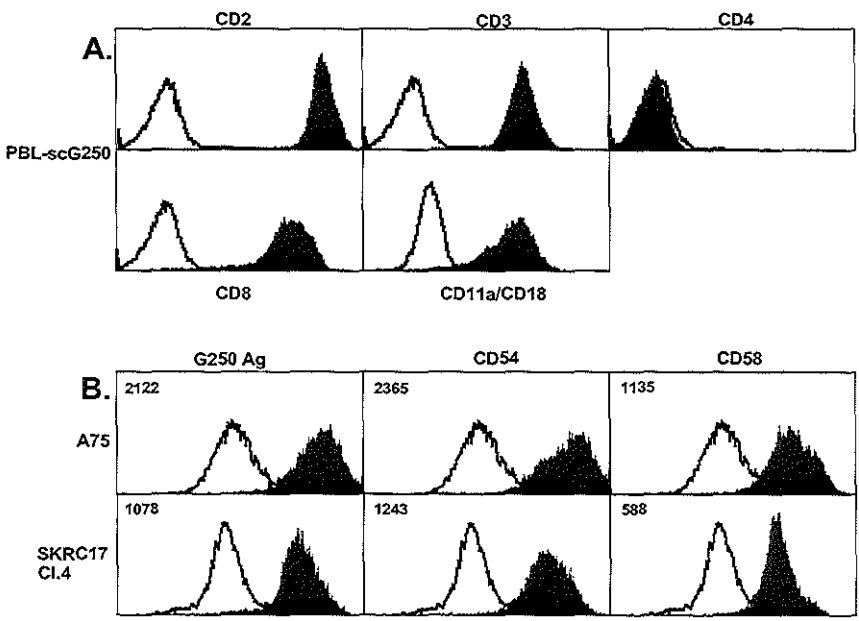

figure 3.2 Cell surface expression of adhesion and accessory molecules on scFv/γ-redirected CTL and RCC. Adhesion and accessory molecules expression was determined by flow cytometry. (A) ScFv/γ^{pos} CTL were stained by indirect immunofluorescence with 2 μg/ml of the following antibodies: anti-CD2 (CLB-CD2), anti-CD3 (CLB-CD3), anti-CD4 (OKT4 culture supernatant), anti-CD8 (B116.1.1) and anti-CD11a/CD18 (CLB-CD11a), followed by incubation with goat-anti-mouse PE-conjugated (GAM-PE). (B) RCC were stained by indirect immunofluorescence with 2 μg/ml of the following antibodies: G250 mAb, anti-CD54 (CLB-CD54) and anti-CD58 (CLB-CD58), followed by incubation with goat-anti-mouse PE-conjugated (GAM-PE). Data are presented in histograms with relative cell number on the y-axis and relative fluorescence intensity (FL2) on the x-axis in log scale. For the RCC A75 and SK-RC-17 cl.4 the mean fluorescence channel is indicated for each sample.

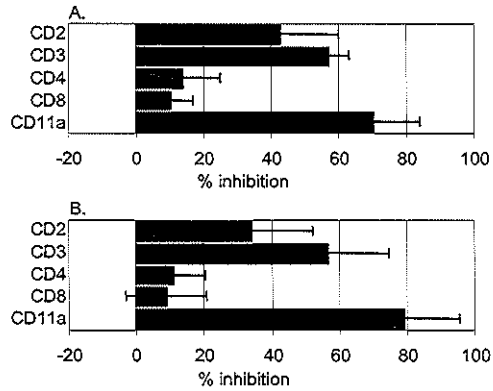

figure 3.3 Effect of mAb binding to scFv/γ-transduced CTL on the scFv/γ-mediated cytolytic activity against RCC A75 (A) and SK-RC-17 cl.4 (B). Percentage lysis in a 5-h ^{51}Cr release assay of A75 was between 55% and 100% (E/T ratio from 20 to 60) and of SK-RC-17 cl.4 between 34% and 100% (E:T ratio from 20 to 60). The following mAb were used: anti-CD2 (CLB-CD2), 10 μg/ml; anti-CD3 (CLB-CD3), 10 μg/ml; anti-CD4 (OKT4 culture supernatant), 1:4; anti-CD8 (B116.1.1), 10 μg/ml and anti-CD11a/CD18 (CLB-CD11a), 10 μg/ml. Mean percentage inhibition of scFv/γ-redirected cytolysis from the control is depicted with standard deviation (n>4).

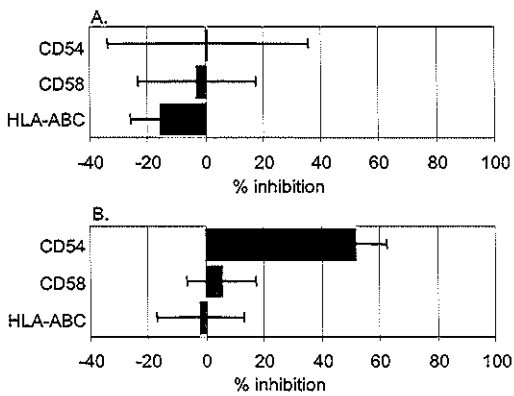

figure 3.4 Effect of mAb binding to the RCC on the cytolytic activity of scFv/γ-transduced CTL against RCC A75 (A) and SK-RC-17 cl.4 (B). Percentage lysis in a 5-h ^{51}Cr release assay of A75 was between 55% and 100% (E:T ratio from 20 to 60) and of SK-RC-17 cl.4 between 34% and 100% (E:T ratio from 20 to 60). The following mAb were used: anti-CD54 (CLB-CD54), 10 μg/ml; anti-CD58 (CLB-CD58), 10 μg/ml and anti-HLA-A,B,C (W6/32), 10 μg/ml. Mean percentage inhibition of scFv/γ-redirected cytolysis from the control is depicted with standard deviation (n>5).

Expression of adhesion and accessory molecules on scFv/γ-redirected CTL and RCC

The expression of adhesion and accessory molecules on scFv/γ-redirected CTL and RCC was analyzed by flow cytometry. All cells in the scFv/γ-transduced bulk population were positively stained with antibodies against CD2, CD3, CD8 and CD11a/CD18 (fig. 3.2A). The RCC A75 and SK-RC-17 cl.4 stained positive with anti-CD54 mAb, anti-CD58 mAb and G250 mAb (fig. 3.2B). RCC A75 showed a higher level of G250 Ag, CD54 and CD58 expression compared to SK-RC-17 cl.4.

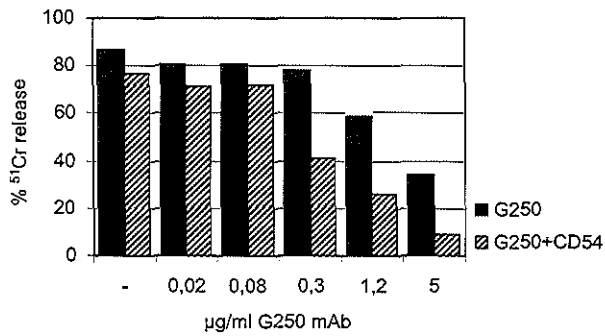

figure 3.5 Binding of G250 mAb to the G250 Ag resulted in anti-CD54 mAb sensitive scFv/γ-redirected lysis of RCC A75. RCC A75 target cells were incubated with or without different concentration of G250 mAb (solid bars) and G250 mAb + anti-CD54 mAb (hatched bars). Cytolysis of RCC by scFv/γ^{pos} CTL was measured in a 5-h ^{51}Cr release assay. Blocking of cytolysis with G250 mAb was performed at the indicated concentrations and blocking with anti-CD54 mAb at 10 µg/ml. The specific ^{51}Cr release is depicted at an E:T of 60. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from two independent experiments.

Addition of anti-CD2, -CD3 and -CD11a/CD18 mAbs to the CTL inhibits scFv/γ-redirected tumor cell lysis

To investigate whether the newly expressed scFv/γ chimeric receptor could functionally interact with adhesion and accessory molecules like the endogenous TCR, we performed studies in which lysis of the RCC A75 and SK-RC-17 cl.4 was performed in the presence of soluble mAbs against CD2, CD3, CD4, CD8 and CD11a/CD18. As shown in fig. 3.3A,B, binding of soluble mAb to CD8, which is involved in binding and signaltransduction of MHC class I-restricted cytolysis, expectedly had no effect on the level of cytolysis, demonstrating the anticipated lack of MHC restriction of scFv/γ-Ag interaction. Anti-CD4 mAb served as negative control in this experiment, since no CD4-positive T cells were present in the culture. The importance of CD2 and CD11a/CD18 adhesion molecules in T cell-mediated RCC lysis was clearly demonstrated by the fact that target cell lysis was indeed inhibited by the relevant mAbs. Interestingly, binding of anti-CD3 mAb to the scFv/γ^{pos} CTL/target cell mixture resulted

in a significant (57%) inhibition of cytolysis of both A75 and SK-RC-17 cl.4 RCC cells (fig. 3.3A,B). Effector/target cell conjugate formation was not affected by addition of these mAbs, suggesting that the inhibition of cytolysis represents the delivery of a negative signal to the CTL which blocked triggering of the cytolytic machinery of the scFv/γ receptor following G250 Ag recognition (data not shown).

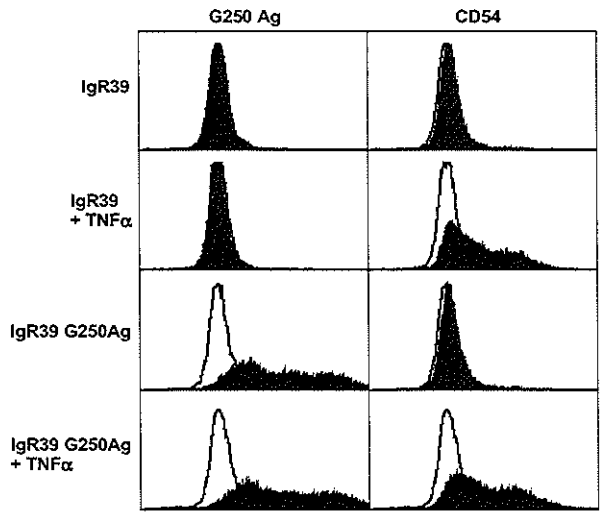

figure 3.6 Cell surface expression of adhesion molecules on melanoma IgR39 and G250 Ag-transfected melanoma IgR39-G250Ag. Adhesion molecules expression was determined by flow cytometry. To induce CD54 expression, IgR39 cells were cultured in the presence of 1000 U/ml recTNFα for 24 h. Cells were stained by indirect immunofluorescence with 2 μg/ml of the following antibodies: anti-CD54 (CLB-CD54) and G250 mAb, followed by incubation with goat-anti-mouse PE-conjugated (GAM-PE). Data are presented in histograms with relative cell number on the y-axis and relative fluorescence intensity (FL2) on the x-axis in log scale.

Addition of anti-CD58 and –CD54 to the target cell inhibits scFv/γ-redirected tumor cell cytolysis, but the level of inhibition depends on the expression level of G250 Ag

Whereas addition of anti-CD58 and anti-HLA-A,B,C to the RCC target cells did not affect cytolysis, anti-CD54 did inhibit cytolysis of RCC SK-RC-17 cl.4 but not of the RCC A75 (fig. 3.4A,B). This differential effect may be due to quantitative differences in tumor cell surface G250 Ag and CD54 expression. Indeed, RCC A75 was found to express high levels of G250 Ag and CD54, whereas RCC SK-RC-17 cl.4 cells exhibited intermediate levels of G250 Ag and CD54 expression. Even the addition of 25 μg/ml of anti-CD54 mAb did not inhibit scFv/γ-redirected cytolysis of RCC A75 (data not shown). This functional correlation between G250 Ag density and susceptibility to lysis was further supported by our finding that, when the levels of free G250 Ag molecules

on RCC A75 by addition of non-saturating concentrations of G250 mAb were decreased, the lysis of RCC A75 became susceptible to inhibition by anti-CD54 mAb (fig. 3.5). Binding of 0,3 μg/ml G250 mAb to RCC A75 resulted in a 50 % decrease in the number of free G250 Ag molecules on RCC A75 as analyzed by FACS with G250 mAb and goat-anti-mouse Ig PE-conjugated (data not shown) and hence A75 now expressed levels of free G250 Ag molecules similar to those expressed by SK-RC-17 cl.4. Binding of anti-CD54 and anti-CD58 mAb to RCC SK-RC-17 cl.4 cells did not affect the percentage of effector/target cell conjugates formed (data not shown).

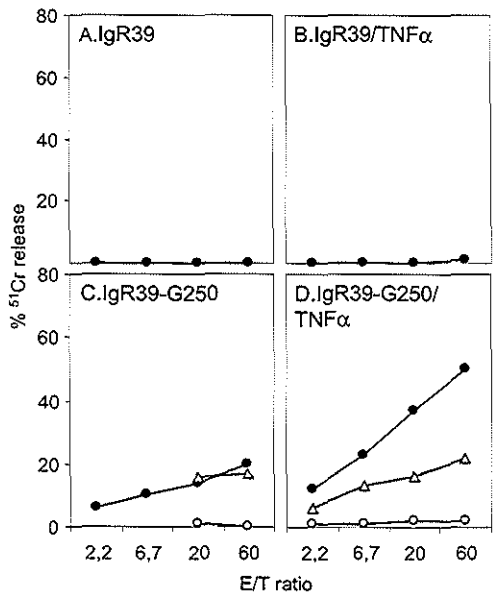

figure 3.7 Cytotoxicity of scFv/γ-transduced CTL against G250 Ag positive and negative melanoma cell lines. ScFv/γ-transduced CTL were incubated with (A) melanoma IgR39 (G250 Ag^{neg}), (B) TNFα-treated IgR39 (G250 Ag^{neg}), (C) IgR39-G250Ag (G250 Ag^{pos}) and (D) TNFα-treated IgR39-G250Ag (G250 Ag^{pos}) and tested in a 4-h ^{51}Cr release assay. For blocking of cytolysis the following mAb were used: anti-CD54 (CLB-CD54; 10 μg/ml) and G250 mAb (5 μg/ml). The specific ^{51}Cr release is depicted at different E:T ratios. (•) scG250-CTL; (o) scG250-CTL + G250 mAb; (Δ) scG250-CTL + CD54 mAb. Experiments were performed in triplicate, and the SD did not exceed 10%.

Cytolysis of G250 Ag^{pos} melanoma cell line by scFv/γ-redirected CTL

To further investigate the coregulation of scFv/γ-mediated CTL cytolysis of target cells, we transfected the G250 Ag gene into melanoma cells which showed no detectable levels of CD54 on the membrane. Transfection of melanoma IgR39 with G250 Ag cDNA resulted in a large percentage (>60%) of cells expressing high levels of G250 Ag (fig. 3.6). At least 25% of the transfected IgR39 showed a G250 Ag expression level that was as high as the dense G250 Ag expression on A75. As expected, parental melanoma

IgR39 cells were not lysed by scFv/γ-redirected CTL, but G250 Ag^{pos} cells were readily lysed by scFv/γ-redirected CTL even in the absence of detectable CD54 (fig. 3.7A,C). This lysis could not be inhibited by the addition of anti-CD54 mAb to the scFv/γ^{pos} CTL/target cell mixture, excluding a role for undetectable low levels of CD54. Addition of tumor necrosis factor α (TNFα) resulted in readily detectable levels of CD54 (fig. 3.6) and, subsequently, the level of scFv/γ-mediated lysis of the G250 Ag^{pos}–$CD54^{pos}$ melanoma cells also increased (fig. 3.7D). To exclude that the induced expression of unknown tumor cell membrane molecules by TNFα was responsible for the increased level of scFv/γ-mediated cytolysis, we added anti-CD54 mAb. The level of cytotoxicity again decreased (fig. 3.7D). The specificity of the interaction was demonstrated by inhibition of scFv/γ-mediated lysis with parental G250 mAb (fig. 3.7C,D).

DISCUSSION

Adhesion and accessory molecules play critical roles in cell-cell interactions in general and in TCR/Ag interactions in particular (35). Here, we identified functional interactions in the cytolytic process of CD2, CD3 and CD11a/CD18 with the chimeric receptor scFv/γ. Moreover, we demonstrated the requirement for CD11a/CD18-CD54 interaction between T cell and target cell in case the expression level of (free) G250 TAA becomes limiting.

Binding of mAbs to T lymphocyte CD2, CD3 or CD11a/CD18 molecules can either enhance or inhibit T cell functions such as activation, conjugate formation with target cells and/or cytolysis (20, 197, 204, 205). Cross-linking of signaling molecules by mAbs or multimeric ligands that bind to accessory molecules induce T lymphocyte activation, whereas the addition of soluble mAbs to these molecules delivers a negative signal to cells (33). For CD2, CD3 or CD11a/CD18 molecules, both stimulatory and inhibitory signaltransducing properties have been described in normal MHC-restricted TCR/Ag interactions (35), NK cells (36) and bsmAb-mediated interaction (37, 198, 199). A similar involvement of CD11a/CD18 in regulation was demonstrated for chimeric Ig/TCR-mediated cytolysis of target cells when Ag was recognized with low avidity (206). We wanted to determine whether the coregulatory signaling pathways of CD2, CD3 and CD11a/CD18 were also functionally related to the artificially induced tumor-specific scFv/γ receptor expressed on human T lymphocytes. Binding of mAbs to CD2, CD3 and CD11a/CD18 molecules on scFv/γ^{pos} human CTL inhibited cytolysis. Because these antibodies did not inhibit the effector/target cell conjugate formation, we suggest that inhibition of lysis was due to the delivery of an inhibitory signal to the CTL cytoplasm via these coactivation molecules.

At the target cell level no inhibition of conjugate formation or cytolysis occurred following binding of anti-CD58 mAb, indicating that the CTL/target cell CD2-CD58 interactions are not limiting in scFv/γ-redirected lysis. Apparently, the inhibitory effect

of anti-CD2 binding is not the result of interference with the CD2-CD58 interaction, but rather the delivery of a negative signal to the T lymphocyte following binding of soluble anti-CD2 to CD2 molecules. Binding of anti-CD54 mAb to RCC target cells expressing intermediate levels of CD54 and G250 Ag (SK-RC-17 cl.4) inhibits cytolysis, demonstrating the importance of CD11a/CD18-CD54 interaction in scFv/γ-mediated lysis. Effector/target cell conjugate formation was not inhibited by anti-CD54 mAb, suggesting that inhibition of cytolysis was due to interference with the CD11a/CD18-CD54 coactivation signal. Since no inhibition of cytolysis was observed on RCC target cells expressing high levels of both CD54 and G250 Ag (A75), even after binding of an equal or even higher concentration of mAb to CD54, it could be argued that the absence of inhibition by anti-CD54 in cytolysis of high CD54/high G250 Ag expressing cells was due to the involvement of other ligands for CD11a/CD18, such as CD50 and CD102. However, a 50% reduction of free Ag on the RCC A75 by addition of parental G250 mAb to the target cells mimics the 'intermediate' G250 Ag expression level as seen for RCC SK-RC-17 cl.4 and at the same time rendered them sensitive to anti-CD54 mAb inhibition of cytolysis. Hence CD11a/CD18-CD54 adhesion interactions are critical in scFv/γ-mediated lysis but only when the number of Ag on the target cell becomes limiting. High levels of G250 Ag on the target cells bypass the requirement for additional CD11a/CD18-CD54 interaction. We previously demonstrated this to be the case for induction of bsmAb-mediated lysis of melanoma cells IgR39, i.e., high concentration of bsmAb could induced lysis of tumor cells without or with only a FACS-undetectable level of CD54 expression (37). This conclusion is further supported by our finding that lysis of G250 Ag gene-transduced, melanoma cell line IgR39 occurred, engineered to express intermediate and high levels of G250 Ag, and low or no CD54 expression. Upregulation of CD54 expression after TNFα treatment resulted in an increase in the level of scFv/γ-mediated cytolysis, most likely because also the intermediate G250 Ag-expressing melanoma cells become lysed. Although additional coregulatory molecules and their ligands may play a role in the lytic CTL/target cell interactions, our results demonstrate the critical involvement of CD2, CD3 and CD11a/CD18, and for CD11a/CD18-CD54 interaction its requirement becomes apparent at limiting tumor Ag concentrations.

We earlier showed that scFv/γ^{pos} CTL produce cytokine upon RCC interaction, specifically lyse RCC and recycle their lytic capacity (163). Here, we demonstrate that this genetically introduced scFv/γ receptor is functionally coregulated by CD2, CD3 and CD11a/CD18 molecules, as described for endogenous NK receptor, αβ and γδ receptors (16, 20, 35, 36, 207). Because the soluble mAb do not affect effector cell-target cell conjugate formation, we conclude that the inhibition of T cell-mediated cytolysis is due to interference with coregulatory signaling. Hence, the γ-chain in the chimeric receptor performs signaltransducing functions that can be coregulated intracellularly via the CD2, CD3 and CD11a/CD18 signaling pathways. Moreover, CTL/target cell CD11a/CD18-CD54 interactions were required only when the RCC expressed low or intermediate levels of G250 Ag but not at high Ag density. For immunotherapy of cancer this may prove an important advantage, since tumor cell

variants express no or low levels of CD54 (208). Furthermore, in patients with RCC metastases, soluble CD54, which may circulate in body fluids (208, 209), might otherwise inhibit normal cell-cell interaction and hence cytolysis (210, 211).

ACKNOWLEDGEMENTS

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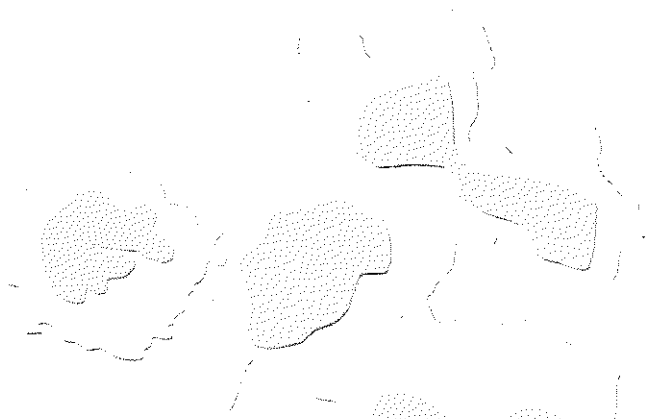

A retroviral vector system 'STITCH' in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T lymphocytes

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SUMMARY

Genetic engineering of T lymphocytes for adoptive clinical immunotherapy calls for efficient gene transduction methods. Therefore, a transient retroviral gene transduction system 'STITCH' was developed comprising pSTITCH retroviral vector encoding the transgene, plasmids encoding Moloney murine leukemia virus gag/pol and gibbon ape leukemia virus envelope, and the human kidney cell line 293T as a packaging line. Co-transfection of retroviral vector and packaging plasmids in 293T cells results in the production of GALV env pseudotyped viral particles with a titer of 10^7 infectious units/ml. The 'STITCH' gene transduction system efficiently transduces genes into activated human T lymphocytes derived from healthy donors and cancer patients. The efficacy of gene transduction is donor-independent. A direct application of 'STITCH' gene transduction system is the genetic engineering of activated human T lymphocytes to induce expression of antibody based chimeric receptors in their membrane. Introduction of these chimeric receptors into activated human T lymphocytes graft these cells with specificity for, for example, renal cell carcinoma. In order to study the effect of the chimeric receptor gene structure on the processes ultimately leading to functional membrane expression, we designed a number of different chimeric receptor gene structures and subsequently compared their membrane expression on 293T cells and activated human T lymphocytes. Distinct membrane expression densities were observed on 293T cells and human T lymphocytes for the different chimeric receptor gene constructs. Gene transduction of activated human T lymphocytes with 4 out of 5 chimeric receptor gene constructs resulted in functional expression of chimeric receptor as demonstrated by specific recognition and cytolysis of renal cell carcinoma.

INTRODUCTION

T lymphocytes are useful as therapeutic agents in viral diseases (212, 213), and cancer (132, 214, 215). Genetic manipulation of T lymphocytes may improve their efficacy in adoptive immunotherapy, e.g. by the introduction of cytokine encoding genes in tumor infiltrating T lymphocytes (216), introduction of the adenosine deaminase (ADA) gene into T lymphocytes from SCID patients (189, 217), expression of chimeric receptors on the membrane of T lymphocytes to endow tumor specificity (155), or the transfer of suicide genes into donor T lymphocytes to control for allogeneic graft-versus-leukemia (218). Up till now, retroviral gene transduction has been the most efficient and safe method for stable introduction of genes into cells (219). The retroviral vector LXSN and derivatives (186) are widespread used vectors for clinical application.

We have successfully engineered anti-CD3 activated human T lymphocytes with antigen receptors genes to redirect T lymphocytes to renal carcinoma cells (RCC) (163). These chimeric antigen receptor genes comprise the binding site of a RCC specific mAb, G250, and the signaltransducing γ-chain molecule of Fc(ε)RI. We have shown that these chimeric receptor transduced activated human T lymphocytes can specifically recognize and lyse RCC target cells via the transduced chimeric Ig receptor (scFvG250) and produce cytokines following specific target cell interaction. However, the gene transduction efficiency obtained with the LXSN vector is low and requires repeated, laborious selection procedures. Moreover, although functional expression of the chimeric receptor is observed, the membrane expression levels are so low that it can not be detected by flow cytometry. A higher expression level of the chimeric receptor would (a) allow rapid screening and immunoselection of scFvG250 positive T lymphocytes, (b) facilitate receptor structure-function analysis, (c) allow monitoring of trafficking and half-life of the gene transduced T lymphocytes following adoptive *in vivo* transfer to patients and overall quality control assessment; and (d) possibly prolong cytolytic capacity of the T lymphocyte.

In the last few years transient gene transduction systems have been reported that resulted in high titer production of retroviral vector particles, but these are either not readily available for clinical use, or not optimized for gene transduction of human T lymphocytes (220-223). Therefore we set out to construct a retroviral gene transduction system with high gene transduction efficiency and functional gene expression in activated human T lymphocytes. The 'STITCH' transient gene transduction system we developed consists of: (a) the retroviral vector pSTITCH encoding the transgene; (b) the plasmids pHIT60 (222) and pCOLT-GALV encoding Moloney murine leukemia virus gag/pol and gibbon ape leukemia virus envelope, respectively; and (c) the human kidney cell line 293T as the packaging cell line. Cotransfection of all three constructs into 293T cells resulted in high titer production of GALV pseudotyped viral particles, which had earlier been shown to infect a wide range of cell types, including human T and B cells (224, 225). In

addition to efficient introduction of the scFvG250 chimeric gene into the activated human T lymphocytes, functional expression of chimeric single chain receptor requires passage through the quality control mechanism of the endoplasmic reticulum and proper protein folding, transportation to the membrane and linkage to the intracellular signaling pathways. In order to study the relation between gene structure and functional membrane expression, we designed 7 different chimeric receptor gene constructs that vary in: (a) the configuration of the variable heavy and variable light chain sequences; (b) the hinge domain sequence; and (c) the sequence of the transmembrane domain and signaltransducing cytoplasmic tail, and tested expression in 293T cells and activated human T lymphocytes using the 'STITCH' gene transduction system.

Here, we present the development of the 'STITCH' gene transduction system. We show that activated human T lymphocytes of multiple donors are efficiently transduced using the 'STITCH' system within 1 week. An increase in membrane expression of the scFvG250 chimeric receptor construct is observed using pSTITCH retroviral vector in comparison with LXSN vector. Different scFvG250 chimeric receptor gene constructs are functionally expressed in activated human T lymphocytes and their membrane expression levels are dependent on the structures of the scFv chimeric receptor gene dictating the protein three-dimensional structure.

MATERIALS AND METHODS

Cells and antibodies

293T cells (kindly provided by Dr. Y. Soneoka, Oxford, UK) and CF2TH cells (ATCC, CRL-1430) were cultured in DMEM medium (BioWhittaker, Verviers, Belgium) containing 10% BCS (HyClone Laboratories, Inc. Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin. PBL of healthy donors were isolated by centrifugation through Lymphoprep (d=1,077 g/cm^3) (Nycomed Pharma, Oslo, Norway) and activated in Mix-med culture medium (78% RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM)(BioWhittaker, Verviers, Belgium), 20% AIM-V (Life Technologies, Grand Island, NY), 2% heat inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin; (8)) with 10 ng/ml OKT3 (Ortho Diagnostic System, Beerse, Belgium) at a density of 0.5x10^6 cells/ml. After 3 days of activation, lymphocytes were washed and cultured in RPMI culture medium (RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 10% heat inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 360 IU/ml human recombinant IL-2 (rIL-2) (Chiron, Amsterdam, The Netherlands). The following cell lines were used as targets in cytotoxicity assays: SK-RC-17 Cl.1 (mock-transfected RCC cell line) and SK-RC-

17 Cl.4 (G250 Ag cDNA-transfected RCC cell line)(kindly provided by Dr. E. Oosterwijk, Nijmegen, The Netherlands). The antibodies used for staining the cells were: anti-idiotype mAb NUH-31 and NUH-82 (Dr. E. Oosterwijk) and anti-CD24 (CLB-CD24; CLB, Amsterdam, The Netherlands).

STITCH gene transduction system

The basis of pSTITCH retroviral vector is SFG (kindly provided by Dr. I. Riviere) a derivative of MFG (64) which contains 5 mutations at the 5' of the gag sequence. To construct the pSTITCH vector the U3 region of the 5' LTR of SFG was replaced by the human CMV IE promoter/enhancer (isolated from PHIT110, kindly provided by Y.Soneoka) and the plasmid backbone contains the SV40 ori.
All construct were all introduced into pSTITCH by using 5' NcoI site and 3' BamHI site. The HKγ construct was made as described (163) and a NcoI site was introduced at the startcodon by using PCR. The CD4 transmembrane region sequence (nucleotides 1258-1329; (226)) and CD3 ζ-chain sequence (nucleotides 228-568; (185)) were derived from mRNA of human PBL and human CTL clone D11, respectively, by reverse transcriptase followed by PCR. Short G250 joining gene region (J) contained only the first 3 of 11 a.a. of G250 J_K and the first 3 of 14 a.a. of G250 J_H. Hinge-CH2-CH3 region DNA (aminoacid 11 of the hinge domain to 107 of the CH3; (227)) was amplified by PCR from a plasmid containing genomic IgG1 DNA (kindly provided by Dr. E. Timmers). To generate the kappa-heavy (KH) configuration, the same strategy as for HKγ construct was followed. The coding sequence of CD24 was generated by PCR using PUC19-CD24 (kindly provided by Dr. K. Humphries, Vancouver, Canada) introducing a NcoI site at the transcription startcodon and a BamHI site after the stopcodon. LacZ sequence was isolated from pSV-β-galactosidase (Promega Corporation, Madison, WI) by PCR. For cloning into pSTITCH, a NcoI site was introduced at the startcodon and a stopcodon and BamHI site was introduced at position 3754. For the transient packaging system the following plasmids are used: pHIT60, a LXSN based vector containing the gag-pol genes of MLV retrovirus, pHIT456, a LXSN based vector containing the MoMLV envelope gene, both containing the CMV IE promoter/enhancer and SV40 ori (both kindly provided by Dr. Y.Soneoka, London UK; (222)), pCOLT-GALV a vector containing GALV env gene, CMV IE promoter/enhancer, β-globulin intron II and SV40 ori.

Transfection and gene transduction methods

Transient transfections were performed in 293T cells by using a $CaPO_4$ transfection kit (Life Technologies). 293T cells ($2x10^6$) were seeded in 10-mm dishes and 24 h later $CaPO_4$-transfected with 20 μg of the pSTITCH vector. Transfected cells were cultured for another 24 h after which the transfection medium was replaced by 10 ml of fresh medium. The following day, cells were analyzed by FACS for membrane expression of the inserted gene. For stable gene transduction, retroviral particles were generated after transfection of 293T cells with 20 μg of pHIT60, pCOLT-GALV

and pSTITCH retroviral vector by using $CaPO_4$ transfection. Twenty-four hours after transfection, cells were irradiated (25 Gy) and co-cultured for 3 days with $0,5x10^6$ anti-CD3 activated PBL/ml. Cocultivation was performed in RPMI culture medium (RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 10% heat inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin) supplemented with 360 IU/ml human recombinant IL-2 (rIL-2) and 4 µg/ml polybrene (Sigma Chemical Co., St. Louis, MO).

Determination of viral titer

For determination of the virus titer, retroviral particles were generated after transfection of 293T cells with 20 µg of pHIT60, pCOLT-GALV and pSTITCH-lacZ retroviral vector by using $CaPO_4$ transfection. Twenty-four hours after transfection, medium was replaced by 5 ml of fresh medium. Retroviral supernatant was harvest after 24 h and filtered through a 0,45 µm filter (Millipore S.A., Molsheim, France) and diluted in medium. CF2TH cells ($2x10^4$) were seeded in 6-wells culture plates and 24 h later medium was replaced by viral supernatant dilutions supplemented with 8 µg/ml polybrene. After 4 days the percentage of positive colonies was counted after staining with X-gal (228).

Immunomagnetic purification of scFvG250^{pos} T lymphocytes

scFvG250^{pos} T lymphocytes were purified by using immunomagnetic isolation with NUH-31 mAb. T lymphocytes ($5x10^6$) were incubated with 10 µl NUH-31 mAb (2 µg/ml) for 30 min at 4 °C. After incubation cells were washed twice and resuspended in 250 µl of culture medium. Magnetic particles (25 µl) (Magnisort, Dupont, Wilmington, DE) were washed twice and also resuspended in 250 µl culture medium. Magnetic particles were added to the cells and incubated on a rollerbank for 30 min at 4 °C. Following incubation, 9,5 ml medium was added and cells were selected against a magnet (Dynal, Oslo, Norway) for 10 min. Medium was replaced by fresh medium and magnet incubation was repeated. Medium from step 1 and 2 was pooled and another 10 min incubation against the magnet was performed. Cells containing the magnetic particles were cultured and 4 days later analyzed on FACS with anti-idiotype mAb NUH-82 and goat-anti-mouse Ig PE-conjugated for expression of the chimeric receptor.

Flow cytometry

The membrane expression of different molecules on 293T cells and T lymphocytes was measured by direct and indirect immunofluorescence and flow cytometry on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cells were washed in PBS containing 1% BSA (PBS/BSA) and resuspended in 50 µl of PBS/1%BSA. Fifty microliter of the diluted mAb was added and incubated for 30 min at 4 °C. Cells were washed once in PBS/1%BSA and for the indirect fluorescence the second step antibody goat-anti-mouse Ig PE-conjugated was added for 30 min at 4 °C. After incubations cells were washed once in PBS/1%BSA, resuspended in 1%

paraformaldehyde containing 1 μg/ml 7-AAD (Brunswig Chemie, Edison, NJ) and analyzed by flow cytometry. Treatment with goat-anti-mouse Ig PE-conjugated served as control staining.

Cytotoxicity assay

Cytotoxic activity was measured in a 4-h ^{51}Cr-release assay. Briefly, varying numbers of effector cells were added in triplicate to 96-well microtiter plates (100 μl/well), followed by the addition of 2500 target cells (100 μl)/well. The target cells were labeled with 50 μCi ^{51}Cr per 0,5x10^6 cells for 2 h at 37 °C. At the end of the 4-h incubation period (37 °C and 5% CO_2), supernatants were collected by using a Skatron harvesting system (Skatron, Lier, Norway) and counted in a gamma-counter (Wallac, Breda, The Netherlands). Percentage specific lysis was calculated as follows: ((test counts - spontaneous counts) / (maximum counts - spontaneous counts)) x 100%.

RESULTS

STITCH transient gene transduction system

The 'STITCH' transient gene transduction system comprises: (a) pSTITCH retroviral vector; (b) plasmids pHIT60 and pCOLT-GALV; and (c) 293T packaging cells. Because clinical application of gene-transduced activated human T lymphocytes requires high gene transduction efficiencies, it was developed to produce high titer of recombinant pseudotyped retroviral vectors in less then one week. pSTITCH retroviral vector contains splice donor/splice acceptor sides and the 5' retroviral gag sequence for stable encapsidation and better transcription and packaging of the transgene as described for the MFG retroviral vector (64). In addition, pSTITCH was constructed to contain both the human CMV IE promoter/enhancer and the SV40 ori to further increase the viral titer (fig. 4.1). In combination with the use of the 293T packaging cell line this was expected to result in high level of expression of the transgene because: (a) constructs containing the human CMV IE promoter/enhancer will be activated by adenovirus early proteins that are present in 293T cells (229); and (b) 293T cells express the SV40 large T antigen which increases the copy number of constructs containing the SV40 ori (222, 230). However, high level of retroviral particles production not only requires efficient expression of the transgene but also of the packaging plasmids. Therefore, the packaging plasmids pHIT60 and pCOLT-GALV also contain the human CMV IE promoter/enhancer and the SV40 ori (fig. 4.1). Indeed high titers of retroviral particles (10^7 infectious units/ml) were obtained by using the pSTITCH vector and 293T packaging cells. This was demonstrated after transfection of pSTITCH-lacZ vector and packaging plasmids in 293T cells and subsequent titration of the obtained

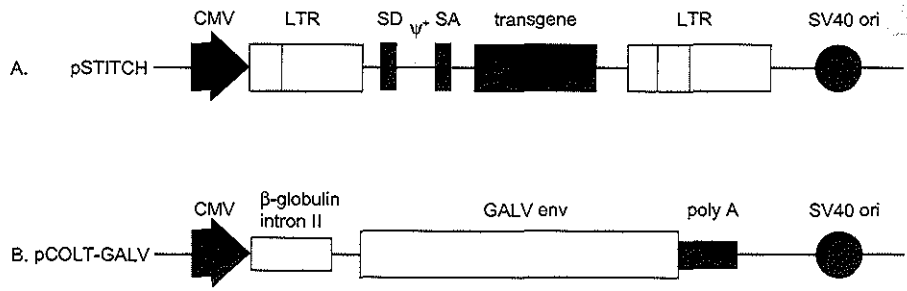

figure 4.1 Retroviral vector and envelope construct. (A) pSTITCH retroviral vector: U3 region of the 5' long terminal repeat (LTR) has been replaced by the human CMV IE promoter/enhancer. Also included in the vector are the extended Moloney Murine Leukemia Virus (MoMuLV) packaging signal (Ψ) splice donor and splice acceptor sides (SD, SA), a cloning site at the envelope gene position, and the 3' MoMuLV LTR. The viral sequences are cloned in an SV40 ori containing plasmid, (B) Gibbon ape Leukemia Virus (GaLV) envelope gene cloned in expression plasmid pCOLT containing the CMV promoter/enhancer, β-globulin intron sequence and SV40 ori.

retroviral particles on CF2TH cells and counting of the gene-transduced cells after X-gal staining (data not shown).

Use of GALV envelope pseudotyped virus particles for gene transduction of activated human T lymphocytes results in higher gene transduction efficiency

To determine the gene transduction efficiency of MoMLV and GALV env pseudotyped retroviral particles on the one hand and to investigate whether the efficiencies are donor-dependent on the other, we set out to transduce activated human T lymphocytes derived from 6 healthy donors with either GALV env pseudotyped or MoMLV retroviral particles. CD24 was used as a marker gene to determine the percentage of gene-transduced T lymphocytes because CD24 can be expressed on the membrane of T lymphocytes and easily be detected by flow cytometry (231). Gene transduction of activated human T lymphocytes was accomplished after cocultivation of anti-CD3 activated PBL with irradiated 293T packaging cells producing pSTITCH-CD24 retroviral virus particles by using either GALV env or MoMLV env. The percentage of $CD24^{Pos}$ T lymphocytes was determined by FACS with anti-CD24 Ig FITC conjugated. FACS analysis showed that the percentage of $CD24^{Pos}$ T lymphocytes from all donors was higher after gene transduction using retroviral particles with GALV env (20%) than with MoMLV env (8%) (table 4.1). It had to be excluded that the observed differences in gene transduction efficiency in T lymphocytes with GALV env pseudotyped and MoMLV

retroviral vector particles were due to differences in viral titers. Hence, the infection of retroviral vector particles on CF2TH cells was determined because these cells are susceptible to gene transduction by GALV as well as MoMLV viruses. Infection of CF2TH cells showed an even better gene transduction efficiency with MoMLV env retroviral particles than with GALV pseudotyped retroviral particles (data not shown).

table 4.1 Increased gene transduction efficiency in activated human T lymphocytes derived from 6 healthy donors with GALV env pseudotyped retroviral vectors

Donor	1	2	3	4	5	6
	% $CD24^{Pos}$ T lymphocytes[a]					
MoMLV env	10	8	8	8	7	6
GALV env	26	21	22	21	17	20

[a]Activated human T lymphocytes were gene transduced by cocultivation with 293T cells producing either CD24 MoMLV env or GALV env pseudotyped retroviral vectors. Three days after gene transduction the percentage of $CD24^{Pos}$ T lymphocytes was determined by FACS with anti-CD24 Ig FITC-conjugated. Percentage $CD24^{Pos}$ T lymphocytes above background staining is presented.

Increased membrane expression of scFvG250 chimeric receptor on activated human T lymphocytes following gene transfer with pSTITCH versus LXSN retroviral vector

The 'STITCH' gene transduction system has been developed to result in both high gene transduction efficiency and functional gene expression in activated human T lymphocytes. A high gene transduction efficiency of activated human T lymphocytes using pSTITCH retroviral vector with GALV env pseudotyped virus particles *versus* MLV env retroviral particles has been demonstrated. We also compared scFvG250 chimeric receptor gene expression in retroviral vector pSTITCH *versus* LXSN. Construct 'A', which contains the variable heavy chain linked to the variable kappa chain gene sequence of the mAb G250 and the γ-chain of the Fc(ε)RI (fig. 4.2A), was used previously for gene transduction of activated human T lymphocytes. Activated human T lymphocytes were transduced with gene construct 'A' in LXSN retroviral vector and showed functional expression of the scFvG250 chimeric receptor, but the level of membrane expression could not be detected by flow cytometry even after G418 selection of the gene-transduced T lymphocytes (163). Transduction of activated human T lymphocytes derived from the same donor with construct 'A' in pSTITCH retroviral vector, however, resulted in a membrane expression of the chimeric receptor on the T lymphocytes as determined by flow cytometry after staining with anti-idiotype mAb (NUH-82) and goat-anti-mouse PE-conjugated (fig. 4.4A).

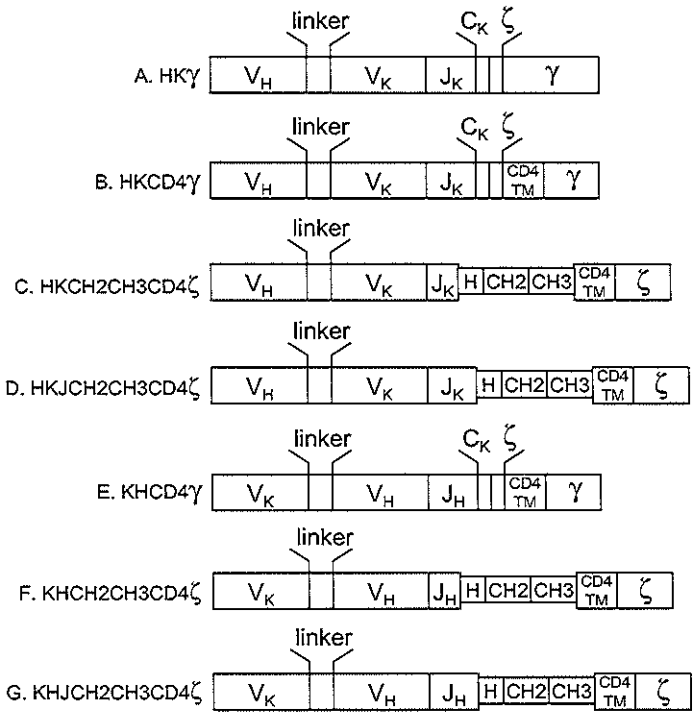

figure 4.2 Single chain G250 chimeric receptor constructs. Single chain G250 constructs comprising the G250 mAb V-heavy/V-kappa (A-D) or the V-kappa/V-heavy (E-G) configuration have been coupled to (A) the complete joining gene segment (J_K), $C_K\zeta$ and the signaltransducing Fc(ε)RI γ-chain; (B) J_K, $C_K\zeta$, CD4 transmembrane region and signaltransducing cytoplasmic γ-chain part; (C) short joining gene segment coupled to Ig hinge region and CH2CH3 regions, CD4 transmembrane region and signaltransducing cytoplasmic ζ-chain part from the TCR/CD3 complex; (D) complete joining region coupled to Ig hinge region and CH2CH3 regions, CD4 transmembrane region and signaltransducing cytoplasmic ζ-chain part; (E) the complete joining gene segment (J_H), $C_H\zeta$, CD4 transmembrane region and signaltransducing cytoplasmic γ-chain part; (F) short joining gene segment (J_H) coupled to Ig hinge region and CH2CH3 regions, CD4 transmembrane region and signaltransducing cytoplasmic ζ-chain part from the TCR/CD3 complex; (G) complete joining region (J_H) coupled to Ig hinge region and CH2CH3 regions, CD4 transmembrane region and signaltransducing cytoplasmic ζ-chain part.

Construction of scFvG250 chimeric receptor genes

To optimize membrane expression which allows immunoselection of receptor expressing T lymphocytes, structure-function analysis and monitoring of gene-transduced T lymphocytes following adoptive *in vivo* transfer, 7 different scFvG250 genes were constructed (fig. 4.2). Replacement of the transmembrane region of the γ-chain by the transmembrane CD4 region in construct 'B' removes the dimerization signal present in construct 'A'. This will result in expression of the scFvG250 as a monomeric molecule (fig. 4.2B). It has been described that a spacer segment

inserted between the scFv and the signaltransducing ζ-chain is a structural prerequisite for antigen binding (232). Constructs 'A' and 'B' contain only a small spacer region (15 a.a.), comprising a part of the constant kappa chain and an extracellular ζ-gene sequence, located between the scFvG250 and the transmembrane region. Thus, constructs 'C' and 'D' were generated to contain a longer spacer by inserting a long hinge region between the scFvG250 and the CD4 transmembrane region. A hinge sequence was selected consisting of the Ig heavy chain constant hinge-CH2-CH3 region to facilitate high level surface expression of chimeric receptors (162). In addition, in construct 'C' the J_K region was shortened from 11 to 3 a.a. to reduce the flexibility of the scFv and to acquire a more rigid, TCR-like structure. These scFvG250 constructs were coupled to the signaltransducing TCR/CD3 ζ-chain (fig. 4.2C and D). Finally, in order to study the effect that scFvG250 gene configuration may have on chimeric receptor protein folding, transport, membrane density and functional T lymphocyte membrane expression, constructs 'E', 'F' and 'G' were generated: the scFvG250 domain was inverted into variable kappa chain linked to the variable heavy chain (fig. 4.2E-G).

Membrane expression levels of distinct scFvG250 molecules on 293T cells

To test the level of membrane expression of the scFvG250 constructs, gene transfection experiments with the pSTITCH scFvG250 constructs were performed in 293T cells. The 293T cells are very suitable to quantify expression of different retroviral vector constructs because transfection efficiencies of 50%-70% can be obtained in these cells using the $CaPO_4$ transfection method. We assumed that protein folding and transport to the membrane of chimeric receptors in 293T cells would be representative for other human cells, including activated human T lymphocytes. $CaPO_4$ transfection of 293T cells with pSTITCH-scFvG250 constructs 'A' to 'G' resulted in membrane expression of scFvG250 receptor with 5 out of 7 pSTITCH-scFvG250 constructs as analyzed by FACS after staining with NUH-31 mAb and goat-anti-ouse Ig PE-conjugated (fig. 4.3).

High level of membrane expression on activated human T lymphocytes by using pSTITCH-HKJCH2CH3CD4ζ construct

To test if the observed difference in membrane expression of the scFvG250 (fig. 4.2) on 293T cells would also be found for activated human T lymphocytes, we compared the membrane expression of different scFvG250 chimeric receptors after gene transduction of activated human T lymphocytes with constructs 'A' to 'E' (fig. 4.2A-E) in pSTITCH. Gene transduction was performed as described by using cocultivation of OKT3-activated PBL with 293T cells which produce GALV env pseudotyped retroviral particles. After gene transduction, clear differences in percentage of scFvG250 chimeric receptor positive T lymphocytes were observed by FACS analysis after staining with anti-idiotype mAb (NUH-31) and goat-anti-mouse Ig PE-conjugated. Similar to the result obtained in 293T cells, activated human T lymphocytes transduced with pSTITCH-HKCH2CH3CD4ζ construct (construct

'C') did not show any membrane expression of the chimeric receptor by FACS analysis, nor RCC specific cytolysis (see below). To compare scFvG250 receptor membrane expression densities on the gene transduced T lymphocytes better we enriched the scFvG250^{pos} population. This was done by positive selection of scFvG250-transduced T lymphocytes with NUH-31 mAb and magnetic beads coated with GAM Ig. Chimeric receptor membrane expression was determined by FACS with anti-idiotype mAb NUH-82 and goat-anti-mouse Ig PE-conjugated after culturing the scFvG250^{pos} enriched T lymphocyte population for 1 week and the mean fluorescence channel number of scFvG250 membrane expression was determined (figs. 4.4A-E). Transduction of activated human T lymphocytes with pSTITCH-HKJCH2CH3CD4ζ construct resulted in the highest density of membrane expression of scFvG250 chimeric receptor on activated human T lymphocytes (fig. 4.2D).

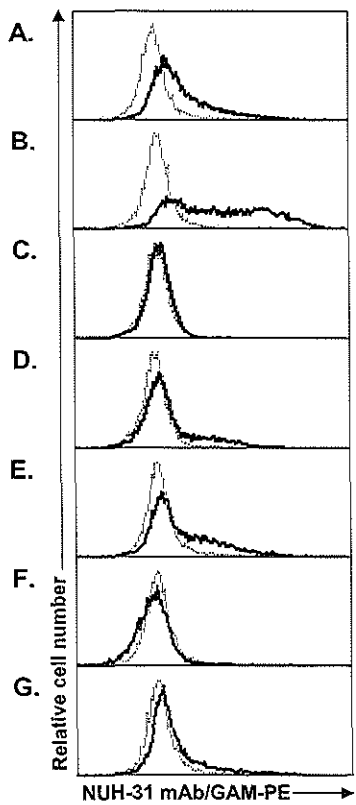

figure 4.3 Cell surface expression of scFvG250 constructs on 293T cells. scFvG250 expression was determined by flow cytometry after transfection of 293T cells with different pSTITCH-scFvG250 constructs ('A'-'G' as indicated in fig. 4.2). Cells were stained by indirect immunofluorescence with 2 μg/ml of the NUH-31 mAb followed by incubation with goat-anti-mouse Ig PE-conjugated (GAM-PE). Data are presented in histograms with relative cell numbers on the y-axis and relative fluorescence intensity (FL2) on the x-axis in log scale.

ScFvG250 chimeric receptor membrane expression on activated human T lymphocytes results in RCC specific cytolysis

Different densities of scFvG250 chimeric receptor membrane expression were observed on scFvG250 gene transduced activated human T lymphocytes (fig. 4.4A-E). Therefore we tested the level of functional expression of scFvG250 constructs 'A' to 'E' on activated human T lymphocytes in cytotoxicity assays after gene transduction and immuno selection with NUH-31 mAb and magnetic beads (fig. 4.5). Activated human T lymphocytes transduced with pSTITCH-HKCH2CH3CD4ζ construct (fig. 4.2C) that did not show membrane expression of the chimeric receptor did not lyse G250 Ag^{pos} RCC. Activated human T lymphocytes transduced with the 4 other constructs demonstrated high levels of G250 Ag^{pos} RCC-specific lysis. Despite the different membrane expression densities, levels of RCC-specific cytolysis were similar.

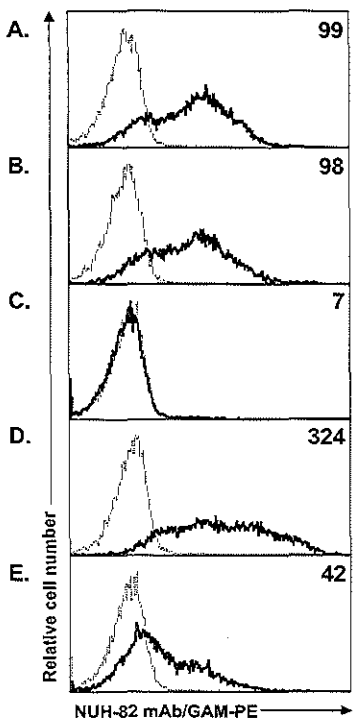

figure 4.4 Cell surface expression of scFvG250 construct on activated human T lymphocytes. scFvG250 expression was determined by flow cytometry after gene transduction of activated human T lymphocytes with different pSTITCH-scFvG250 constructs ('A'-'E' as indicated in fig. 4.2) after NUH-31 immunomagnetic selection. Gene-transduced T lymphocytes were stained by indirect immunofluorescence with NUH-82 mAb (20 µl culture supernatant) followed by incubation with GAM-PE. Data are presented in histograms with relative cell number on the y-axis and relative fluorescence intensity (FL2) on the x-axis in log scale and the mean fluorescence channel is indicated for each sample.

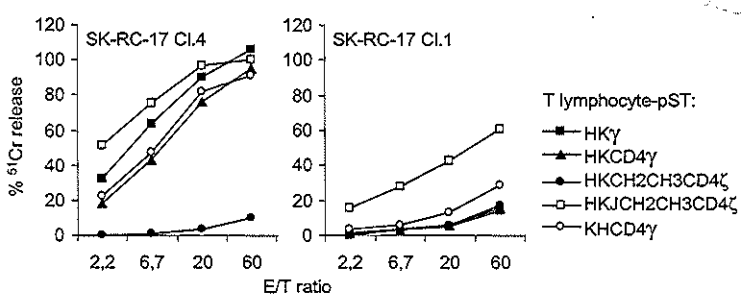

figure 4.5 Cytotoxicity of pSTITCH-scFvG250-transduced activated human T lymphocytes against RCC. Activated human T lymphocytes, transduced with different pSTITCH-scFvG250 constructs ('A'-'E' as indicated in fig. 4.2) and NUH-31 immunomagnetic selected for scFvG250 membrane expression, were incubated with RCC (SK-RC-17 cl.4, a G250 Ag^{pos} RCC and SK-RC-17 cl.1, a G250 Ag^{neg} RCC) and tested in a 4-h ^{51}Cr release assay. The specific ^{51}Cr release is depicted at different E:T ratios. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from at least two independent experiments.

DISCUSSION

Genetic modification of activated human T lymphocytes allows receptor structure analysis in relation to its function and its signaling pathways. Moreover, it broadens application of human T lymphocytes in clinical adoptive immunotherapy. To this end, an efficient gene transduction system is required. Here we describe the development of a transient gene transduction system 'STITCH' that efficiently introduces genes into activated human T lymphocytes. This will ultimately allow to study: the influence of scFv gene structure; the distinct phases of gene transduction efficiency; DNA transcription; protein folding; intracellular transport; membrane expression; affinity of Ag recognition and signaltransduction mechanisms. Transfection of pSTITCH retroviral vector, a GALV env gene construct and MoMLV gag-pol gene plasmids into 293T cells, resulted in production of retroviral vector particles with a titer of 10^7 c.f.u./ml in less than one week. High titer production of retroviral vector particles was the result of the combination of vector and packaging cell line in a transient virus producing system. Transient systems have been described earlier but are either not available for clinical applications or could be improved by increase of virus titer by incorporation of the SV40 ori in the retroviral vector (220-223).

pSTITCH was constructed to contain the SFG vector regions that increases the titer of virus production, as well as expression level of the gene transduced in the cells as earlier described for MFG retroviral vector (64, 233). Indeed, comparison of scFvG250 chimeric receptor gene expression in both LXSN and pSTITCH retroviral vector clearly showed a higher level of chimeric receptor expression using pSTITCH

retroviral vector. Addition of CMV promoter and SV40 ori in pSTITCH was expected to result in high level of protein production due to efficient CMV promoter regulated transcription and SV40 large T induced amplification of plasmid template in 293T cells (222, 229). Together these features make pSTITCH vector very applicable for use in a transient packaging system using 293T cells. In addition to high titer production of retroviral particles, the efficiency of gene transduction is determined by the susceptibility of the type of target cell, e.g. activated human T lymphocytes, for gene transduction with the retrovirus. The receptor for GALV env viruses is expressed on hemopoietic stemcells at high levels and better gene transduction efficiencies were demonstrated in activated human T lymphocytes (224, 225). We showed higher gene transduction efficiency with GALV env pseudotyped retroviral particles in activated human T lymphocytes derived from 6 individuals, demonstrating that this increased gene transduction efficiency is not donor-dependent.

We reported earlier the construction and functional expression of a scFvG250 construct in activated human T lymphocytes using the retroviral vector LXSN (163). Although we observed high levels of RCC-specific scFvG250-redirected cytolysis, the level of chimeric receptor surface expression on the gene-transduced T lymphocytes was too low for detection by FACS analysis with anti-idiotype mAb. High level expression of the chimeric receptor would have several advantages because it will allow immunoselection of receptor expressing cells and structure-function analysis. Furthermore, it may improve cytolytic recycling capacity of the T lymphocyte. Functional membrane expression of chimeric receptors is a result of both gene transcription regulated by the retroviral vector and structure of the inserted chimeric receptor gene. The inserted gene must be translated into a protein, folded into the right configuration, passed through the endoplasmic reticulum control mechanism, transported to the membrane, expressed on the membrane and finally recognize Ag and transduce a functional signal into the T lymphocyte. We assume that man-made molecules like scFv chimeric receptors might not always fold in the right configuration and therefore may become degraded in the endoplasmic reticulum (234). To study the effect of scFv gene structure on the membrane expression of the chimeric Ig receptor receptors we generated 7 different scFvG250 constructs and tested membrane expression on 293T cells ('A' to 'G') and activated human T lymphocytes ('A' to 'E') with pSTITCH retroviral vector. For 5 out of the 7 scFvG250 gene constructs, gene transfers resulted in membrane expression on 293T cells, showing that these 5 gene products were properly folded. ScFvG250 membrane expression on 293T cells appeared representative for expression on the membrane of activated human T lymphocytes. To study membrane expression profiles of the different scFvG250 gene structures, transduced T lymphocytes were enriched using immuno selection with anti-idiotype mAb coated magnetic beads. FACS staining with anti-idiotype mAb of the enriched fractions showed different fluorescence profiles. Activated human T lymphocytes transduced with pSTITCH-HKJCH2CH3CD4ζ (construct 'D') showed the highest density of chimeric receptor

membrane expression. Removal of 8 a.a. of the G250 J_K region in this scFvG250 construct completely abrogated scFvG250 membrane expression and function (construct 'C'). Hence, the presence of the complete J_K gene region appears essential for functional membrane expression of scFvG250 chimeric receptors. Replacement the 15 a.a. spacer region in construct 'A' by an Ig heavy chain constant hinge-CH2-CH3 region (construct 'D') resulted in an even more dense membrane expression on activated human T lymphocytes. We conclude that a small spacer sequence (15 a.a.) together with the complete Ig joining region is already sufficient for functional membrane expression of scFvG250 chimeric receptors. However, other, larger hinge regions, may result in distinct and higher levels of membrane expression. Moreover, the different profiles in membrane expression of the scFvG250 may also reflect differences in gene transfer efficiencies for the scFvG250 constructs.

ScFvG250 mediated antigen recognition and signaltransduction triggered specific cytolysis by these T lymphocytes as was determined in short term ^{51}Cr release assays. Four out of five scFvG250 chimeric receptors were functionally expressed on the activated human T lymphocytes and triggered T cell cytotoxicity. The levels of lytic activity were similar despite the differences in membrane density. These findings confirm our earlier observation that even at FACS undetectable levels of scFvG250 chimeric receptor membrane expression on T lymphocytes of a panel of five different RCC lines were specifically lysed by these scFvG250 gene-transduced T lymphocytes (163).

In conclusion, the 'STITCH' gene transduction system is efficient in (a) expressing gene constructs in 293T cells for rapid screening of membrane expression; (b) production of high titer of retroviral vectors; and (c) introduction of genes and relatively high levels of gene expression in activated human T lymphocytes. We demonstrated efficient gene transduction of optimized scFvG250 chimeric gene constructs in these activated human T lymphocytes, resulting in high density of chimeric receptor membrane expression. Specific cytolysis of cancer cells expressing the relevant antigens was observed. These findings open the way to clinical application with chimeric receptors engineered human T lymphocytes. A clinical phase I/II study is in preparation.

5

Functional balance between T cell chimeric receptor density and tumor associated antigen density: CTL mediated cytolysis and lymphokine production

Mo Weijtens, Emmy Hart, Reinder Bolhuis

Gene Therapy 7: 35-42 (2000)

SUMMARY

Genetically engineered expression of tumor-specific single chain antibody chimeric receptors (ch-Rec) on human T lymphocytes endow these cells with the parental monoclonal antibody (mAb) dictated tumor specificity and may be useful for clinical immuno-gene therapy. Therefore it was of importance to assess how the densities of tumor-specific receptors and tumor associated antigens (TAA), respectively, affect primary human T lymphocyte functions in relation to target cell susceptibilities to lysis. We therefore studied the functional balance between ch-Rec densities on human T lymphocytes and TAA on tumor cells. The gene construct encoding a ch-Rec derived from (a) a renal carcinoma cell (RCC) specific mouse mAb (G250), and (b) the human signal transducing Fc(ε)RI γ-chain was used. To obtain ch-Rec$^{HIGH-POS}$ and ch-Rec$^{LOW-POS}$ T lymphocytes, 2 distinct retroviral vectors were used to introduce the gene constructs into primary human T lymphocytes. Levels of ch-Rec-redirected T lymphocyte mediated tumor cell lysis as well as lymphokine production were determined using RCC lines as target/stimulator cells, which express either no or increasing densities of the TAA. A functional and dynamic balance between ch-Rec densities on cytotoxic T lymphocytes (CTLs) on the one hand and TAA densities on RCCs on the other, was found. In short, ch-Rec$^{HIGH-POS}$ CTLs are triggered by TAA$^{HIGH-POS}$ as well as TAA$^{LOW-POS}$ RCCs to lyse tumor cells and produce (IFN-γ and TNF-α) lymphokine. In contrast, ch-Rec$^{LOW-POS}$ T lymphocytes are only triggered for cytolysis and lymphokine production by relatively TAA$^{HIGH-POS}$ RCCs. In conclusion, (a) the activation of T lymphocyte responses is co-determined by the expression levels of the ch-Rec on T lymphocytes and the TAA on tumor cells and (b) at relatively high T lymphocyte ch-Rec expression levels the CTLs lyse tumor cells with a wide range of TAA densities.

INTRODUCTION

Immune responses of T lymphocytes result from binding of T cell receptors (TCRs) on T lymphocytes to major histocompatibility complex (MHC) / antigen complexes on target cells. This binding leads to TCR-mediated signal transduction and activation of effector functions such as cytolysis and lymphokine production (35, 235). The quantity as well as quality of the functional immune responses are affected by these TCR / antigen interactions. In a model system, T lymphocyte function was triggered *in vitro* by bispecific mAbs (bsmAbs), that interact with the CD3 complex on T lymphocytes and TAA on tumor cells, respectively (138, 145). We and others showed that the level of activation of a T lymphocyte function, e.g. cytolysis, is positively correlated with the amount of bsmAb cross-linking TCRs and activation / target antigens (36, 37, 236), and that following TCR/CD3 complex engagement with these antigens, TCRs on a single CTL that were triggered, become inactivated and degraded (150, 237). Hence, CTLs serially use sets of TCR/CD3 complex for antigen engagement and can serially lyse multiple target cells (150). However, T lymphocytes are not triggered to exert the full range of T cell functions at very low antigen densities. Also, different antigen densities or potencies result in different levels of TCR engagements and are elicit for the triggering of distinct effector functions (150, 238-241). The limited data that are available on the effect of low versus high TCR densities on antigen reactive T lymphocyte responses show a correlation with T lymphocyte activities (239, 241, 242). A detailed understanding of the functional balance between TCR and TAA densities is therefore essential for the generation of optimally anti-tumor reactive T lymphocytes for cancer gene-immunotherapy now that adoptive transfer of tumor-specific CTLs to patients has demonstrated clinical benefit. However, specific CTLs from patients are difficult to isolate and cumbersome to expand to therapeutic numbers and this critically limits their clinical application (44). Fortunately genetic programming of $CD3^{POS}$ T lymphocytes specificity for TAA can be achieved by retroviral transfer of transgenes encoding for a tumor specific mAb-derived ch-Rec (154, 243, 244). Such ch-Rec consists of a mAb-based single chain antibody (scFv), coupled to a Fc(ε)RI γ-chain or CD3ζ-chain, and these ch-Rec can functionally be expressed in the membrane of primary human T lymphocytes (161-163, 245, 246). As for endogenous TCRs, binding of ch-Rec to relevant TAA results in activation of lymphocyte functions, e.g. target cell lysis and lymphokine production (155, 161-163, 245, 246). The ch-Rec-mediated antigen binding is MHC-unrestricted and of high affinity. Moreover, adoptive transfer of genetically engineered $ch\text{-}Rec^{POS}$ CTLs has shown *in vivo* anti-tumor activity in mice (166-168).

We used a chimeric mAb-based receptor derived from the G250 mAb which specifically recognizes G250 TAA on RCCs (247). Ch-Rec gene-transduced primary human $CD3^{POS}$ T lymphocytes specifically: (a) recognize and lyse RCCs in an MHC-unrestricted fashion, and (b) produce lymphokines during interaction with RCCs, in

spite of the fact that the ch-Rec density was too low to be detected by flow cytometric analysis (163, 245, 246). In order to optimize the generation of primary human CTLs with genetically engineered tumor specificity and anti-tumor activity, we developed a retroviral vector system that produces high gene expression and high membrane expression of the ch-Rec by primary human T lymphocytes (246). Because tumor cells *in vivo* express TAA at varying densities we determined how different densities of TAA tumor cells on the one hand and ch-Rec and T lymphocytes on the other affect the quantity and quality of T lymphocyte responses, which are important in anti-cancer immune activities.

We demonstrated that distinct TAA surface expression levels on tumor cells are required for triggering of ch-Rec^{LOW}-versus$^{HIGH-POS}$ primary T lymphocyte functions. In short, ch-Rec$^{HIGH-POS}$ T lymphocytes can be triggered for cytolysis and lymphokine production by both G250 TAA$^{HIGH-POS}$ as well as $^{LOW-POS}$ RCCs. In contrast, ch-Rec$^{LOW-POS}$ T lymphocytes are only activated by TAA$^{HIGH-POS}$ but not $^{LOW-POS}$ RCCs. Hence, the activation level of a CTL is co-determined by TAA as well as ch-Rec cell surface densities. To minimize the chance that TAA$^{LOW-POS}$ tumor cells escape from immune recognition by adoptively transferred T lymphocytes which are genetically programmed with tumor cell specificity, ch-Rec$^{HIGH-POS}$ CTLs, i.e. in the range of physiological TCR densities, are recommended for gene-immunotherapy of cancer.

MATERIALS AND METHODS

Cells and antibodies.

Peripheral blood lymphocytes (PBL) of healthy donors were isolated by centrifugation through Lymphoprep (d=1,077 g/cm^3) (Nycomed, Oslo, Norway) and activated in Mix-med culture medium (78% RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 20% AIM-V (Gibco BRL, Paisley, UK), 2% heat inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin), with 10 ng/ml OKT3 (Ortho, Beerse, Belgium) at a density of 0.5x10^6 cells/ml. Three days after activation, lymphocytes were washed and cultured in RPMI culture medium (RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 10% heat inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin), supplemented with 360 IU/ml human recombinant IL-2 (rIL-2) (Chiron, Amsterdam, the Netherlands). 293T cells (kindly provided by Y. Soneoka, Oxford, UK) were cultured in DMEM medium (BioWhittaker, Verviers, Belgium) containing 10% BCS (HyClone Laboratories, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. The following cell lines were used as targets in cytotoxicity assays: 3 clones of MHC-identical SKRC17 RCC (SKRC17-1: mock-transfected, G250 TAA^{NEG} RCC; SKRC17-2 and –3: G250 TAA cDNA transfected RCC), and 4 clones of MHC-identical SKRC59 cell line (SKRC59-1: G250 TAA^{NEG}

RCC; SKRC59-2, -3 and –4: G250 TAA cDNA transfected RCC) (kindly provided by E. Oosterwijk, Nijmegen, the Netherlands). The antibodies used for FACS staining were: RCC antigen-specific G250 mAb (kindly provided by S. O. Warnaar, Centocor, Leiden, the Netherlands) (247) and anti-idiotype G250 mAb NUH-31 and NUH-82 (kindly provided by E. Oosterwijk, Nijmegen, the Netherlands)(190).

Construction of ch-Rec retroviral vectors

The ch-Rec gene construct HKγ was made as described elsewhere (163) and cloned into the retroviral vector LXSN (186) and pSTITCH (246). For cloning into the vector pSTITCH, a NcoI site was introduced at the start codon using PCR.

ch-Rec gene transduction and selection of gene-transduced human T lymphocytes

Anti-CD3 activated T lymphocytes were gene-transduced with the either the LXSN or pSTITCH retroviral vector containing the ch-Rec construct. Gene transduction using LXSN was performed as described earlier (163). In short, activated human T lymphocytes were co-cultivated for 3 days with the irradiated L(ch-Rec)SN-producing amphotropic packaging cell line PA317 in the presence of 360 IU/ml rIL-2 and 4 μg/ml polybrene (Sigma St. Louis, MO, USA), followed by 9 days G418 selection. LXSN-mediated ch-Rec transduction generates ch-Rec$^{LOW-POS}$ T lymphocytes. For pSTITCH gene transduction, retroviral particles were generated after transfection of 293T cells with 20 μg of pHIT60, pCOLT-GALV and pSTITCH(ch-Rec) retroviral vector by using $CaPO_4$ transfection (246). After 24 hours, transfected cells were irradiated (25 Gy) and co-cultured for 3 days with $0,5x10^6$ anti-CD3 activated human T lymphocytes/ml. Co-cultivation was performed in RPMI culture medium (RPMI 1640 buffered with bicarbonate (2 g/l) and HEPES (25 mM), 10% heat inactivated human serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) supplemented with 360 IU/ml rIL-2 and 4 μg/ml polybrene. ch-Rec^{POS} human T lymphocytes were purified by using immunomagnetic isolation with NUH-31 mAb (248). Gene-transduced human T lymphocytes were expanded in the presence of feeder cells as described previously (7, 187).

Flow cytometry

The membrane expression of G250 TAA on RCC cells and ch-Rec on human T lymphocytes was measured by indirect immunofluorescence and flow cytometry on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Cells were washed in PBS containing 1% BSA (PBS/BSA) and resuspended in 50 μl of PBS/1%BSA. Fifty microliter of the diluted mAb was added and incubated for 30 min at 4 °C. Cells were washed once in PBS/1%BSA and the second step antibody goat-anti-mouse Ig PE conjugated was added for 30 min at 4 °C. After incubations cells were washed once in PBS/1%BSA, resuspended in 1% paraformaldehyde containing 1 μg/ml 7-AAD (Sigma, St. Louis, MO, USA) and analyzed by flow

cytometry. Treatment with goat-anti-mouse Ig PE conjugated served as control staining.

Cytotoxicity assay

Cytotoxic activity was measured in a ^{51}Cr-release assay. Briefly, varying numbers of effector cells were added in triplicate to 96-well microtiter plates (100 µl/well), followed by the addition of 2500 target cells (100 µl)/well. The target cells were labeled with 50 µCi ^{51}Cr per $0{,}5x10^6$ cells for 2 h at 37 °C. At the end of the 4 h incubation period (37 °C and 5% CO_2), supernatants were collected by using a Skatron harvesting system (Skatron, Lier, Norway) and counted in a gamma-counter (Wallac, Breda, the Netherlands). Percentage specific lysis was calculated as follows: ((test counts - spontaneous counts) / (maximum counts - spontaneous counts)) x 100%. Unless otherwise indicated four effector target cells (E/T) ratios were tested (60, 20, 6.7 and 2.2).

Measurement of lymphokine production

To determine lymphokine production by gene-transduced human T lymphocytes upon antigen stimulation, $6x10^4$ gene-transduced human T lymphocytes were cultured for 24 h either in the presence or absence of $2x10^4$ adherent tumor cells in RPMI culture medium containing 360 IU/ml rIL-2. Supernatant was harvested and levels of TNF-α and IFN-γ were measured by ELISA (Medgenix, Fleuris, Belgium) according to suppliers' specification.

RESULTS

Ch-Rec$^{LOW-POS}$ CTLs can only lyse relatively G250 TAA$^{HIGH-POS}$ RCC

G250 TAA densities on different RCC cell lines are shown in figure 5.1. The number of G250 TAA on SKRC17-2 is 16.400 (G250 TAA$^{LOW-POS}$) and on SKRC17-3 465.000 (G250 TAA$^{HIGH-POS}$) as determined by scatchart (Dr. E. Oosterwijk, personal communication). Two distinct viral vectors for ch-Rec gene transduction, LXSN and pSTITCH, yielded 'low' versus high expression levels of ch-Rec on primary human T lymphocytes (246) ('low': negative in flowcytometry but positive in ch-Rec-mediated cytolytic assays (163)). Immunoselection of ch-Rec$^{LOW-POS}$ CTL did not yield ch-Rec$^{HIGH-POS}$ CTL as determined by flowcytometry (data not shown). Therefore LXSN transduced CTL were selected using G418 containing medium since the transgene also comprised the neomycine selection marker. Ch-Rec$^{HIGH-POS}$ T lymphocytes were selected with anti-idiotype coated magnetic beads (fig. 5.2). How TAA density affects signaling strength of CTL was determined by measuring the levels of RCC cytolysis produced by ch-Rec$^{LOW-POS}$ versus$^{HIGH-POS}$ human CTLs. The following cell lines were used as targets: (a) G250 TAA^{NEG} RCCs (SKRC17-1 and

SKRC59-1); (b) G250 TAA$^{LOW-POS}$ (SKRC17-2) and (c) HIGH^{-POS} RCCs (SKRC17-3, SKRC59-2, SKRC59-3 and SKRC59-4; fig. 5.1). As expected, mock transduced CTLs did not lyse G250 TAA^{POS} RCCs and vice versa, G250 TAA^{NEG} RCCs were not lysed by ch-Rec^{POS} CTLs (fig. 5.3A). In contrast, all relatively G250 TAA$^{HIGH-POS}$ RCCs were readily and equally well lysed by ch-Rec$^{LOW-POS}$, i.e. no TAA dose response was observed (fig. 5.3A).

Only a marginal level of lysis of G250 TAA$^{LOW-POS}$ RCCs by ch-Rec$^{LOW-POS}$ CTLs was observed (fig. 5.3B). To exclude that G250 TAA$^{LOW-POS}$ SKRC17-2 cells showed a G250 TAA independent resistance to CTL lysis we added bsmAb CD3xG250 to the CTL/RCC mixture, because bsmAbs cross-link CD3 on CTLs to G250 TAA on RCCs and trigger cytolysis (140). G250 TAA$^{LOW-POS}$ RCCs were readily lysed by the ch-Rec$^{LOW-POS}$ CTLs sensitized with bsmAbs (fig. 5.3B). These results demonstrate that: (a) no 'overall resistance' explained the lack of lysis by ch-Rec$^{LOW-POS}$ CTLs, and (b) G250 TAA were expressed by SKRC17-2 RCCs, as already demonstrated by scatchart analysis (E. Oosterwijk, personal communication). The G250 TAA$^{HIGH-POS}$ RCC cell lines showed increased densities of G250 TAA, but no TAA dose response was observed in combination with ch-Rec$^{LOW-POS}$ CTLs.

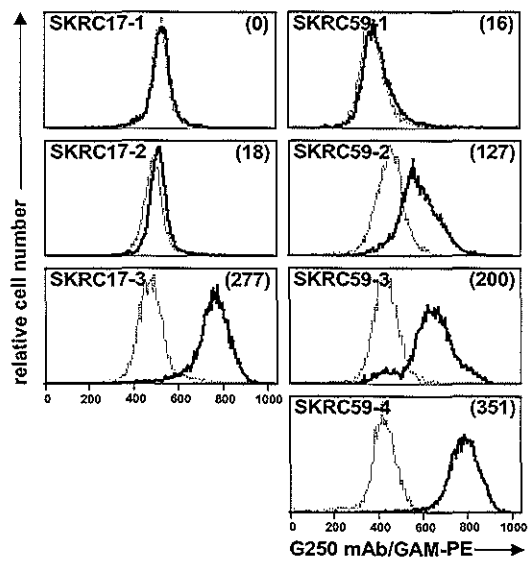

figure 5.1 Cell surface expression densities of G250 TAA on RCC. G250 TAA expression was determined by flow cytometry on the following RCCs SKRC17-1, SKRC17-2, SKRC17-3, SKRC59-1, SKRC59-2, SKRC59-3 and SKRC59-4. RCCs were stained by indirect immunofluorescence with 2 μg/ml of G250 mAb followed by incubation with goat-anti-mouse PE conjugated (GAM-PE) (solid line) or by incubation with GAM-PE alone (dotted line). The difference in linear channel number is indicated for each sample (top right corner). Data are presented in histograms with relative cell number on the y-axis and relative fluorescence intensity (FL2) on the x-axis in log scale.

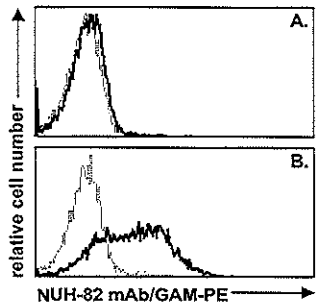

figure 5.2 Different retroviral vectors result in distinct ch-Rec receptor expression levels on human T lymphocytes. ch-Rec expression was determined by flow cytometry after gene transduction of human T lymphocytes with (A) LXSN-HKγ and (B) pSTITCH-HKγ. Gene-transduced human T lymphocytes were stained by indirect immunofluorescence with 2 μg/ml of the NUH-82 mAb followed by incubation with goat-anti-mouse PE conjugated (GAM-PE) (solid line) or by incubation with GAM-PE alone (dotted line). Data are presented in histograms with relative cell number on the y-axis and relative fluorescence intensity (FL2) on the x-axis in log scale.

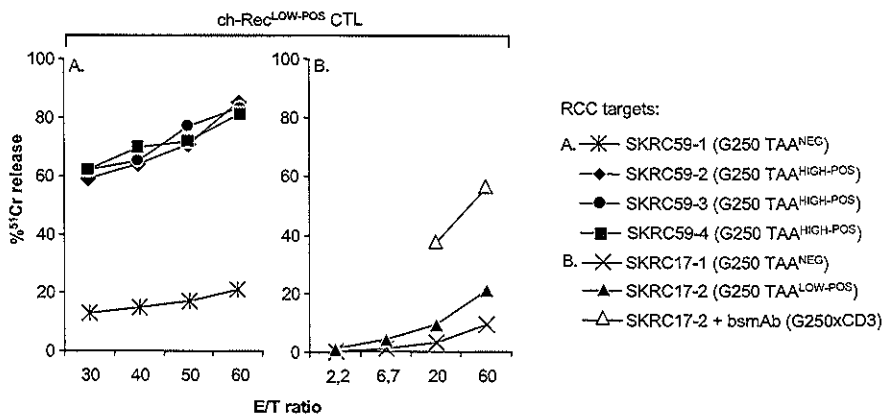

figure 5.3 Cytotoxicity of ch-Rec gene-transduced human T lymphocytes against RCCs with distinct G250 TAA membrane expression densities. LXSN-HKγ gene-transduced human T lymphocytes (ch-Rec$^{LOW-POS}$ CTL) were incubated with the following RCCs: (A) SKRC59-1 (G250 TAA^{NEG} RCC), SKRC59-2, SKRC59-3 and SKRC59-4 (G250 TAA^{POS}, gene transfected RCC), and (B) SKRC17-1 (mock-transfected G250 TAA^{NEG} RCCs), SKRC17-2 (G250 TAA ^{POS}, gene transfected RCCs) and tested in a 4-h ^{51}Cr release assay. BsmAb CD3xG250 (1:5000) was added to the effector/target (E/T) reaction mixture where indicated. The specific ^{51}Cr release is depicted at different E/T ratios. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from at least two independent experiments.

Ch-Rec$^{HIGH-POS}$ CTLs lyse G250 TAA$^{LOW-POS}$ as well as G250 TAA$^{HIGH-POS}$ RCCs

The lack of significant lysis of G250 TAA$^{LOW-POS}$ RCCs by ch-Rec$^{LOW-POS}$ CTLs might be compensated for by using CTLs with increased ch-Rec surface densities. Therefore two retroviral vectors were used in parallel to introduce the same ch-Rec

gene construct into human T lymphocytes derived from the same donor blood sample, LXSN and pSTITCH, respectively (163, 246). The ch-Rec$^{LOW-POS}$ and $^{HIGH-POS}$ primary human T lymphocyte populations were then used as effector cells against G250 (a) TAA^{NEG}, (b) TAA$^{LOW-POS}$ and (c) TAA$^{HIGH-POS}$ target cells (fig. 5.2). G250 TAA$^{LOW-POS}$ RCCs, that were not lysed by ch-Rec$^{LOW-POS}$ CTLs (see above), were efficiently lysed by ch-Rec$^{HIGH-POS}$ CTLs (fig. 5.4), whereas G250 TAA$^{HIGH-POS}$ RCCs were readily lysed by both ch-Rec$^{LOW-POS}$ and$^{HIGH-POS}$ CTLs. In the combination ch-Rec$^{HIGH-POS}$ CTL G250 TAA$^{HIGH-POS}$ RCC lines with increasing TAA densities a TAA dose response curve for CTL lytic activity is observed: the higher the TAA density the higher the percent RCC lysis (fig. 5.4B). Expectedly, mock transduced CTLs did not lyse G250 TAA$^{HIGH-POS}$ or $^{LOW-POS}$ RCCs and ch-Rec^{POS} CTLs did not lyse G250 TAA^{NEG} RCCs.

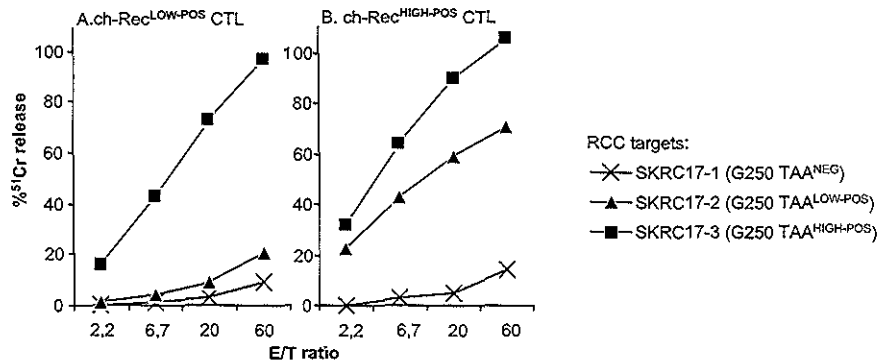

figure 5.4 ch-Rec$^{HIGH-POS}$ CTLs can lyse TAA$^{LOW-POS}$ tumor cells. LXSN-HKγ (A) and pSTITCH-HKγ (B) gene-transduced human T lymphocytes expressing low or high ch-Rec receptor densities, respectively, were incubated with RCC SKRC17-1 (mock-transfected G250 TAA^{NEG} RCC), SKRC17-2 (G250 TAA$^{LOW-POS}$, gene transfected RCC) and SKRC17-3 (G250 TAA$^{HIGH-POS}$, gene transfected RCC), and tested in a 4-h ^{51}Cr release assay. The specific ^{51}Cr release is depicted at different E/T ratios. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from at least two independent experiments.

To illustrate further the functional balance between ch-Rec and G250 TAA densities, we determined the levels of G250 TAA$^{HIGH-POS}$ RCC lysis in the absence and presence of increasing concentrations of parental G250 mAb. G250 TAA^{NEG}, TAA$^{LOW-POS}$ and TAA$^{HIGH-POS}$ RCC cell lines cloned from parental SKRC17 cell line, and hence MHC identical, were used as target cells. By adding increasing concentrations of parental G250 mAb the number of free G250 TAA accessible for ch-Rec^{POS} receptor engagement becomes gradually decreased mimicking decreasing TAA densities. Even high concentrations (10-50 μg) of 'blocking' G250 mAb cannot totally block the lysis of G250 TAA$^{HIGH-POS}$ RCC by ch-Rec$^{HIGH-POS}$ CTLs. In conclusion, the higher the concentrations of blocking G250 mAb present the less G250 TAA is accessible for ch-Rec engagement as reflected by lower levels of target cell lysis (fig. 5.5). This is

further substantiated by the fact that lysis of TAA$^{LOW-POS}$ RCC by ch-Rec$^{HIGH-POS}$ CTL can be blocked by as little as 5 μg/ml of G250 mAb. This observed TAA dose response curve for ch-Rec$^{HIGH-POS}$ CTL lytic activity confirms the dose-response results presented in figs 4A and 4B. From the results shown in figure 5.5 (hatched bars) it can also be concluded that the more TAA are available for ch-Rec engagement the higher the percent tumor cell lysis occurs per unit time, provided that the density of free TCRs on CTLs is not limiting, i.e. only in combination with ch-Rec$^{HIGH-POS}$ CTLs. Lysis of G250 TAA$^{HIGH-POS}$ RCCs by ch-Rec$^{LOW-POS}$ CTL was already blocked at 1 μg/ml of G250 mAb. For comparison, only partial inhibition of lysis of G250 TAA$^{HIGH-POS}$ RCCs (SKRC17-3) by ch-Rec$^{HIGH-POS}$ CTLs already required a 50 times higher concentration of G250 mAb.

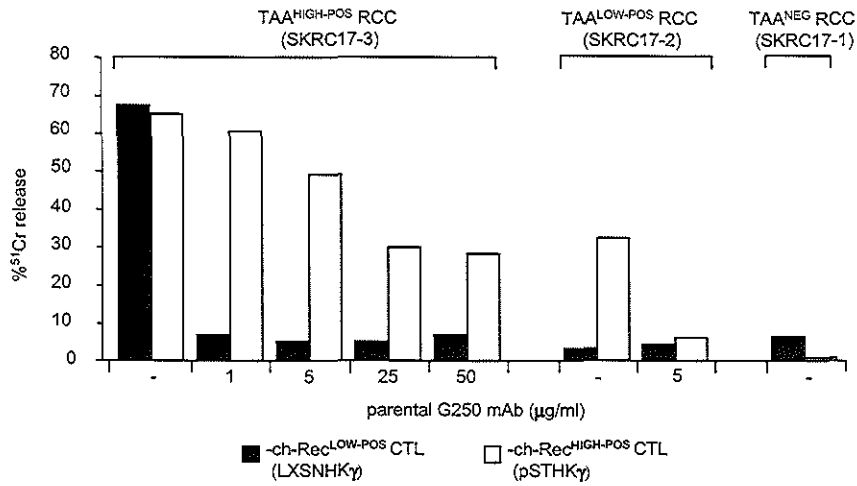

figure 5.5 Gene-transduced T lymphocytes with low ch-Rec membrane density are more sensitive to inhibition of cytolysis with G250 mAb. RCCs SKRC17-3 and SKRC17-2 target cells were incubated with or without various concentration of G250 mAb. Cytolysis of SKRC17-1 (mock-transfected G250 TAA^{NEG} RCC), SKRC17-2 (G250 TAA$^{LOW-POS}$, gene-transfected RCCs) and SKRC17-3 (G250 TAA$^{HIGH-POS}$, gene-transfected RCCs) by ch-Rec$^{LOW-POS}$ and ch-Rec$^{HIGH-POS}$ CTL was measured in a 4-h ^{51}Cr release assay. Blocking of cytolysis with G250 mAb was performed at the indicated concentrations. The specific ^{51}Cr release is depicted at an E/T of 20. Experiments were performed in triplicate, and the SD did not exceed 10%. Similar results were obtained from two independent experiments.

Kinetics of tumor cell lysis do not depend on chimeric receptor density

Now that the functional balance between ch-Rec densities on CTLs and of TAA on tumor cells in relation to triggering of effector functions has been shown, we investigated the effect of TAA density on the kinetics of target cell lysis. Ch-Rec^{LOW} and $^{HIGH-POS}$ CTLs were mixed with G250 TAA^{NEG}, G250 TAA$^{LOW-POS}$ and G250 TAA$^{HIGH-POS}$ RCCs for 2, 4, 6 and 8 h respectively (fig. 5.6). Again, the percentage of RCCs lysed per unit of time correlated with the G250 TAA density on the RCCs,

provided the ch-Rec density was not limiting, i.e. in combination with ch-Rec$^{HIGH-POS}$ CTLs. Neither ch-Rec$^{LOW-POS}$ nor $^{HIGH-POS}$ CTLs reached plateau levels of RCC lysis during up to 8 hrs of incubation with RCC, irrespective of their G250 TAA expression level.

Chimeric receptor and TAA densities also co-determine levels of T lymphocyte IFN-γ and TNF-α production

Individual CTLs can exercise distinct biological responses as a function of percentage of TCR occupancy (150, 238-242), which in turn is determined by ligand density and potency (240). Therefore we studied the capacity of ch-Rec^{LOW} and $^{HIGH-POS}$ T lymphocytes to secrete INF-γ and TNF-α in response to G250 TAA^{LOW} and $^{HIGH-POS}$ RCCs. For lymphokine production the same functional balance was seen between the densities of ch-Rec receptors and G250 TAA as for cytolysis. G250 TAA^{LOW} and $^{HIGH-POS}$ RCCs induce IFN-γ and TNF-α production in ch-Rec$^{HIGH-POS}$ CTLs. G250 TAA$^{HIGH-POS}$ induced higher lymphokine concentrations than G250 TAA$^{LOW-POS}$ RCCs (table 5.1). Only G250 TAA$^{HIGH-POS}$ RCC but not G250 TAA$^{LOW-POS}$ RCCs induced IFN-γ and TNF-α production in ch-Rec$^{LOW-POS}$ CTLs. G250 TAA^{NEG} RCCs did not induce lymphokines in any of the ch-Rec^{POS} CTLs.

table 5.1 TNF-α and IFN-γ secretion of gene-transduced T lymphocytes upon target cell interaction[a]

	TNF-α			
effector	G250 TAA^{NEG} (SKRC17-1)	G250 TAA$^{LOW-POS}$ (SKRC17-2)	G250 TAA$^{HIGH-POS}$ (SKRC17-3)	-
ch-Rec$^{LOW-POS}$ CTLs (LXSNHKγ)	<12	25	480	14
ch-Rec$^{HIGH-POS}$ CTLs (pSTHKγ)	35	274	4440	145
	IFN-γ			
effector	G250 TAA^{NEG} (SKRC17-1)	G250 TAA$^{LOW-POS}$ (SKRC17-2)	G250 TAA$^{HIGH-POS}$ (SKRC17-3)	-
ch-Rec$^{LOW-POS}$ CTLs (LXSNHKγ)	<12	<12	125	<12
Ch-Rec$^{HIGH-POS}$ CTLs (pSTHKγ)	<12	143	346	17

[a]Six times 10^4 T lymphocytes transduced with ch-Rec/γ (ch-Rec$^{LOW-POS}$ and $^{HIGH\ POS}$ CTLs) were cultured in medium or with $2x10^4$ G250 mAb-binding or non-binding tumor cells, for 24 h. Lymphokine secretion in supernatant was measured by ELISA (Medgenix, Brussels, Belgium). Background lymphokine secretion by tumor cells was < 1 pg/ml for TNF-α and < 0,2 IU/ml for IFN-γ. Production of TNF-α is in pg/ml/$3x10^5$ cells/24 h and of IFN-γ in IU/ml/$3x10^5$ cells/24 h. Results were obtained from 2 independent experiments. [a)]stimulator: SKRC17-1 = a G250 TAA^{NEG} human renal carcinoma cell clone; SKRC17-2 = G250 TAA$^{LOW-POS}$ human renal carcinoma cell clone; SKRC17-3 = G250 TAAHIGH^{-POS} human RCC clone; - = no stimulator cell.

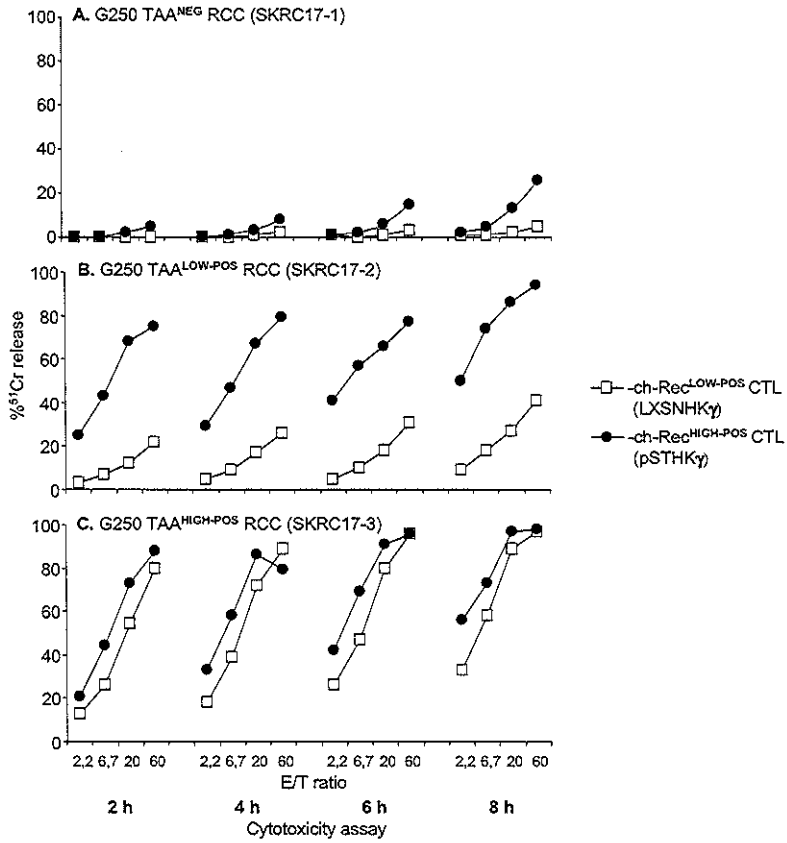

figure 5.6 Kinetics of RCC specific lysis by T lymphocytes with low and high density of ch-Rec membrane expression. Ch-Rec$^{LOW-POS}$ and ch-Rec$^{HIGH-POS}$ CTL were incubated with (A) SKRC17-1 (mock-transfected G250 TAA^{NEG} RCC), (B) SKRC17-2 (G250 TAA$^{LOW-POS}$, gene transfected RCC) and (C) SKRC17-3 (G250 TAA$^{HIGH-POS}$, gene transfected RCC), and tested in a 2, 4, 6 and 8h ^{51}Cr release assay. The specific ^{51}Cr release is depicted at different E/T ratios. Experiments were performed in triplicate, and SD did not exceed 10%.

DISCUSSION

Major functions of CD8^{POS} T cells are to exert cellular activity in order to kill cancer cells or virally infected cells and to produce cytokines that influence both their own performances as well as those of other cell types (35). The different T lymphocyte effector responses that can be elicited, i.e. quality and magnitude of the response, depend on the density of the T lymphocyte receptors as well as on target cell density (238-242). A detailed understanding of antigen-receptor / antigen relationships is

important for the efficient generation and expansion of optimally reactive, tumor-specific CTLs for immunotherapy, since adoptive transfer of tumor-specific CTLs has shown significant clinical benefit (44, 244). However, tumor-specific CTLs are difficult to isolate from cancer patients and their expansion to therapeutic numbers is time-consuming and the succes rate unpredictable.

Fortunately, genetic programming of T lymphocyte specificity is now possible by (retro)viral transfer of genes encoding mAb-based ch-Rec with a chosen TAA specificity. Engagement of such ch-Rec with TAA results in triggering of human T lymphocyte responses such as tumor cell lysis and tumor cells triggered lymphokine production (155, 161-163, 245, 246). This molecular approach offers fundamental new opportunities for gene-immunotherapy (244). In an earlier report on native TCR/CD3 complex functions we showed that individual human CTLs serially use sets of TCR/CD3 complexes to engage target TAA and that following signal transduction the disengaged TCR/CD3 complexes become inactivated and degraded (150, 237). Prior unengaged TCRs on the same T lymphocyte can engage with antigens on new target cells. We proposed that this serial use of sets of TCRs may underlie the ability of individual CTL to serially lyse multiple target cells (150). Further studies showed that already small numbers of target antigens can repeatedly trigger increasing numbers of TCRs on an individual CTL (193, 242). The few reports available in the literature on the functional balance between TCR density and T lymphocyte response to antigens show that a decrease in TCR density results in reduced T lymphocyte responses (239, 241). Hence, quality and quantity of T lymphocyte responses depend on and differ in (a) required thresholds of TCR occupancies; (b) antigen densities; and (c) antigen potencies. Different T lymphocyte responses may require distinct signaling thresholds, in a T cell clone-specific hierarchy (239-242, 249). During the preparation of this manuscript we became aware of the data recently published by Alvarez and Russell (250), who used a model system using Jurkat T cells with tetracycline-regulable ch-Rec densities to study their level of IL-2 response as a function of plastic coated antigen density. They concluded that Jurkat T cells can be engineered to discriminate between different antigen densities allowing optimization of the Jurkat T cells' response to a fixed concentration of antigen. Here we indeed demonstrate that their conclusion holds for primary human T lymphocytes expressing ch-Rec specific for native TAA at the level of (a) specific tumor target cell lysis; and (b) lymphokine production (TNF-α and IFN-γ). We further investigated this functional balance between ch-Rec and TAA densities in relation to quantity and quality of CD8 T lymphocyte responses.

Primary human $CD3^{POS}$ T lymphocytes transduced with ch-Rec genes using the LXSN retroviral vector subsequently express very low densities of ch-Rec, not detectable by flow cytometric analysis using anti-idiotype mAbs. Presence of the ch-Rec was however readily detected in a sensitive ^{51}Cr release assay by measuring tumor-specific cell lysis, and by quantitative assessment of lymphokine production (163). Here we determined the levels of both (a) tumor target cell lysis by ch-Rec^{POS}

CTL; and (b) lymphokine production in combinations of G250 $TAA^{NEG, LOW}$ and ^{HIGH} RCC populations and ch-$Rec^{LOW-POS}$ and $^{HIGH-POS}$ CTL populations. A high expression level of ch-Rec by CTLs was accomplished by using our novel pSTITCH viral vector (246). Now, aliquots of T lymphocytes from the same donors were gene transduced with the two viral vectors LXSN and pSTITCH. These two vectors yielded low and high expression levels of ch-Rec in the membrane of human T lymphocytes. Because no qualitative or quantitative differences in the gene transduction were observed between lymphocyte donors the results obtained for one representative donor are presented. It appeared that G250 $TAA^{LOW-POS}$ RCCs were only lysed by ch-$Rec^{HIGH-POS}$ CTLs, not by ch-$Rec^{LOW-POS}$ CTLs. In contrast, G250 $TAA^{HIGH-POS}$ RCCs were lysed by both ch-$Rec^{LOW-POS}$ and $^{HIGH-POS}$ CTLs. For the induction of the lymphokines INF-γ and TNF-α the same functional balance between ch-Rec versus TAA densities were found. We excluded that the G250 $TAA^{LOW-POS}$ RCCs would in fact be: (a) G250 TAA^{NEG} or, (b) that these RCCs showed a G250 TAA independent overall resistance to CTL mediated lysis. The RCCs which were functionally labeled G250 $TAA^{'LOW-POS'}$, in cytolytic assays, but G250 TAA^{NEG} in flowcytometric analysis, were indeed shown to be G250 $TAA^{LOW-POS}$ in (a) scatchart analysis (E. Oosterwijk, personal communication) and in (b) bsmAb-mediated cytolysis assays (fig. 5.3B). Differences in levels of lysis could not be due to alloreactivities generated in our T lymphocyte culture system since all SKRC cell lines used were cloned from one RCC line and hence MHC-identical.

We earlier reported that both bsmAb and ch-Rec-mediated TAA recognition and tumor cell lysis may involve adhesion molecules such as CD54 and CD58, depending on their cell surface densities (36, 37, 245). Therefore we also needed to exclude that suboptimal levels of expression of CD54 and / or CD58 caused (a) the lack of ch-Rec-mediated lysis of G250 $TAA^{LOW-POS}$ RCC and / or (b) the lack of a G250 TAA dose response in combination ch-$Rec^{HIGH-POS}$ CTLs. Indeed, all cloned RCC lines tested expressed identical levels of CD54 and CD58 as determined by flow cytometry using the appropriate fluorochrome labeled mAbs. One could further speculate that only the minor 2% fraction of ch-$Rec^{HIGH-POS}$ CTL within the ch-$Rec^{LOW-POS}$ CTL population was responsible for the lysis of G250 $TAA^{HIGH-POS}$ RCCs. However, if this were true then, based on the E/T ratios presented and from cytotoxicity data shown in fig. 5.4, by extrapolation it follows that at an 50-fold lower E/T ratio (of about 0.4) 70% lysis of RCCs could be induced. But as shown in fig. 5.4, for a level of 70% lysis of RCCs by ch-$Rec^{HIGH-POS}$ CTLs a 10-fold higher E/T ratio is required (i.e. about 4). Based on these considerations and calculations we propose that above a density threshold, TAA are spatially positioned in the membrane, that they can be optimally engaged by ch-Rec on CTLs. This hypothesis may find experimental support in our observations that for combinations of ch-$Rec^{HIGH-POS}$ CTLs where ch-Rec density is not limiting clear TAA dose responses were observed: higher levels of lysis and lymphokine production are induced by higher TAA densities.

It is noteworthy that a relative over-expression of (ch)-Rec in combination with high antigen density can even induce T cell apoptosis rather then T cell activation (250).

In short, we showed that quantity and quality of the T lymphocyte responses is co-determined by (a) the ch-Rec density on CTLs and (b) by the TAA density on target cells. This functional balance is reciprocal and dynamic: high CTLs ch-Rec density compensates for low TAA. The use of ch-Rec$^{HIGH-POS}$ T lymphocytes for gene-immunotherapy is recommended to prevent TAA$^{LOW-POS}$ tumor cells from escaping immune recognition and destruction (100).

ACKNOWLEDGEMENTS

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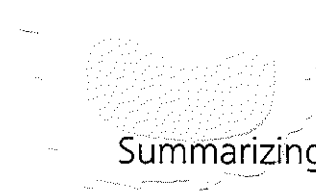

Summarizing Discussion

Adoptive cellular immunotherapy

Adoptive cellular immunotherapy in cancer treatment refers to the transfer of cultured immune cells with anti-tumor reactivity into patients. A variety of adoptive immunotherapeutical strategies have been employed including infusion of lymphokine-activated killer (LAK) cells, tumor-infiltrating lymphocytes (TIL) and tumor-specific MHC-restricted cytotoxic T lymphocytes showing therapeutic responses in some cancer patients (122, 132, 178). Improving this response and broadening of the range of tumor specificity has been obtained using bispecific monoclonal antibodies (bsmAb) that combine lymphokine production, homing and lytic capacity of T lymphocytes with the selectivity of tumor-recognizing monoclonal antibodies (137-139). In a clinical study involving locoregional treatment of ovarian cancer patients, with bsmAb against CD3 on T lymphocytes and the folate receptor on the ovarian carcinoma cells, an overall anti-tumor response of 27% was observed (144, 145). Although promising, the use of bsmAb may be hampered by the inaccessibility of solid tumors to Ab penetration (99) and the limited time of antibody binding to the T lymphocytes (139, 150). To circumvent the limitations associated with bsmAb, T lymphocytes can be grafted with permanent Ab-dictated specificity (155, 158). This approach combines the humoral and cellular arm of the immune system by genetically grafting T lymphocytes with chimeric receptors that consist of the binding site of a tumor selective antibody linked to an intracellular signaling domain from a membrane-bound receptor involved in cellular activation. Binding of the chimeric receptor to antigen on the tumor cell results in activation of the T lymphocytes and subsequently lymphokine production and cytolytic activity. The chimeric receptor application should enable the production of specific T lymphocytes against any antigen to which antibodies exist and broaden the use of specific T lymphocytes in adoptive immunotherapy for cancer and viral diseases.
In this thesis the construction and functional characterization of a chimeric receptor against renal cell carcinoma is described.

Functional expression of chimeric receptors on T lymphocytes

Chimeric receptor gene structure

To enable construction of cytotoxic T lymphocytes (CTL) with known predefined antibody (Ab) specificity for adoptive immunotherapy, we constructed a chimeric scFv/γ gene composed of the variable regions of a renal cell carcinoma selective mAb G250 (179, 180). These variable domains were coupled via a linker designated 212 (156) that consisted of glycine and serine aminoacids. The transmembrane and signal-transducing molecule we used was the γ-chain from the mast cell Fc(ε)RI (181). Introduction of this chimeric receptor into CTL rendered these lymphocytes specific for renal cell carcinoma. We demonstrated that the transduced CTL

functionally express the scFv/γ receptor for a prolonged period of time (4.5 month of *in vitro* culture), showed high levels of Ab-dictated lysis of renal cell carcinoma similar to that of normal CTL, and importantly, we demonstrated that these CTL can recycle their lytic activity. Moreover, these scFv/γ-expressing T lymphocytes produce lymphokines upon stimulation with the relevant target cell (chapter 2).
Although functional membrane expression was demonstrated, membrane expression of the chimeric receptor at the protein level could not be detected by flow cytometry. For screening and detection of gene-transduced T lymphocytes it would be advantageous to have a higher expression level of the chimeric receptor. Both retroviral vector and chimeric receptor gene structure can determine membrane expression level (162, 232, 246, 251). We designed a transient retroviral gene transduction system 'STITCH' comprising pSTITCH retroviral vector encoding the transgene, plasmids encoding Moloney murine leukemia virus gag/pol and gibbon ape leukemia virus envelope, and the human kidney cell line 293T as a packaging line (Chapter 4). Co-transfection of retroviral vector and packaging plasmids in 293T cells results in the production of GALV env pseudotyped viral particles with a titer of 10^7 infectious units/ml. The 'STITCH' gene transduction system efficiently transduces genes into activated human T lymphocytes resulting in high expression levels of the inserted gene. In addition, we designed a number of different chimeric receptor gene structures that vary in (a) the configuration of the variable heavy and variable light chain sequences; (b) the hinge domain sequence; and (c) the sequence of the transmembrane domain and signal-transducing cytoplasmic tail, and subsequently compared their membrane expression on activated human T lymphocytes (Chapter 4). We conclude that a small spacer sequence (15 a.a.) together with the complete Ig joining region is already sufficient for functional membrane expression of scFvG250 chimeric receptors. However, other, larger hinge regions, may result in distinct and higher levels of membrane expression. Furthermore, both γ and ζ-chain signaling molecules successfully transduced activation signals to the T lymphocyte signal-transducing machinery.

The role of chimeric receptor membrane expression levels

For T cell receptors it has been described that the quality and magnitude of the T lymphocyte response depends on the density of the T lymphocyte receptors as well as on target cell antigen density (238-242). A decrease in T cell receptor (TCR) density has been demonstrated to result in reduced T lymphocyte responses (239, 241). Furthermore, in a study using Jurkat T cells with tetracycline-regulable chimeric receptor densities it has been demonstrated that Jurkat T cells can discriminate between different antigen densities resulting in different T cell responses (250, 252). Because tumor cells *in vivo* express TAA at varying densities we determined how different densities of TAA on tumor cells on the one hand and chimeric receptor on T lymphocytes on the other affect the quantity and quality of T lymphocyte responses, which are important in anti-cancer immune activities.

Therefore we investigated the functional balance between chimeric receptor expression and TAA densities in relation to quantity and quality of CD8 T lymphocyte responses using T lymphocytes expressing either low or high densities of chimeric receptors in the membrane (Chapter 5). It appeared that tumor cells expressing a low level of TAA were only lysed by T lymphocytes with high density expression of chimeric receptors, not by T lymphocytes with only few chimeric receptors. In contrast, tumor cells with large numbers of antigen molecules on their membrane were lysed by T lymphocytes with either high or low densities of chimeric receptor. For the induction of the lymphokines INF-γ and TNF-α the same functional balance between chimeric receptor versus TAA densities were found.
The use of T lymphocytes with high density expression of chimeric receptor may be advantageous in clinical application to prevent that tumor cells with downregulated antigen expression may escape immune recognition and destruction (100).

Interaction of chimeric receptor with adhesion and co-stimulatory molecules on the T lymphocytes

T lymphocytes recognize their target cells via the TCR. Interaction of CTL with target cells requires specific recognition of the TCR with Ag in the context of MHC molecules resulting in CD3 signaling, activation and subsequent effector functions of T lymphocytes. This specific target cell recognition is preceded by non-specific conjugate formation, primarily mediated by non-polymorphic cell-surface receptors such as CD4, CD8, CD2 and CD11a/CD18 on the T lymphocytes with their ligands on the target cells, i.e., MHC Class II and I, CD58 and CD54, respectively (33, 195). In addition to an adhesion function, the CD8, CD2 and CD11a/CD18 receptors play an important role in T lymphocyte activation as costimulatory molecules. T lymphocytes can be activated by cross-linking of CD2, CD3 or CD11a/CD18 molecules without requiring specific TCR/CD3 interaction with MHC-peptide complex (16, 20, 34-38). Since adhesion and co-stimulatory molecules play a critical role in T lymphocyte activation and effector function, we investigated the contribution of CD2, CD3, CD11a/CD18, CD54 and CD58 molecules in T lymphocyte-tumor interactions via the chimeric receptor (Chapter 3). A co-regulatory role for CD2, CD3 and CD11a/CD18 molecules in chimeric receptor mediated lysis was demonstrated as the binding of mAbs, specific for these surface markers, inhibited chimeric receptor-mediated lysis of RCC. This indicated that chimeric receptors interact with other molecules on T lymphocytes in a similar fashion as the native T cell receptor. Moreover, CTL/target cell CD11a/CD18-CD54 interactions were required only when the RCC expressed low or intermediate levels of G250 Ag but not at high Ag density. For immunotherapy of cancer this may prove an important advantage, for it allows killing of tumor cells in patient's with circulating CD54 and of tumor cells with low or no CD54 expression (208, 209).

Therapeutic application of chimeric receptor-redirected T lymphocytes

Experimental data from this thesis and others have demonstrated that chimeric receptors can redirect T lymphocytes towards tumor cells and subsequently trigger cytolysis and lymphokine production. To test the *in vivo* function and efficacy of the chimeric receptor approach, suitable animal models have been developed that showed inhibition of tumor growth, reduction in lung metastases, increased survival and homing to the tumor and even complete tumor regression in nude and syngeneic mice models (165, 166, 168, 170-172). Both *in vitro* and *in vivo* experiments form the basis of the development of protocols for the treatment of human tumors.

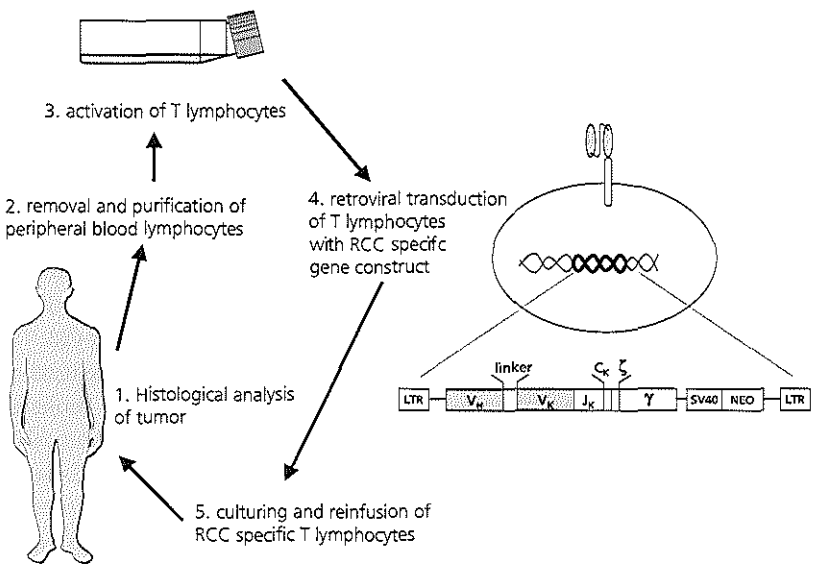

figure 6.1 Immuno-gene therapy protocol for the treatment of RCC with chimeric receptor gene – transduced T lymphocytes. Peripheral blood lymphocytes are isolated from the patients, activated and gene transduced with retroviral vector containing the chimeric receptor gene. Gene-transduced T lymphocytes are cultured and reinfused into the patient were they should home to the tumor and eliminated the tumor cells.

Immuno-gene therapy protocol

A possible protocol for the treatment of human cancer with redirected autologous T lymphocytes is depicted in figure 6.1. The presence of a particular tumor antigen should be confirmed histologically and the expression of this antigen should be restricted to tumor cells to prevent induction of possible autoimmune reaction against self-antigens (253). With the available tumor-selective mAb or TCR a

chimeric receptor construct can be composed and cloned into a retroviral vector. The availability of a vector with a universal framework structure for chimeric receptors would make the gene construction of chimeric receptors fast and easy (244). Next, peripheral blood lymphocytes will be taken from the patients, stimulated *in vitro* using anti-CD3 antibodies and transduced with a retroviral vector containing the chimeric receptor gene specific for the patients TAA. The use of retroviral vectors with high transduction efficiencies in human PBL (224, 246, 254, 255) would be advantageous for it would allow direct reinfusion of the redirected T lymphocytes that shortens the overall procedure and the heterogenicity of the PBL population will be conserved (256). After reinfusion, the redirected T lymphocytes should home to the tumor site and, upon tumor cell encounter, produce lymphokines to recruit other components of the immune system and kill tumor cells.

Future prospects

T lymphocytes can now be grafted with mAb-based specificity as well as TCR-based specificity redirecting them to antigens on target cells and MHC class I presented peptides respectively (163, 176). Depending on the tumor antigen expression, i.e. MHC-restricted or not, each tumor demands a different chimeric receptor type. However, the choice of chimeric receptors is limited by the availability of tumor-specific antibodies and of TCR from tumor-specific T lymphocytes that are still difficult to generate due to their low frequency. The phage display technique can provide a pool of tumor antigen reactive molecules with potential use in the chimeric receptor approach (257). Large antibody Fab fragments and TCR libraries can be screened for molecules that bind to tumor cells (258, 259). These molecules can be genetically coupled to a T lymphocyte intracellular signaling molecule and transduced into T lymphocytes conferring either MHC-restricted and non-MHC-restricted tumor specificity. The use of a pool of various chimeric receptors against different tumor antigens would be advantageous for treatment since it allows the elimination of tumor cells with heterogenous antigen expression patterns and of antigen-loss variants that might otherwise escape immune rejection.

The clinical applicability of chimeric receptors could further be extended by using, in addition to T lymphocytes as anti-tumor effector cell, other cells such as neutrophils, NK cells, γδ T lymphocytes and monocytes (173). Their homing pattern, cytokine repertoire and cytolytic mechanism differ from T lymphocytes and thereby can target different tumor cells and attract particular components of the immune system. In a mouse model the transduction of bone marrow cells with chimeric receptor genes resulted in expression of the chimeric receptor in T lymphocytes, NK cells, granulocytes and macrophages, and a significant *in vivo* anti-tumor activity was observed. *In vivo* depletion of T lymphocytes did not diminish the anti-tumor reactivity suggesting that the non-T lymphocytes play an important role in the tumor regression (172).

Ongoing studies on the efficacy of the combination of multiple chimeric receptors and various effector cells will demonstrate their potential in cancer treatment

Final remarks

Chimeric receptors with specificity for defined tumor antigens are valuable tools for targeting T lymphocytes to tumor cells. Now functional construction of chimeric receptors is technically possible, efficient gene-transduction systems are available and experimental animal studies have shown anti-tumor reactivity, the first clinical trials with retargeted T lymphocytes have started. In these trials the safety of infusions with chimeric receptor gene-transduced T lymphocytes will be determined and appearance, persistence and homing to the tumor of retargeted T lymphocytes in the body will be studied. These clinical trials will contribute to the design of optimal chimeric receptors and treatment protocols.
The coming years will reveal whether the chimeric receptor approach has potential in the treatment of cancer patients.

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Nederlandse Samenvatting

Immunotherapie van kanker

Het afweersysteem biedt bescherming tegen infectieuze agentia zoals bacteriën, virussen en parasieten. Vreemde moleculen (antigenen) kunnen worden herkend door het immuunsysteem en veroorzaken een afweerrespons die leidt tot de vernietiging van de indringers of zieke cellen. Tumorcellen kunnen zich ook onderscheiden van gezonde cellen door afwijkende expressie van genetische informatie. Desondanks worden de tumorcellen soms genegeerd door het afweersysteem en kan er kanker ontstaan. Immunotherapie als behandelingsmethode is er op gericht het natuurlijke afweersysteem van de patiënt zo te stimuleren dat de tumorcellen opgeruimd worden.

Het afweersysteem

Het afweersysteem bestaat uit een humorale en een cellulaire component. De humorale component levert antistoffen die een belangrijke rol spelen bij de afweer tegen bacteriën en andere lichaamsvreemde stoffen en organismen. De cellulaire component bestaat onder meer uit T-lymfocyten die een rol spelen bij de afweer tegen micro-organismen zoals virussen die de cel indringen en waarvan alleen kleine stukjes eiwit op de celwand zichtbaar zijn. Beide componenten kunnen ook een rol spelen in de tumorafweer. Antistoffen tegen tumorcellen zijn eenvoudig te verkrijgen door muizen te immuniseren met humane tumorcellen en vervolgens antistoffen uit het serum te isoleren. Helaas zijn antistoffen vaak niet werkzaam gebleken in de behandeling van solide tumoren. T-lymfocyten zijn daarentegen beter in staat solide tumoren binnen te dringen maar het is vaak moeilijk om T-lymfocyten specifiek voor een bepaalde tumor te isoleren uit het bloed en op te kweken voor therapie. Een combinatie van antistofherkenning en T-lymfocyt-effectiviteit zou kunnen leiden tot een krachtig anti-tumoreffect.

Functionele expressie van chimere receptoren op T-lymfocyten

In dit proefschrift wordt een mogelijke therapeutische benadering beschreven voor de behandeling van nierkanker. De therapie combineert het humorale (antistoffen) en cellulaire (T-lymfocyten) afweersysteem om een optimaal anti-tumoreffect te verkrijgen. Door op het celmembraan van T-lymfocyten een niercarcinoom-specifieke receptor te plaatsen kunnen deze T-lymfocyten niertumorcellen herkennen en vervolgens opruimen door de cellen te lyseren of stoffen te produceren die weer andere afweercellen kunnen aantrekken. Zo'n niercarcinoom-specifieke receptor moet instaat zijn een eiwit op de niertumorcellen te herkennen, te binden en een signaal door te geven naar de T-lymfocyt zodat die zijn opruimfunctie kan uitoefenen. Om deze receptor permanent op de T-lymfocyten tot expressie te brengen wordt de T-lymfocyt genetisch veranderd m.b.v. retrovirale gentransductie. Een methode waarbij een ongevaarlijk retrovirus de genetische informatie bevat van de receptor en T-lymfocyten kunnen worden geïnfecteerd met dit virus. Bij groei van de T-lymfocyten wordt de genetische informatie doorgegeven aan de dochtercellen zodat de nieuwe specificiteit aanwezig blijft. In hoofdstuk 2 is

beschreven hoe zo'n retrovirale vector gemaakt wordt voor een specifieke receptor tegen niertumorcellen. Er is gebruik gemaakt van de niercarcinoom-specifieke antistof G250 gekoppeld aan de signaaltransducerende γ-keten. Samen vormen deze de zogenaamde chimere receptor. T-lymfocyten, die na transductie met retrovirale vectoren de chimere receptor tot expressie brengen, zijn in staat om *in vitro* niertumorcellen te herkennen en te lyseren.

Voor optimale activatie en functie van T-lymfocyten spelen meerdere moleculen op de celmembraan een belangrijke rol. Dit zijn de zogenaamde adhesie en co-stimulatoire moleculen die samen met de endogene T cel receptor (TCR) de werking van de T-lymfocyt bepalen. In hoofdstuk 3 is de interactie van de chimere receptor met andere eiwitten op de celmembraan onderzocht. Hieruit is gebleken dat de chimere receptor interactie heeft met andere moleculen maar dat deze interacties afhankelijk zijn van het aantal tumorantigenen die op de tumorcel tot expressie komen. Bij een hoge dichtheid van tumorantigeen is de interactie van de adhesie moleculen CD11a/CD18-CD54 niet meer van belang. Dit zou een voordeel kunnen zijn bij de behandeling van tumoren omdat sommige tumoren weinig of geen expressie vertonen van het adhesiemolecuul CD54. Ook zijn in patiënten oplosbare CD54 moleculen aangetoond die de interactie van CD11a/CD18 zou kunnen blokkeren en daarmee ook de activatie en functie van de T-lymfocyten.

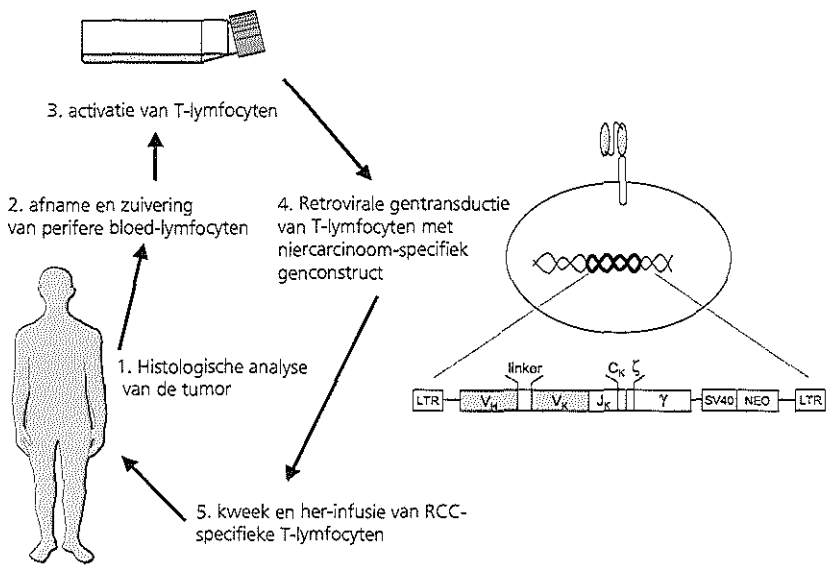
Voor screening en detectie van getransduceerde T-lymfocyten is een hoge dichtheid van chimere receptoren op T-lymfocyt van belang. Expressie van chimere receptoren kan mede bepaald worden door het ontwerp van de receptor. Dit kan leiden tot een stabielere expressie en mogelijk een verbeterde functie. Daarom is het effect van de chimere receptor genstructuur op de functionele expressie bestudeerd (hoofdstuk 4). Hiertoe is een nieuw retroviraal gentransductie systeem ontwikkeld (STITCH). STITCH bestaat uit de retrovirale vector pSTITCH, de retrovirale genen gag en pol, een gen coderend voor het envelop eiwit van gibbon ape leukemia virus, en de cellijn 293T. Dit systeem resulteerde in verhoogde expressie van de chimere receptor in T-lymfocyten en een verbeterde transductie-efficiëntie. Daarnaast is een aantal verschillende chimere receptor genstructuren ontworpen en is de membraanexpressie op T-lymfocyten bekeken. Hieruit bleek dat een kleine brugregio tussen het immunoglobuline gen en het transmembraan gen al voldoende was voor membraan-expressie. Dit sluit niet uit dat andere, misschien langere, brugregio's leiden tot hogere dichtheid van de chimere receptor op de celmembraan.

Voor de TCR is beschreven dat de kwaliteit en sterkte van de T-lymfocytrespons afhankelijk is van de dichtheid van de TCR en het tumorantigeen (TAA). Omdat de dichtheid van antigenen op tumorcellen *in vivo* sterk kan verschillen is er onderzocht hoe verschillende dichtheden van TAA op tumorcellen enerzijds en van chimere receptoren op T-lymfocyten anderzijds, invloed kunnen hebben op de T-lymfocytrespons (hoofdstuk 5). Door het gebruik van het retrovirale transductie systeem STITCH zijn er T-lymfocyten beschikbaar met dezelfde chimere receptor maar met verschillende membraandichtheden van de chimere receptor. Tumorcellen met een lage dichtheid van het TAA worden alleen vernietigd door

T-lymfocyten met een hoge dichtheid van de chimere receptor. Dit in tegenstelling tot tumorcellen met veel antigenen op de celmembraan die vernietigd worden door T-lymfocyten met een lage of hoge dichtheid van de chimere receptor. Om te voorkomen dat tumorcellen met lage antigeendichtheid ontsnappen aan de therapie is het gebruik van T-lymfocyten met hoge dichtheid van de chimere receptor aan te raden.

Therapeutische toepassing van T-lymfocyten met chimere receptoren

De resultaten uit dit proefschrift en andere *in vitro* studies hebben aangetoond dat T-lymfocyten met chimere receptoren in staat zijn tumorcellen te herkennen en deze vervolgens te vernietigen. Om deze functie van T-lymfocyten met chimere receptoren *in vivo* te testen zijn verschillende diermodellen ontwikkeld. Hierin is aangetoond dat injectie van T-lymfocyten met chimere receptoren, specifiek voor een bepaalde tumorsoort, kan leiden tot remming van tumorgroei, afname van uitzaaiingen in de long, verlenging van de levensduur, en ophoping van de T-lymfocyten in naar de tumor. Deze experimenten vormen de basis voor de ontwikkeling van een protocol voor de behandeling van humane tumoren. Een mogelijk protocol dat gebruikt kan worden in de behandeling van humane tumoren



figuur 6.1 Immuno-gentherapie protocol voor de behandeling van nierkanker met chimere receptor getransduceerde T-lymfocyten. Perifere bloed-lymfocyten worden afgenomen van de patiënt, geactiveerd en vervolgens getransduceerd met retrovirale vectoren die het gen voor de chimere receptor bevatten. Deze getransduceerde T-lymfocyten worden verder opgekweekt en teruggegeven aan de patiënt.

is afgebeeld in figuur 6.1. Als de tumor is getest voor de expressie van tumorspecifieke antigenen op de celmembraan waartegen antistoffen gemaakt zijn of gemaakt kunnen worden, kan de chimere receptor geconstrueerd en gekloneerd worden in de retrovirale vector. Perifere bloed-lymfocyten worden geïsoleerd uit de patiënt, geactiveerd en getransduceerd met de retrovirale vector die het chimere receptor genconstruct bevat, specifiek voor niertumorcellen. Hierbij is het gebruik van retrovirale vectoren met hoge transductie-efficiëntie een voordeel omdat de getransduceerde T-lymfocyten dan direct terug gespoten kunnen worden zonder verdere selectie. Dit verkort de behandeling en behoudt de heterogeniteit van de T-lymfocyten populatie. Na her-infusie moeten de getransduceerde T-lymfocyten zich specifiek binden aan de niertumorcellen en worden aangezet tot cytokinen productie en tumorcelvernietiging.

Tenslotte

Chimere receptoren met specificiteit voor tumor antigenen leveren een grote bijdrage aan het specifiek richten van T-lymfocyten tegen tumorcellen. Nu het technisch mogelijk is om chimere receptoren te maken, er efficiënte gentransductie systemen zijn ontwikkeld en met experimentele dierstudies de anti-tumoreffectiviteit van T-lymfocyten met chimere receptoren is aangetoond, zijn de eerste klinische studies gestart. Hierin zullen veiligheidsaspecten van de infusie van genetisch gemodificeerde T-lymfocyten worden onderzocht en de aanwezigheid en lokalisatie van de T-lymfocyten worden bekeken. Deze eerste studies zullen bijdragen aan het ontwerp van optimale chimere receptoren en behandelingsprotocollen. In de komende jaren zal duidelijk worden of de chimere receptor benadering kan bijdragen aan de behandeling van kanker.

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Mo

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 10 mei 1966 te Eindhoven. Na het behalen van het VWO diploma aan de Gemeentelijke Scholengemeenschap Genderdal te Eindhoven begon zij in 1984 aan de studie biologie aan de Rijksuniversiteit Utrecht. In 1991 werd het doctoraalexamen gehaald met als specialisatierichtingen Immunologie (Dr. B. de Geus, T.N.O. Rijswijk) (Prof. Dr. A. Zapata, Complutense Universidad, Madrid) en Embryologie (Prof. Dr. J.A.M. van de Biggelaar en Dr. A.E. van Loon, Utrecht). In 1991 werd begonnen als wetenschappelijk onderzoeker bij de afdeling Medische en Tumor Immunologie van de Dr. Daniel den Hoed Kliniek op een door de Nierstichting Nederland en Stichting Technische Wetenschappen gefinancierd project. Gedurende deze periode werd onder leiding van Dr. R.L.H. Bolhuis het onderzoek verricht wat heeft geleid tot de totstandkoming van dit proefschrift. Sinds 1997 is zij werkzaam als wetenschappelijk onderzoeker bij de vakgroep Haematologie van het Universitair Medisch Centrum Utrecht in het Jordan laboratorium voor Haemato-oncologie van Prof. dr. A. Hagenbeek.