Transfer and Targeting of the Human CD20 Molecule

New perspectives in immuno-gene therapy of hematological malignancies

Tom van Meerten

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Transfer en Targeten van het Humane CD20 Molecuul

Nieuwe inzichten in immuno-gentherapie voor hematologische maligniteiten

(met een samenvatting in het Nederlands)

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“I don’t know how long the city will stand (…)

if the Greeks get inside the walls, it’s over.”

Hector

Voor mijn ouders

Commissie: Prof.dr. F. Miedema

Prof.dr. M. Theobald

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List of abbreviations

ABC	antibodies-bound-per-cell
ADA-SCID	adenosine deaminase-deficient severe combined immunodeficiency
ADCC	antibody-dependent cellular cytotoxicity
allo-SCT	allogeneic stem cell transplantation
APC	allophycocyanin
Bcl-2 gene	B-cell lymphoma 2 gene
BLI	Bioluminescent Imaging
BM	bone marrow
Cbp	C-terminal src kinase-binding protein
CD	Clusters of Differentiation
CDC	complement-dependent cytotoxicity
cDNA	complementary DNA
CFSE	carboxyfluorescein diacetate succinimidyl ester
CHO cells	Chinese hamster ovary cells
cHS4	Chicken hypersensitive site 4
CLL	Chronic Lymphocytic Leukemia
CMP	cow milk peptide
CMV	Cytomegalovirus
CRP	complement regulatory protein
Csk	C-terminal Src kinases
C-terminal	Carboxy-terminal
CV	coefficient of variation
DLBCL	diffuse large B-cell lymphoma
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EGFP	enhanced green fluorescence protein
FACS	fluorescence-activated cell sorter
FcεRI β	β chain of the high affinity immunoglobulin E receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FKBP	FK506-binding protein
FL	follicular lymphoma

GCV	ganciclovir
GMP	good manufacturing practice
GvHD	Graft versus Host Disease
GvL	Graft versus Leukemia
HLA	Human Leukocyte Antigen
HSC	hematopoietic stem cells
HSVtk	Herpes Simplex Virus thymidine kinase
ICE	insulator core element
IFN-SAR	interferon-β scaffold attachment region
Ig	immunoglobulin
IL	interleukin
INS	cHS4 insulator element
INF	interferon
IRES	internal ribosome entry site
iv	intravenously
kDa	kilodalton
LTR	long terminal repeat
mAb	monoclonal antibody
MACS	magnetic cell sorting
MFI	mean fluorescence intensity
MHC II	major histocompatibility complex class II
MLV	Moloney Murine Leukemia Virus
MOI	multiplicity of retroviral infection
MS4A	Membrane-Spanning 4A
mRNA	messenger RNA
NEO	Neomycin
NGFR	Nerve Growth Factor receptor
N-terminal	amino-terminal
NHL	Non-Hodgkin's Lymphoma
OS	Overall Survival
PAG	phosphoprotein associated with glycophospholipid-enriched membrane microdomain
PBMC	peripheral blood mononucleated cells
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin

PI	propidium iodide
PLC	phospholipase C-γ1 and γ2
PMA	phorbol myristate acetate
RNA	Ribo Nucleic Acid
RSV	respiratory syncytial virus
SD	Standard Deviation
TCR	T cell receptor
TIL	tumor-infiltrating lymphocytes
TU	transduction units
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
XL	Cross-linking

Chapter 1

Introduction

Immunotherapy and gene therapy are the two most innovative treatment modalities for hematological malignancies that have been developed over the last years. Both strategies are marked by a rapid translation from bench towards clinical application.
The use of anti-CD20 monoclonal antibodies in the Non-Hodgkin's lymphomas (NHL) can be considered the most successful and worldwide accepted form of immunotherapy so far. The field of gene therapy has yielded great results for treatment of single genetic disorders but has also encountered complications that have shifted its current focus towards the incorporation of safety measures into clinical application.

This thesis combines both fields and aims to improve antibody-mediated immunotherapy and gene therapy by investigation of the human CD20 molecule.
The following part of the introduction describes the role of the human CD20 molecule, anti-CD20 immunotherapy and CD20 gene therapy. At the end the purpose of the research in this thesis is described.

The human CD20 molecule

CD20 expression

Expression of the human CD20 molecule is restricted to the lineage of B lymphocytes. B cell precursors and mature B cells express CD20 on their cell surface, but it is lost upon plasma cell differentiation (1-3).

CD20 gene

The human CD20 gene is found on chromosome 11, encoding eight exons (4). The CD20 gene shares a common chromosomal location with the β chain of the high affinity Immunoglobulin (Ig) E receptor (FcεRI), which is expressed on mast cells (4, 5). CD20 and FcεRI β also have a comparable overall structure. FcεRI β, involved in signaling in mast cells, and CD20 are both members of the membrane-spanning 4A (MS4A) gene family. The MS4A family components are oligomeric cell surface complexes involved in signal transduction in diverse cell lineages (5, 6).

CD20 protein

Translation and transcription of the CD20 gene results in a protein of 297 amino acids with a molecular weight of ~ 33 kDa. The protein has an amino and carboxy terminus located within the cytoplasm (7, 8). The intracellular parts are rich in threonine and serine, which can be phosphorylated upon B cell stimulation (9, 10). No tyrosine residues are found in the cytoplasmic regions. The CD20 molecule can be immunoprecipitated from B cells or CD20 transfected T cells, leaving three isoforms (33, 35 and 37 kDa) (9, 11, 12). The isoform depends on the phosphorylation of the threonine and serine residues. Activation of B cells or mitogenic stimulation of B cells with, for example, phorbol myristate acetate (PMA) results in an increase of the 35 and 37 kDa forms (9, 12, 13).

CD20 spans the membrane four times (7, 14-16). Despite the small extracellular region of CD20, there is a varied degree of epitope recognition among anti-CD20 monoclonal antibodies (17, 18). Two residues are important, alanine and proline at position 170 and 172, respectively. Mutation of these amino acids results in the impairment of antibody binding, without the loss of the oligomeric complex (18) (figure 1).

The CD20 molecule is associated with other B cell membrane proteins and intracellular proteins. Co-precipitation of surface and intracellular B cell proteins suggests that the CD20 molecule is part of a multimeric cell surface receptor complex (see below).

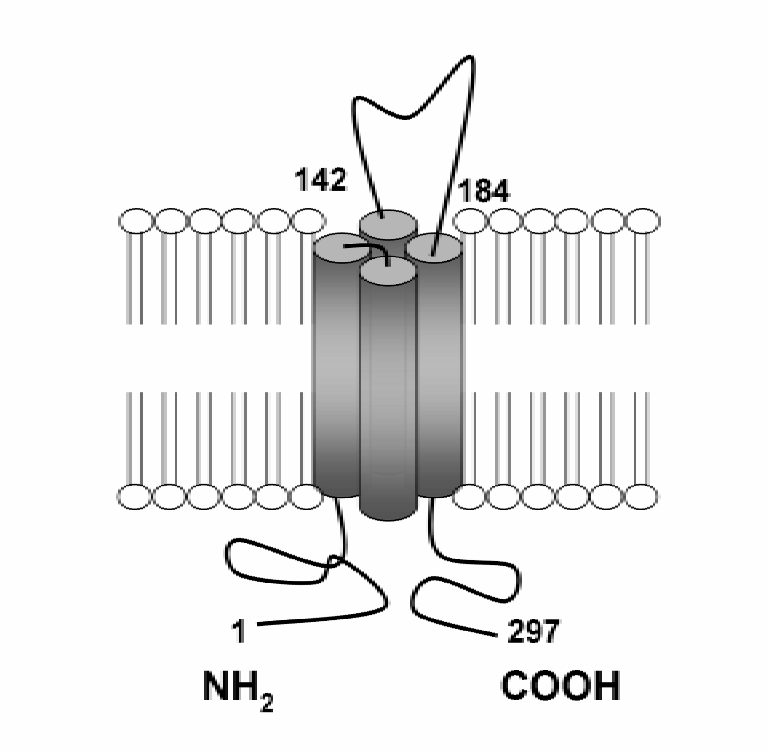

Figure 1. The human CD20 molecule.
Human CD20 is 297 amino acids long and is located within the lipid bilayer of the plasma membrane of B cells. The N- and C-terminal are located in the cytoplasm and only a small portion of the molecule is located outside the membrane.

CD20 function

The function of the CD20 molecule is not yet completely understood. Actually, the CD20 molecule was the first B cell antigen discovered through a generated monoclonal antibody. Balb/c mice were immunized with Burkitt's lymphoma cells and a novel antibody was found after the fusion, called anti-B1 that recognizes CD20 (1). No natural ligand is known for CD20 and our current understanding of the function of the CD20 molecule comes from ligation with different antibodies to CD20 (17-20).

Expression of CD20 seems unnecessary for B cell development, as engineered CD20-deficient mice have normal B cells (21). Co-expression of other MSA4 related genes on the B cells may be responsible for normal B cell development in the CD20-deficient mice (6, 21, 22). In addition, the homology of the amino acid sequence of murine and human CD20

molecules differs for some critical amino acids. Murine CD20 may therefore have a different function compared to human CD20 (17).

CD20 probably functions as a B cell activating and/or differentiating molecule. Different antibodies have distinct effects on B cell proliferation, and some antibodies are even able to block B cell growth (reviewed in Deans et al (17)). CD20 seems to function as a transmembrane Ca^{2+} channel. For example, it was found that ectopic expression of the CD20 molecule on T cells showed Ca^{2+} activity, not found on CD20-negative cells. Increased steady-state $[Ca^{2+}]_i$ levels were found in these CD20 expressing T cells. The ligation of antibodies to CD20 also leads to an enhanced Ca^{2+} conductance in B cells (11, 23, 24). In addition, reducing CD20 expression by antisense CD20 sequences decreased the Ca^{2+} entry across the plasma membrane (17, 25).

CD20 signaling

The binding of monoclonal antibodies to CD20 generates transmembrane signals. Different effects have been observed, ranging from phosphorylation of the molecule (9) to up-regulation of the c-myc and b-myc oncogene expression (26, 27). Signaling through CD20 is associated with the transactivation of tyrosine kinase-dependent pathways (figure 2). This was first demonstrated by the use of tyrosine kinase inhibitors, which inhibited the induction of homotypic aggregation triggered by the CD20 monoclonal antibody B1 (28). Up-regulation of c-myc by the 1F5 anti-CD20 antibody was shown to be tyrosine kinase dependent (10). Anti-CD20 monoclonal antibody 2H7 induced tyrosine kinase activation leading to phosphorylation of multiple tyrosine kinase substrates including phospholipase C-γ2 (10).

Upon anti-CD20 ligation, CD20 is translocated into lipid rafts (29) (figure 2). Lipid rafts, consisting of sphingolipids and cholesterol, are signaling platforms that selectively group receptors and signaling molecules (30, 31). Like FcεRI β, CD20 is insoluble using low-stringency detergent conditions, but after antibody binding CD20 becomes insoluble in high stringency Triton X-100 (17, 32). Upon antibody ligation, CD20 becomes associated with C-terminal src kinase-binding protein (Cbp) (also known as phosphoprotein associated with glycophospholipid-enriched membrane microdomain, PAG), which is selectively localized in lipid rafts (17). Cbp is a highly tyrosine phosphorylated adaptor protein that recruits the C-terminal Src kinases (Csk) such as Lyn, Fyn, or Lck. Csk phosphorylates Cbp, leading to induction of kinase activity and mediates downstream signaling (33). In B-cell lines, (hyper)crosslinking of CD20 (trans)activates Csk and Cbp and causes the release of

intracellular calcium stores, up-regulation of FAS expression, phosphorylation of phospholipase C-γ1 and phospholipase C-γ2 and activation of caspase-3 to induce apoptotic cell death (10, 17, 33-36).

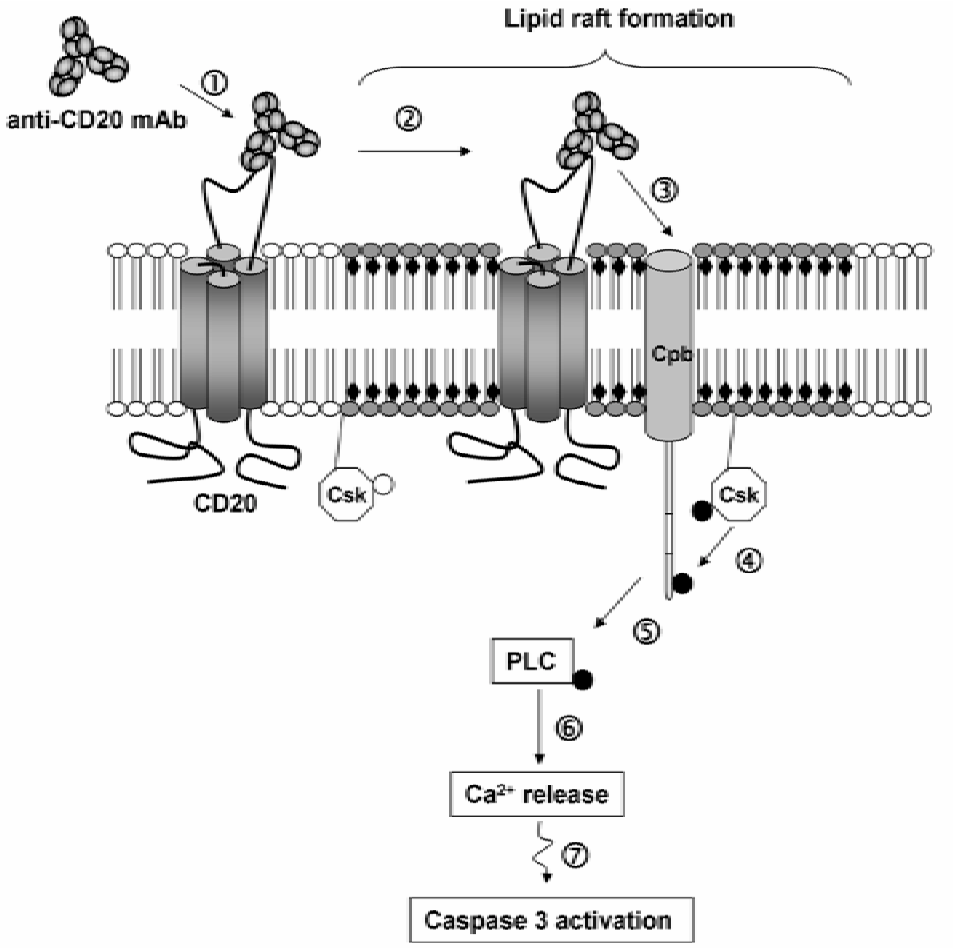

Figure 2. Signaling events upon CD20 antibody binding.
(1) Anti-CD20 monoclonal antibodies bind the extracellular loop of CD20. **(2)** Upon antibody ligation CD20 migrates into lipid microdomains, consisting of sphingolipids and cholesterol. Within these lipid rafts several other (signaling) molecules reside, for example the C-terminal Src kinase-binding protein (Cbp) (also called the phosphoprotein associated with glycophospholipid-enriched membrane microdomain (PAG)), CD40 (not shown), and major histocompatibility complex class II proteins (MHC II) (not shown). **(3)** CD20 binding (trans)activates the C-terminal Src kinases (Csk) such as Lyn, Fyn, or Lck, **(4)** which phosphorylate Cbp, leading to induction of kinase activity. **(5)** Activation of Csk leads to phosphorylation of phospholipase C-γ1 and γ2 (PLC), **(6)** which induces a Calcium influx and **(7)** activation of caspase-3.
Figure 2 is modified according to Cartron et al, Blood. 2004;104:2635-2642 (37).

CD20 immunotherapy

Anti-CD20 antibody therapy

Antibodies in general

Antibodies are produced by plasma cells, the final stage of the B cell lineage. When a B cell recognizes a danger signal (antigen), it starts to multiply and can differentiate into a plasma cell. Plasma cells produce immunoglobulins or antibodies, which are large, glycosylated proteins that are specific for the antigen they encountered. Antibodies can be found in serum, tissue fluid and on the surface of B cells, where they act as receptors for antigens.

There are 5 classes of antibodies: IgM, IgG, IgA, IgD and IgE. Within each class of antibodies there are also subclasses. All classes of antibodies differ in their structure and effector function. In general, the basic structure of the antibody monomer consists of two identical halves connected by two disulfide bonds. Each half consists of a heavy chain and a light chain, joined together by a disulfide bond.

Antibodies are bi-functional molecules. One part specifically binds the antigen (variable region) and the other part mediates the effector function (constant region). Some antibodies mediate a direct effect on their target cell, but generally they bind receptors on the surface of immunomodulatory cells such as macrophages and stimulate the release of cytokines that regulate the immune response, or they bind C1q, the first component of the complement system (figure 3).

Rituximab

Development of the anti-CD20 antibody rituximab

The first monoclonal antibody (mAb) that recognized CD20, anti-CD20 B1, was generated in 1980, by immunizing mice with human lymphoma cells (1). Because of its potential for the treatment of B cell disorders, in the years thereafter anti-CD20 antibodies were genetically engineered for clinical application. In 1997, rituximab ((MabThera®, Rituxan®) was the first monoclonal antibody approved specifically for the treatment of relapsed or refractory CD20-positive low-grade (follicular) Non-Hodgkin's Lymphoma (NHL). Rituximab is a chimeric monoclonal anti-CD20 antibody that is engineered as follows: the light and heavy chain variable regions from the murine 2B8 anti-CD20 antibody (IDEC-

2B8), generated by immunizing mice with a CD20-positive human lymphoma, are amplified by PCR and inserted into a cDNA mammalian chimeric antibody expression vector, which also contains the neomycin phosphotransferase gene (NEO). This vector is electroporated into CHO cells and under antibiotic pressure the cells stably secrete Ig levels (38). The resulting chimeric antibody is purified and consists of a human kappa constant region, a human IgG Fc portion (IgG1), and a murine variable region, recognizing the human CD20 protein (38).

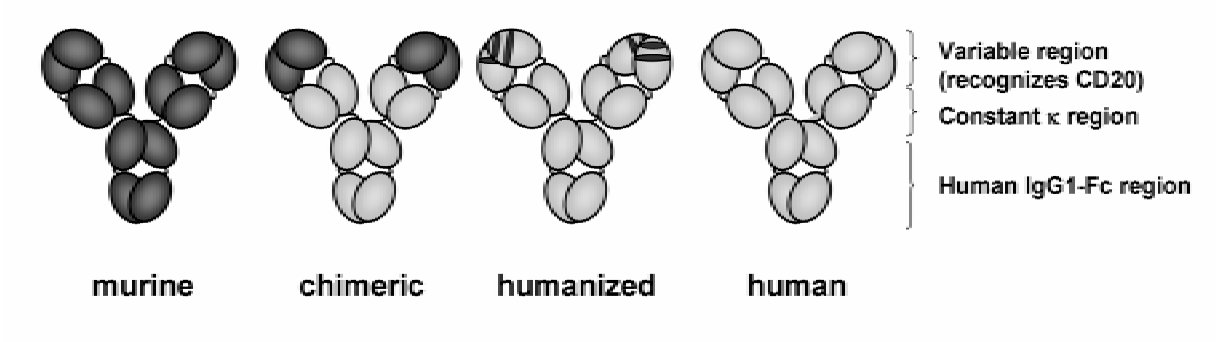

Figure 3. Development of monoclonal antibodies recognizing CD20.
Murine anti-CD20 mAbs are generated by immunization of mice with CD20-positive cells. In chimeric antibodies, the 30% that is of murine origin, is the variable region that recognizes the CD20 antigen. The variable regions are cloned into a chimeric antibody expression vector, resulting in an antibody which contains the constant κ region and the IgG1-Fc region of human origin. For humanized antibodies, also with cloning techniques, the variable region is modified to be more human. Humanized antibodies contain complementary-determining regions of murine origin, which recognize the CD20 antigen. Only 10% of the antibody is of murine origin. Human anti-CD20 mAbs are derived from human immunoglobulin transgenic mice. The latter antibodies are likely to be non-immunogenic in men.

Mechanisms of action of rituximab

In vitro results

CD20 is an ideal target for antibody-mediated therapy because, in general, CD20 does not circulate in the plasma (39), is not shed from the cell surface (14) and is not internalized (40) after antibody binding. Upon ligation of CD20, rituximab triggers different effector mechanisms. Many *in vitro* and *in vivo* studies have been conducted to explore the most

important effector mechanism. *In vitro*, it is well established that there are three main modes of action of rituximab: 1) induction of apoptosis 2) complement-dependent cytotoxicity (CDC) and 3) antibody-dependent cellular cytotoxicity (ADCC), as described below (figure 4).

In the first place, apoptosis may be induced upon CD20 ligation. Data concerning the mechanism of the apoptotic effect of rituximab are conflicting. Different groups obtained different results, even if they used similar target cell lines (17, 41, 42). It has been suggested that one of the late apoptotic pathways, caspase-3, is activated (43). However, others documented that the apoptotic pathways are caspase or Fas ligand/ Fas death pathway and mitochondria independent, and do not require lipid raft formation (41, 44). Important to note is that hyper-crosslinking of rituximab, either by a secondary antibody or by Fc bearing effector cells generally increased the apoptosis (17). Also important is the fact that within a treated cell population not all cells uniformly undergo apoptosis. This finding is also the focus of research of many groups that study rituximab resistance.

Secondly, the Fc portion of rituximab is able to trigger the classic complement system, resulting in CDC. *In vitro*, C1q is bound efficiently by rituximab (38, 45) and simple CDC-assays demonstrated that complement activation induced cell kill mediated by rituximab (42, 46, 47). Rituximab-induced CDC has a variable degree of efficiency, which has been associated with expression of complement regulatory proteins (CRP) CD55 and CD59 (42, 47, 48). Contradictory results have been published on the relationship of CD20 expression level and CDC efficiency of rituximab (42, 48-50).

Finally, ADCC is mediated by effector cells expressing FcγRI (CD64), FcγRII (CD32) or FcγRIII (CD16). Effector cells, such as NK cells, granulocytes or macrophages, are able to recognize the Fc portion of rituximab, and kill the ligated cells by phagocytosis or the release of cytotoxic granules (38, 42, 51, 52). For ADCC, it has been demonstrated that the efficacy depends on polymorphisms of the effector cells (52, 53).

In vivo results

In the *in vitro* studies it was possible to investigate the mechanisms of rituximab separately, but this is more complex for the *in vivo* studies. In several murine studies it was attempted to clarify the effector mechanisms. Elegant mouse models using FcγR deficient mice pointed out that CD20 expressing cells clearing of was FcγR dependent for a panel of

murine anti-CD20 monoclonal antibodies (54) and rituximab (55). Others nicely demonstrated that complement was responsible for CD20 positive tumor clearance by rituximab (19, 56, 57). However, there is no agreement in the literature about the dominant *in vivo* effector mechanism.

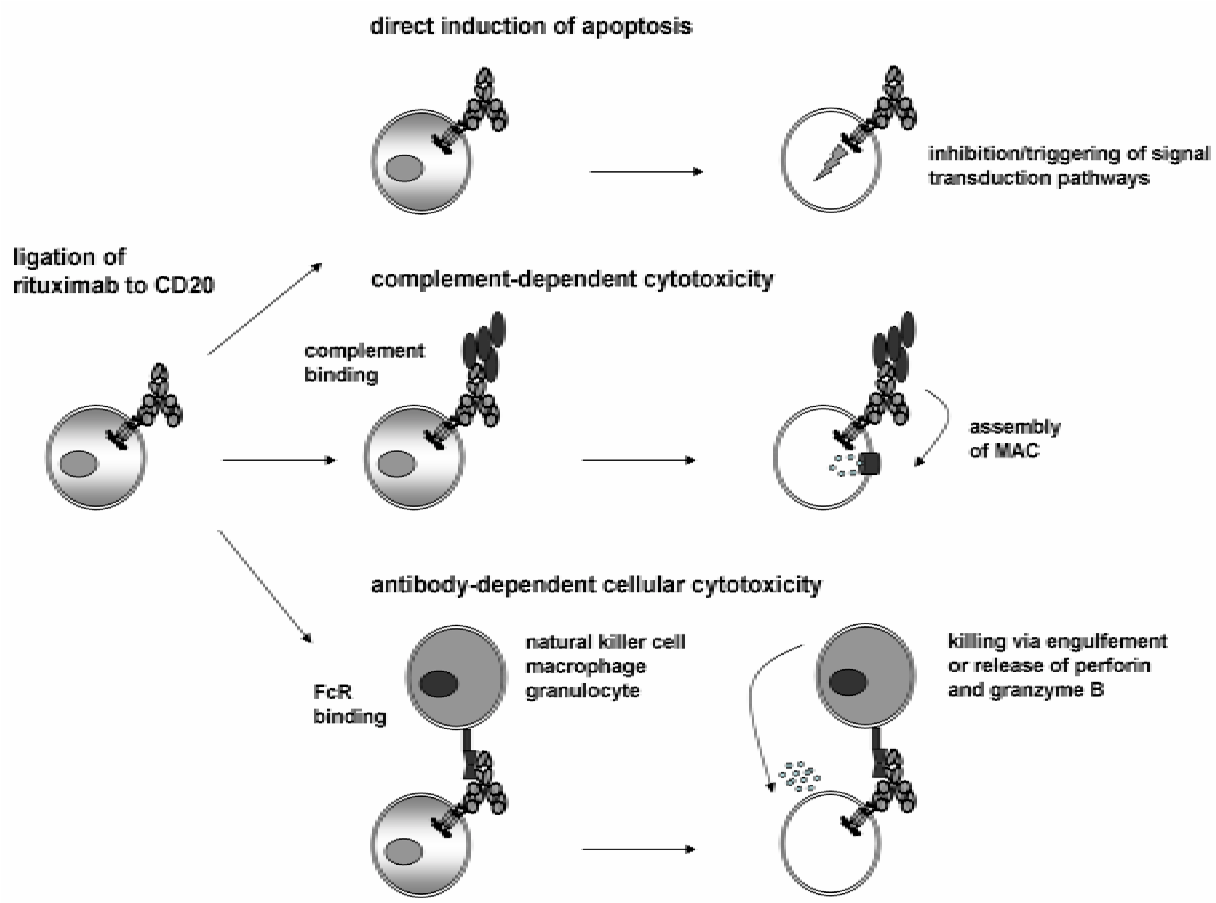

Figure 4. The three main mechanisms of action of rituximab.
After rituximab ligation to CD20, three different effector mechanisms can be activated. **(1)** Direct induction of apoptosis may be caused by the activation of the death receptor pathway or the mitochondrial pathway by modulation of the *bcl-2* gene family. But apoptosis-induced death can also bypass the mitochondrial pathway or caspases. **(2)** The Fc portion of the IgG1 tail of rituximab is able to bind C1q, the first component of the classical complement pathway. Binding of C1q triggers a proteolytic cascade, resulting in accumulation of C3b. C3b molecules act as opsonins for phagocyting cells but also bind to the C3 convertase to form a C5 convertase, leading to the generation of the membrane attack complex (MAC), which kills the cell. **(3)** The Fc portion is also recognized by cells expressing the Fc receptors (NK cells, granulocytes and macrophages). Cell killing is mediated by phagocytosis or the release of cytotoxic granules.

Patients

Also some evidence concerning the mechanism of rituximab has been obtained in patients. One of the infusion related side effects of rituximab is the complement consumption after administration (58, 59), indirectly confirming CDC. On the other hand, clinical responses have been correlated to polymorphisms in the FcγRIIIA gene (53, 60, 61), indirectly confirming ADCC. In addition, a significant direct effect of rituximab cell kill by activating caspase-3 was demonstrated *in vivo* in patients with chronic lymphocytic leukemia (CLL) (43).

Clinical application of rituximab

The first phase I trial with rituximab as a single agent was conducted in 1993 for patients with relapsed low-grade B-cell lymphoma (62). Within five single-agent trials, no severe toxicities were found and only infusion-related adverse events occurred within the first hours and only after the first infusion. The most common side effects were chills, fever, nausea, fatigue, headache and angioedema (63). Several phase II and III trials studied the optimal schedules and dosing with or without chemotherapy, biologicals, and radiotherapy (63). After approval in 1997 in the US and in 1998 in Europe, rituximab is included in the standard treatment of NHL. Rituximab works very efficiently in combination with chemotherapy. For diffuse large B cell lymphoma (DLBCL) the seven-year overall survival (OS) improved from 36% to 53% by the addition of rituximab to chemotherapy (64, 65). For low-grade lymphoma, with a median follow-up of 53 months, the OS improved from 71% to 81% with the combination of rituximab and chemotherapy (66-68). Moreover, if patients with low-grade lymphoma respond to single-agent rituximab therapy, progression free survival and overall survival is substantially prolonged with scheduled maintenance treatment (69, 70). In addition, if these patients achieved complete or partial remission after the combination of chemotherapy and rituximab, maintenance with rituximab increased the overall and progression free survival (70, 71).

The therapeutic effect of rituximab, by the depletion of B cells, has also proven to be successful for B-cell related auto-immune diseases (72, 73) and chronic Graft versus Host Disease after allogeneic stem cell transplantation (74-77).

However, despite the success, resistance of lymphoma B cells towards rituximab is still being observed in about half of the patients. The precise mechanism of resistance to rituximab is still unknown. Improving the effect of rituximab by enhancing CDC or ADCC or the development of improved antibodies directed to CD20 is the current focus of many research groups.

New anti-CD20 antibodies

For improving anti-CD20 therapy, construction of second generation anti-CD20 mAb is desired. Different murine (78), humanized (79, 80) and completely human (20, 81) CD20 monoclonal antibodies have been developed and were compared *in vitro* to rituximab (see also figure 3). Especially, the completely human CD20 antibodies proved to be more effective than rituximab *in vitro*. These antibodies, generated in human immunoglobulin transgenic mice, bind a different epitope on the CD20 molecule compared to rituximab and have a slower off rate. These mechanisms are thought to be responsible for the improved CDC (20, 81). First clinical data with these agents showed safe application and responses in rituximab resistant patients (82-86). However, currently ongoing clinical trials will point out their efficacy in the future.

CD20 gene therapy

Gene therapy in general

The goal of gene therapy is the introduction of new genetic material into a cell in order to change that cell's phenotype and/or function for a therapeutic purpose.

The first clinical trial with genetically-modified T cells was conducted in 1989 by Rosenberg et al., who inserted a neomycin resistance gene into human tumor-infiltrating lymphocytes (TILs) for patients with metastatic melanoma to track down and study the *in vivo* distribution of these TILs (87). In 1990, the first clinical trial was performed, in which a single genetic defect was successfully corrected for the treatment of adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID) (88).

Since then, several pre-clinical studies and clinical trials have been conducted for the correction of defective or missing genes in various congenital disorders of hematopoietic origin. In general, the missing or defective gene was inserted into autologous stem cells and the transgenic cells were re-administered to the patient. Examples are X-linked SCID (89, 90), ADA-SCID (88, 91), Fanconi's anemia (92), chronic granulomatous disease (93), hemophilia A and B (94) and hemoglobinopathies (95).

Besides the correction of congenital defects, gene therapy can also be used to challenge acquired diseases, such as cancer, viral infections or T cell disorders. One way is redirection of (tumor or viral) antigen-specific T cells by genetic transfer of a T cell

receptor (TCR), which has been shown to enhance the immune response to malignant cells (96) or to viral infections like cytomegalovirus (97), Epstein-Barr virus (EBV) (98) and the human immunodeficiency virus (99).

Another interesting application of T cell gene therapy is the introduction of a suicide gene into donor T cells in the setting of allogeneic stem cell transplantation (allo-SCT) or donor lymphocyte infusion (DLI), which is explained below.

Suicide gene therapy

For many hematological malignancies, allogeneic stem cell transplantation is the only curative option. In allo-SCT, the patient is conditioned with chemotherapy, immunosuppressive drugs and total body irradiation, after which hematopoietic stem cells (HSC) together with T cells from an HLA-identical donor are administered (100, 101).

Alloreactivity of donor T cells

Donor T cells play an important role in allo-SCT. They promote the engraftment of HSC and help to protect against viral infections in the immunocompromised patient. In addition, a crucial beneficial effect of these T cells is their ability to eliminate leukemic cells, the so-called Graft versus Leukemia (GvL) effect. The most direct evidence comes from donor lymphocyte infusions, which significantly decrease leukemia relapse (102-104) (figure 5).

In GvL, circulating T cells recognize intracellularly derived tumor antigen fragments that are presented by the major histocompatibility complex (MHC) on the cell surface of cells. Recognition of the tumor antigen then leads to clonal expansion of antigen-specific T cells and elimination of the leukemic target cells.

Unfortunately, the donor T cells may also recognize the MHC-presented peptides from normal tissues, leading to possibly life-threatening Graft-versus-Host Disease (GvHD) (102, 105-107).
At present, it is difficult to separate the detrimental and beneficial effects of donor T cells. For example, immune suppression reduces GvHD, but also leads to a delay in immune reconstitution, resulting in considerable morbidity and mortality due to infections (108). Similarly, T cell depletion of the graft reduces the incidence of GvHD, but this has been associated with increased graft rejection and leukemia relapse (109, 110).

Suicide gene therapy to control GvHD

A promising and specific strategy to control GvHD, while utilizing the GvL effect as long as possible, is the genetic modification of human donor T cells through introduction of a suicide gene (figure 5). The transgenic expression in donor T cells of a gene encoding a susceptibility factor (suicide gene) incorporates sensitivity to a selective agent that kills only the transgenic T cells.

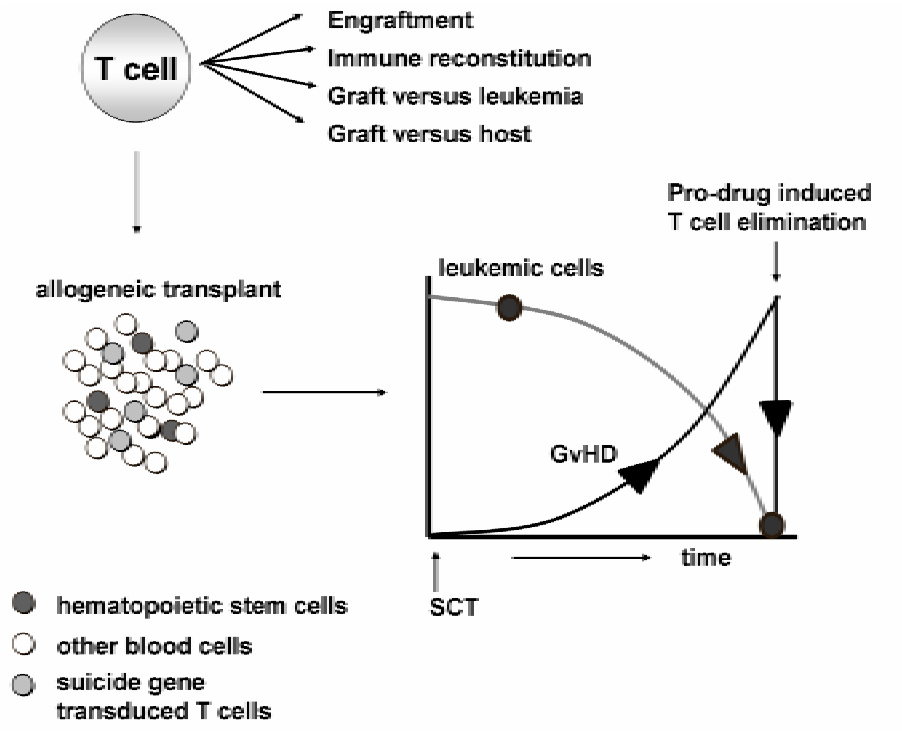

Figure 5. Principle of suicide gene therapy

T cells play an important role in allogeneic stem cell transplantation. They are supportive to the engraftment of the transplant, they reconstitute the immune system and are active against early viral infections post-transplantation and they recognize and eliminate leukemic cells that have escaped chemotherapy and irradiation. Unfortunately, the same T cells also respond to the patient's own tissue, called Graft versus Host Disease (GvHD).

An effective strategy to control GvHD is the introduction of a suicide gene into the T cells of the allogeneic graft. As indicated above, the T cells attack the leukemic cells and the number of leukemic cells decline. If the patient's normal tissue is attacked and GvHD develops, administration of a non-toxic pro-drug will allow the specific elimination of the alloreactive T cells, thereby abrogating GvHD.

At present, the Herpes Simplex Virus thymidine kinase (HSVtk) suicide gene from the herpes simplex virus 1 is the most effective suicide gene and is already used successfully in early clinical trials (111, 112). HSVtk sensitizes cells to specific elimination by ganciclovir (GCV), a thymidine analogue that is phosphorylated by HSVtk, resulting in a toxic metabolite (113). Results of the trials showed that GvHD could be efficiently controlled by the administration of GCV.

However, several limitations of the HSVtk/GCV suicide system have been observed.
In the first place, reactivation of cytomegalovirus disease can occur after allo-SCT and is commonly treated with GCV, leading to a premature elimination of HSVtk-expressing T cells (114, 115). Secondly, months after allo-SCT, patients who have regained immune competence may develop an immune response towards the HSVtk-positive T cells (111, 116, 117). In addition, resistance of HSVtk-positive T cells against GCV has been reported, which is caused by a truncated, non-functional HSVtk gene product (118, 119). Finally, the activity of GCV is limited to dividing cells only, resulting in partial resistance of resting HSVtk-positive cells against GCV (120).

Therefore, other suicide genes have been proposed to overcome the limitations of the HSVtk/GCV system. An important additional requirement for a safe clinical application of the suicide system is that the genetically manipulated cell population has to be purified prior to administration into the patient. In this way, it is ensured that only transgenic and therefore controllable T cells will be administered. Table 1 shows the proposed suicide genes to date.

Thomis et al. developed a suicide system based on the human Fas receptor (121). Cross-linking of the Fas molecule results in the recruitment of a death-inducing signaling cascade leading to apoptosis (122). Cell death is accomplished by crosslinking of the transgenic expression of chimeric protein containing the membrane-anchored intracellular domain of Fas fused to two copies of the FK506-binding protein (FKBP). A bivalent dimerizer drug, AP1903, binds FKBP and induces FAS cross-linking, leading to apoptosis (121, 123, 124).

Table 1. Suicide genes to control GvHD

Suicide gene	Elimination agent	Co-expression of a selection marker needed?	origin	Reference
Herpes Simplex Virus thymidine kinase	Ganciclovir	Yes	Viral	(111, 125, 126)
Fas ligand/ FK506-binding protein	AP1903	Yes	Human	(121)
Cytosine Deaminase	5-FC	No	Bacterial	(127, 128)
Reduced folate carrier gene	Methotrexate	No	Murine	(129)
CD20	Rituximab	No	Human	(130, 131)

Expression of the HSVtk and FAS gene can not be used as a tool to purify the genetically manipulated population prior to administration to the patient. Co-expression of a so-called selection marker is therefore needed. This leads to larger retroviral vectors, which are more difficult to transcribe at therapeutic levels (132).

An ideal suicide gene would be a selection marker and suicide gene in one. An example of this is the Murine-Reduced Folate Carrier Gene (129). Transgenic cells expressing the reduced folate carrier gene can be eliminated *in vivo* by methotrexate, and can be selected by trimetrexate *in vitro*, a lipid-soluble antifolate drug (129). However, this system is of murine origin and the transgenic cells will be immunogenic in patients, leading to premature elimination of the T cells *in vivo*.
The human CD20 gene is another candidate to act as a suicide gene as well as a selection marker (130, 131). CD20 transgenic T cells can be purified by monoclonal antibodies. *In vivo*, CD20-expressing cells can be eliminated by the anti-CD20 antibody rituximab.

Importantly, CD20 is a non-immunogenic self-antigen to the human immune system, and rituximab eliminates both dividing and non-dividing cells.

Retroviral vector production

Expression of a suicide gene depends on the successful transfer of the gene to the target cells. Still the best way to transfer the genes is by use of retroviral vectors.
Much research in the field of gene therapy has focused on the optimization of retroviral vector design and transduction of target cells to obtain stable, safe, long-term and sufficient expression for therapeutic use.

Moloney-Murine-Leukemia-Virus (MLV) based vectors are generally used as constructs to carry the transgene. Before transduction of T cells, retroviral particles have to be made by transfecting packaging cells with the retroviral vector (figure 6). These packaging cells contain the viral transgenes *gag*, *pol*, and *env*. These viral genes are required for packaging, processing, reverse transcription, and integration into the chromosomes. The viral gene *gag* encodes the viral matrix, capsid and nucleocapsid proteins, *pol* encodes a protease, reverse transcriptase and an integrase, and *env* encodes a membrane anchoring protein for cellular uptake. The RNA molecules that carry the Psi (Ψ) packaging signal *in cis* are packaged into mature virions. The retroviral vectors contain such a Ψ packaging signal. Because the viral vectors do not contain the *gag*, *pol* and *env* genes, generation of viral particles by the packaging cells will lead to replication-defective viral particles. Viral particles can be harvested from the culture supernatant (133-136).

T cell culture conditions

For retroviral transduction, T cells need to be activated *in vitro* by mitogens or TCR (CD3) stimulation. Protocols have been designed to optimize T cell expansion and gene transfer efficiency, but also to maintain T cell function. *In vitro* culturing and transduction lead to impaired T cell function and culture time must be as short as possible (137-139). For example, stimulation with OKT-3 and interleukin-2 (IL-2) leads to preferential expansion of CD8-positive T cells and inversion of the CD4:CD8 ratio (140). This leads to TCR Vβ skewing, or preferential clonal expansion. In addition, CD3/IL-2 stimulated T cells have impaired anti-EBV function (141).

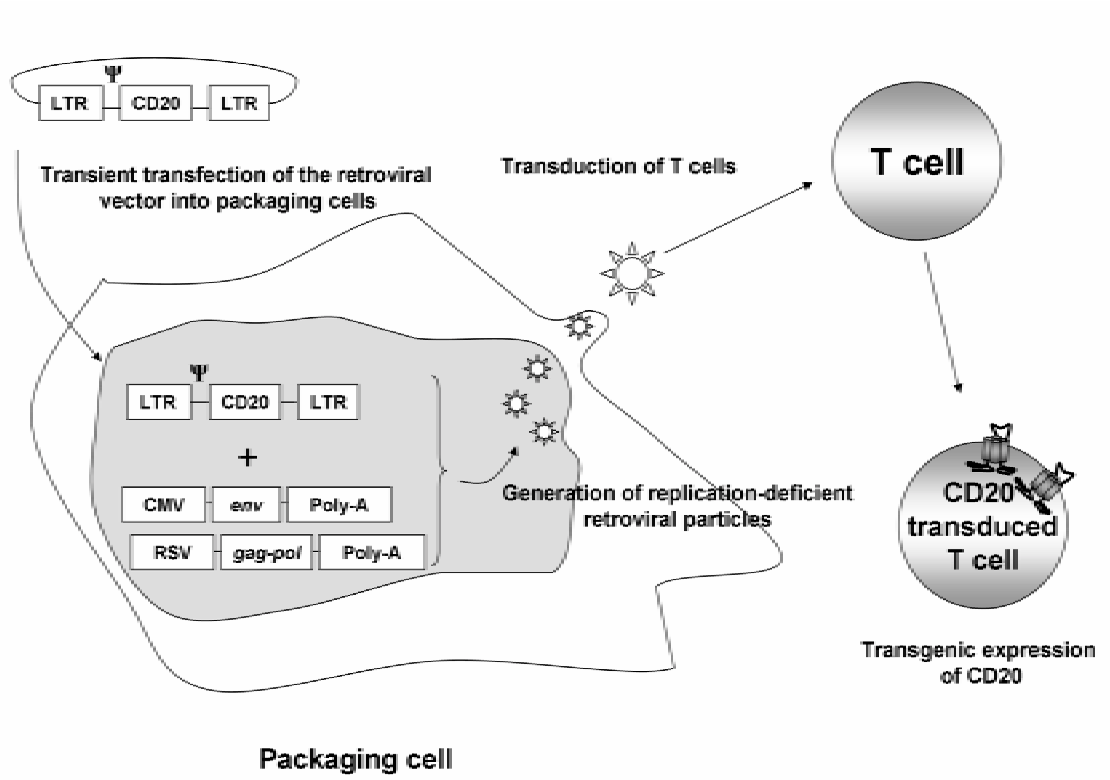

Figure 6. Replication-deficient retrovirus generation and transduction of T cells.
Replication-competent retroviruses contain three essential genes required for integration, reverse transcription and expression of the genes and for generation of new viral particles. These genes are *gag*, *pol* and *env* and are required for packaging, processing, reverse transcription, and integration into the chromosomes. A packaging signal, Ψ, is required for assembly of new viral particles. The *gag*, *pol* and *env* genes are replaced by the therapeutic gene within the retroviral vector. For the generation of replication-defective retroviral particles, the retroviral vectors are transfected into packaging cell lines. These cells express the *gag* and *pol* gene in one construct under control of the respiratory syncytial virus (RSV) promoter and the *env* gene in a different construct under control of the cytomegalovirus (CMV) promoter. Introducing the retroviral vector, containing the Ψ signal, into the packaging cells, results in the generation of retroviral particles. The retroviral particles are transported to the membrane of the packaging cells and liberated into the supernatant. These retroviral particles can transduce T cells by binding to the cell surface and internalize into the cytoplasm via a specific receptor. The RNA is released and the RNA is reverse transcribed via reverse transcriptase leading to double-stranded DNA. The DNA integrates into the chromosomes. Expression of the proteins is accomplished by the transcription and translation of the transgenes under control of the viral promoter within the long terminal repeat (LTR).

By the addition of a co-stimulatory signal (CD28) to the stimulation, gene transfer and transgene expression on the T cells is enhanced (142-144). Other effects of CD28 costimulation are enhanced expansion of $CD4^+$ T cells resulting in preservation of the CD4:CD8 ratio (145), reduction of apoptotic-induced cell death (146) and less TCR Vβ skewing (147, 148).

Retroviral constructs

Upon reverse transcription and insertion into the chromosomes, the transgene is transcribed from the Long Terminal Repeat (LTR). Purification of the transduced cells is warranted and therefore a selection marker is included into the retroviral vector. Inclusion of a selection marker often needs another internal promoter or an internal ribosome entry site.

One potential problem with retroviral vectors containing multiple transcription units is that if selection is applied for one gene, expression of the other gene can be reduced or lost completely (149, 150). Transgene size in the retroviral vector must not be too large, since this will reduce the viral titer and transgene expression (132). However, the development of fusion proteins may overcome these limitations (151).

Silencing and variegation (varied transgene expression in daughter cells) of gene expression can be overcome by the insertion of post-transcriptional regulatory elements. One such element is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). Enhanced transgene expression by improved post-transcriptional processing, transport of mRNA from the nucleus to the cytoplasm and improved translation of the mRNA, have been attributed to the activity of the WPRE element (152-154). The interferon-β scaffold attachment region (IFN-SAR), can also increase the transgene expression (155).

The use of insulators in retroviral vectors is associated with stable transgene expression. Insulators protect retroviral transgene expression from position effects through definition of the boundary between differentially regulated loci. In addition, they shield promoters from the influence of neighbouring regulatory elements and protect retroviral transgenes from silencing (156-159).

Safety of gene therapy

Infection with wild-type replication competent retroviruses can lead to malignant transformation of the infected cells (160-162). The uncontrolled proliferation is induced in large part by the strong enhancer within the U3 region of the LTR (162).

However, recently it has been demonstrated that insertion of a recombinant replication-defective retrovirus can lead to insertional mutagenesis, or the malignant transformation of the transgenic cells (163, 164). The complication of genotoxicity has made safety a main topic in the field of gene therapy. For safety of clinical application different strategies have been proposed. Transductions should be performed at a low multiplicity of retroviral infection (MOI), reducing the viral copies per cell (165). Inclusion of an insulator element into the retroviral vector may prevent oncogenic activation following chromosomal insertion of a retrovirus (166, 167). Also, the addition of a safety switch (suicide gene) will be required in case malignant transformation would occur.

Aims of this thesis

In the first part of this thesis, the results of the investigation of the suitability of CD20 as a suicide gene are described. Different retroviral vectors were constructed to study the best vector configuration for stable and long term CD20 expression on T cells. After transduction, cells expressing the transgene had to be purified from the non-transduced cells. Different selection markers and purification methods were compared. CD20 has signaling properties, and therefore, the functionality of CD20-expressing T cells was studied.
Part two describes the use of CD20 transgenic T cells to investigate the different mechanisms of rituximab. The role of CD20 expression-related resistance of anti-CD20 antibody therapy is studied and new antibodies directed to CD20 are compared with rituximab.

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Chapter 2

The CD20/αCD20 ‘suicide’ system: novel vectors with improved safety and expression profiles and efficient elimination of CD20-transgenic T cells

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Abstract

Adoptive transfer of T lymphocytes is an attractive strategy for many experimental treatment strategies for cancer. Unfortunately, manipulated T cells could be responsible for serious adverse events. Retroviral CD20-transduced T cells may be able to control these unwanted effects. CD20-positive cells are sensitive to rituximab, a monoclonal antibody specific for CD20. This permits their selective elimination *in vivo* in case of adverse events. To this end a system is required that permits efficient and safe transduction of donor T cells and effective elimination of CD20-positive T cells.

We constructed different CD20-encoding retroviral vectors and investigated the impact of inclusion of the woodchuck post-transcriptional regulatory element (WPRE) and the chicken hypersensitivity site 4 insulator elements on the levels, homogeneity, and stability of CD20 expression. Importantly, inclusion of either WPRE or insulator elements in the retroviral vector resulted in a dramatic improvement in the stability of CD20 expression. The insulator element also led to a much more homogeneous level of CD20 expression. We also show the efficient elimination of the CD20-transgenic T cells via rituximab by different effector mechanisms.

In conclusion, we have constructed CD20-encoding retroviral vectors with improved efficiency and safety profiles, which can be used as a suicide strategy.

Introduction

Adoptive transfer of T lymphocytes is an attractive approach in many experimental treatment strategies for cancer. However, these therapeutic T cells could also be responsible for unwanted effects. For example, in hematology allogeneic donor T cells may respond to the patient's own tissue resulting in the life-threatening complication Graft versus Host Disease (GvHD). An innovative strategy to control these unwanted effects is the genetic manipulation of these donor T cells with a 'suicide' gene. Introduction of a suicide gene into these T cells prior to transplant allows their selective elimination in case of uncontrollable GvHD (1, 2). Recently, the B cell-restricted CD20 molecule has been proposed as a novel suicide gene (3, 4). CD20-positive cells can be killed effectively via an anti-CD20 monoclonal antibody. Rituximab is a monoclonal antibody that recognizes the human CD20 molecule and is joined to a human IgG1 constant region. This IgG1 region is responsible for the activation of the complement system (CDC) and the recruitment of effector cells (ADCC) to kill the rituximab-ligated cells. It has also been shown that rituximab can induce apoptosis and growth arrest of CD20-positive cells (5-8). Importantly, the CD20 molecule may also be used as a selection marker to purify CD20-transgenic cells, which obviates the need to include selection genes (3, 4).

For stable expression of a transgene in T cells retroviral vectors are still the most effective tools. Particularly so, since most T cells proliferate vigorously after adoptive transfer, necessitating insertion of the transgene into the cell's genome. However, retroviral vectors also bear some disadvantages. For example, loss of transgene expression (9) and the potential activation of oncogenes (10). Moreover, for efficient elimination of CD20-transgenic T cells high CD20 expression level is necessary (11).

We now report the construction and analysis of a set of CD20-encoding retroviral vectors with various configurations. Two *cis*-acting elements were inserted into the vector in order to evaluate their effectiveness in mediating a homogeneous, stable, safe and high level of CD20 expression. The first is the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), which has been used widely in retroviral vectors and has been proven to increase transgene expression in different cell types *in vitro* and *in vivo*. Improved post-transcriptional processing, transport of mRNA from the nucleus to the cytoplasm and improved translation of the mRNA have been attributed to the activity of the WPRE element (12-14).

Secondly, we included insulator elements in the CD20 vectors. Chicken hypersensitive site 4 (cHS4) insulators have been used in retroviral vectors for the treatment of β-chain hemoglobinopathies for stable and long-term expression of the β-globin gene. Insulators protect retroviral transgene expression from position effects through definition of the boundary between differentially regulated loci. In addition, they shield promoters from the influence of neighboring regulatory elements and protect retroviral transgenes from silencing. Therefore, it is generally expected that insulators will also prevent oncogene activation following chromosomal insertion of a retrovirus. Insulators therefore represent an attractive safety tool for retrovirus-mediated gene therapy approaches (13, 15-17).

In this study we demonstrate the importance of the WPRE and insulator elements for homogeneous, stable and high level expression of CD20. Moreover, we show for the first time that CD20-transgenic T cells can efficiently be killed via rituximab by activating different human effector mechanisms.

Materials and Methods

Subcloning of the CD20 cDNA and the WPRE fragment.
The human CD20 cDNA was amplified by PCR from the pCMV-CD20-expression vector and the WPRE fragment was amplified from the pSFβ-EGFP-WPRE retroviral vector (18), by using the following primers:
CD20 FW 5'-GGG CCG CGG CCG CCG CCA TGA CAA CAC CCA GAA ATT CAG TA - 3'
(Kozak-sequence underlined); CD20-SalI RV 5'-GGG GTC GAC AAA TCA CTT AAG GAG AGC TGT CAT-3'; CD20-NotI RV 5'-GGG GCG GCC GCA AAT CAC TTA AGG AGA GCT GTC AT-3'; WPRE-NotI FW 5'-GCG GCC GCG AAT TCG AGC ATC TTA CCG CCA T-3'; WPRE-SalI RV 5'-GTC GAC TTG GCA TGC CAA GTT GAC GAT-3'.
The amplified CD20 fragment and WPRE were ligated into a pCRII TOPO cloning vector (Invitrogen, Paisley, UK) according to manufacturer's instructions, creating the following constructs: pCRII-CD20-BamHI-NotI, pCRII-CD20-BamHI-SalI, pCRII-WPRE-NotI-SalI.

Construction of CD20 retroviral vectors and generation of virus particles
The Moloney Murine leukemia virus (MLV)-based vector pMX-α-IRES-β (19), was used for the insertion of the CD20 gene, WPRE and the insulator elements. The pMX-CD20

vector (CD20 vector) was generated by replacing the α-IRES-β fragment for the CD20 gene after digesting pMX-α-IRES-β and pCRII-CD20-BamHI-SalI with BamHI and SalI. The CD20W vector was generated by first inserting the CD20 gene from the pCRII-CD20 vector into the BamHI and NotI site of the pMX-α-IRES-β, substituting the α fragment for the CD20 gene. Next, the IRES-β fragment was substituted for the WPRE element from pCRII-WPRE-NotI-SalI, by digesting with NotI and SalI. CD20-INS, CD20W-INS, CD20-ICE and CD20W-ICE vectors were generated as followed: the double copy of the insulator core element (ICE) and the 1.2kb cHS4 insulator element (INS) were obtained from the pNI-CD expression vector by digesting with KpnI (ICE) or with XbaI (INS). These fragments were blunted and cloned into the blunted 3'LTR NheI restriction site of the different CD20 retroviral vectors. CD20 virus particles were produced by calcium phosphate transfection of amphotropic Phoenix packaging cells as previously described (20). Viral titers were determined by serially diluted virus supernatant on CEM T cells.

Cell culture

The CEM T cell line was cultured in RPMI (Gibco-BRL, Paisly, Scotland), 10% fetal calf serum (FCS, Integro, Zaandam, the Netherlands), penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco-BRL), 5 x 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany), further called as culture medium. Amphotropic Phoenix cells were cultured in DMEM (Gibco-BRL), 10% FCS, penicillin, streptomycin and 2-mercaptoethanol. PBMCs were isolated by centrifugation through Ficoll (Amersham Pharmacia, Uppsala, Sweden) and stimulated with 300 U/ml human recombinant interleukin 2 (hurIL-2) (Proleukin, Chiron, Amsterdam, the Netherlands) for 24 hrs to generate ADCC effector cells. All cells were cultured at 37°C in a 5% CO_2 atmosphere.

Flow cytometry and analysis

CD20 expression was determined by monoclonal antibodies specific for the human CD20 molecule conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (BD Biosciences, San Jose, CA). Monoclonal antibody anti-CD20 Mabthera (Rituximab) was purchased from Roche. WinMDI 2.8 software was used to analyze CD20 expression level (mean fluorescence intensity, MFI) and homogeneity of expression of different membrane proteins. Homogeneity of CD20 expression was determined by the value of the percent coefficient of variation (CV).

Transduction of T cell lines, selection and clonal expansion

To study CD20 expression in T cells CEM T cells were transduced with different the viral supernatants. In a Costar 12-wells plate (Corning Incorporated, Cirus, USA) 10^5 cells were resuspended in 2 ml viral supernatant and culture medium (MOI of 1) in the presence 6 μg/ml of polybrene (Sigam-Alldrich, Steinheim, Germany). After culturing for 24h the infection medium was refreshed with 2 ml culture medium. Transduction efficiency was determined after 5 or 6 days of culture. CD20 positive transduced T cells were selected with monoclonal antibodies against CD20, conjugated with magnetic beads according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). CD20-positive cells were selected by passing the labelled cells through a magnetic cell sorting column using positive selection program on an autoMACS cell separation device (Miltenyi Biotec, Bergisch Gladbach, Germany).

rituximab-mediated killing assays

For CDC assays, 10^6 CEM-CD20-positive cells were resuspended in various concentrations of human or rabbit serum with increasing concentration of rituximab at 37°C at different time points in a 5 ml Falcon tube (Becton Dickinson Labware, Franklin Lake, USA). Cells were washed twice with PBS and resuspended in 0.5 ml of PBS/1%BSA and propidium iodide (PI) (1 μg/ml), allowing the discrimination of living from dead cells. As negative controls cells in the presence or absence of human serum with or without rituximab were used.

The ADCC assay was based on a newly developed cytotoxicity assay by Jedema et al (21). CD20-positive cells were washed in PBS and resuspended at $1.0x10^6$ cells/ml. Next, the cells were stained with 5 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Europe BV, Leiden, The Netherlands) for 10 min at 37°C. Subsequently, an equal volume of FCS was added and the cells were left at room temperature for 5 min. Cells were washed twice with PBS and the cell concentration was adjusted to 0.25×10^6 cells/ml in culture medium. 100 μl of the CD20-positive CFSE-labelled cells were plated in 96-roundbottom microtiter plate per well in the absence or presence of 10 μg/ml rituximab. IL-2 (300 U/ml) stimulated PBMCs were added at 1:10 target/effector ratio (ADCC) or with 100 μl of human serum (CDC). To study the effect of ADCC and CDC on rituximab-mediated cell kill simultaneously both effector cells and human serum were added. After incubating the plates for 4 hours at 37°C the wells were harvested and washed with PBS. Cells were resuspended in 200 μl of PBS, 1 μg/ml propidium iodide and 5,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, Florida) were added, allowing quantitative

analysis of the cell populations using flowcytometry. In order to determine the absolute number of surviving cells acquisition was stopped after taking up 2,500 Fluorospheres and the CFSE-positive and PI-negative target cells were counted. The percentage rituximab-mediated cell survival was calculated as follows:

$$\%\ \text{rituximab survival} = \frac{\text{with rituximab absolute viable CFSE+ PI- cells}}{\text{without rituximab absolute viable CFSE+ PI- cells}}$$

For all rituximab-mediated killing experiments three independent experiments were performed and all experiments were performed in triplicate.

Statistical Analysis

Statistical analyses were performed with SPSS 11.5. To compare the homogeneity and MFI of CD20-positive cells transduced with the different CD20 vectors a Dunnett's t-test was performed. A Student's t-test was used to analyze rituximab-mediated cell kill of the cells transduced with the different constructs and the killing efficiencies of the different effector mechanisms. A p value <0.05 is considered to be significant.

Results

Construction of CD20-encoding retroviral vectors

CD20-encoding retroviral vectors were constructed to obtain a high, stable, and homogeneous CD20 expression in T cells. The human CD20 cDNA was cloned into a MLV-based backbone under transcriptional control of the LTR promoter. A Kozak sequence was included within the first AUG codon of the CD20 gene to favor ribosome binding for the start of translation (22). A second construct was made through insertion of the WPRE element downstream of the CD20 cDNA, resulting in the CD20W vector. A double copy of the 250 bp cHS4 insulator core element (ICE) or a single copy of the complete 1.2 kb cHS4 insulator element (INS) was inserted into the U3 region of the 3' LTR of the CD20- and CD20W-vectors, resulting in the CD20-ICE, CD20-INS, CD20W ICE and CD20W-INS vectors, respectively (Figure 1).

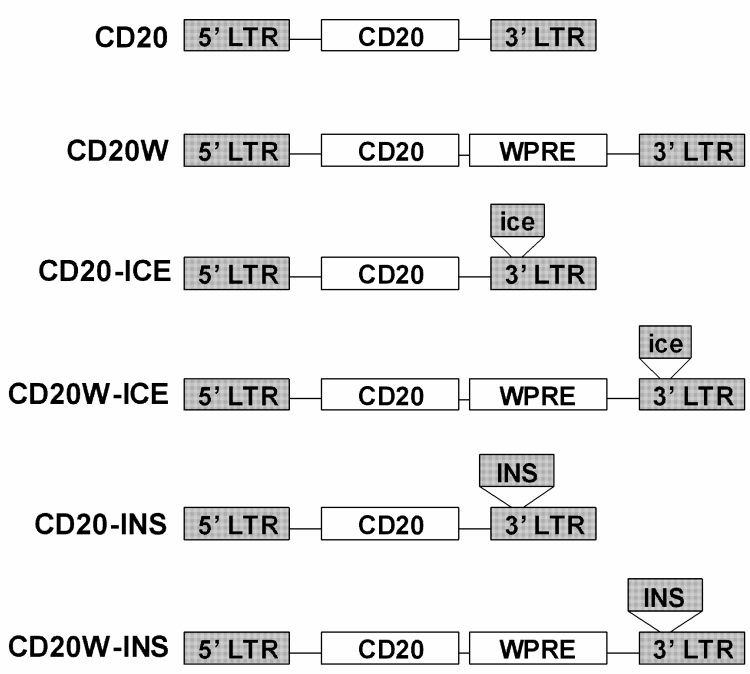

Figure 1. Schematic representation of the CD20 retroviral constructs
CD20 cDNA was cloned into the pMX backbone under transcriptional control of the MLV-LTR. The WPRE was inserted downstream of the CD20 cDNA. The 1.2 kb cHS4 insulator (INS) or the insulator core element (ICE) was inserted into the 3' U3 region.

Viral titers and CD20-expression patterns
Several independent transient transfection experiments were performed in amphotropic Phoenix cells to generate various batches of viral preparations. Average titers of 0.4 x 10^6 (ranging from 0.6 x 10^5 – 1.5 x 10^6, n = 8) transduction units (TU)/ml were obtained with both the CD20 and CD20W vectors. Inclusion of the insulator elements reduced the viral titers two-six fold, confirming previous observations (data not shown) (23, 24).

To compare the level of CD20 expression provided by the individual constructs, the CD20-negative human T cell line CEM was transduced in triplicate with each of the CD20-encoding vectors. For application of gene-modified cells in clinical settings, it is desirable to minimize the number of viral integrations per cell, thereby reducing the chance of insertional mutagenesis. Therefore we used an MOI of 1 in all experiments, routinely leading to transduction efficiencies of < 30%, as expected (25) (Figure 2A). After transduction, CD20-positive cells were purified via immunomagnetic beads and kept in culture for at least three months. CD20 expression was studied by flow cytometry shortly after transduction, after immunomagnetic bead selection and after long term culture. The level of CD20 expression is represented by the MFI. Figure 2 displays a typical example of

a transduction, purification and long-term culture of one experiment. Figure 3A shows the MFI of CEM cells transduced with the different CD20 vectors of three independent transduction experiments performed in triplicate. The analyses were restricted to the positive fraction only. The CD20 vector (MFI = 386 ± 29) and the vectors containing the WPRE element, CD20W, CD20W-ICE and CD20W-INS, provided the highest level of CD20 expression (351 ± 66, 378 ± 42 and 309 ± 14, respectively). In contrast, the MFI of cells transduced with CD20-ICE and CD20-INS vectors were 2- to 3-fold reduced (198 ± 3 and 118 ± 1, respectively) compared with the CD20 vector ($p < 0.001$). These data suggest that insulators alone exert a negative effect on the level of CD20 expression, confirming previous reports (23, 24). The additional presence of the WPRE element seems to compensate for the negative insulator effect.

Homogeneity and stability of CD20 expression

The homogeneity of CD20 expression is represented by the coefficient of variation (CV), that represents the variability of CD20 expression within a given cell population (23). Consequently, the lower the CV value, the more homogeneous the levels of CD20 expression. The CV values are represented by the mean ± s.d. of three different transduction experiments performed in triplicate (Figure 3B). Cells transduced with the CD20 vector displayed a broad CD20 expression pattern (CV = 26.8 ± 1.6). Inclusion of the WPRE element (CD20W) did not improve the homogeneity (CV = 27.0 ± 1.9). In contrast, a significant positive effect ($p<0.001$) on the CV value was observed in cells transduced with all vectors containing the insulator elements, compared to the CD20 vector. For the CD20-ICE and CD20-INS vectors the CV values were 22.8 ± 1.7 and 22.1 ± 2.0, respectively. Inclusion of both the WPRE element and the insulator did not result in more homogeneous CD20- expression pattern, compared with vectors containing the insulators but without the WPRE element (CV = 22.5 ± 1.9 for CD20W-ICE and 21.0 ± 1.9 for CD20W-INS These results indicate that insulator elements lead to a more homogeneous CD20-expression pattern.

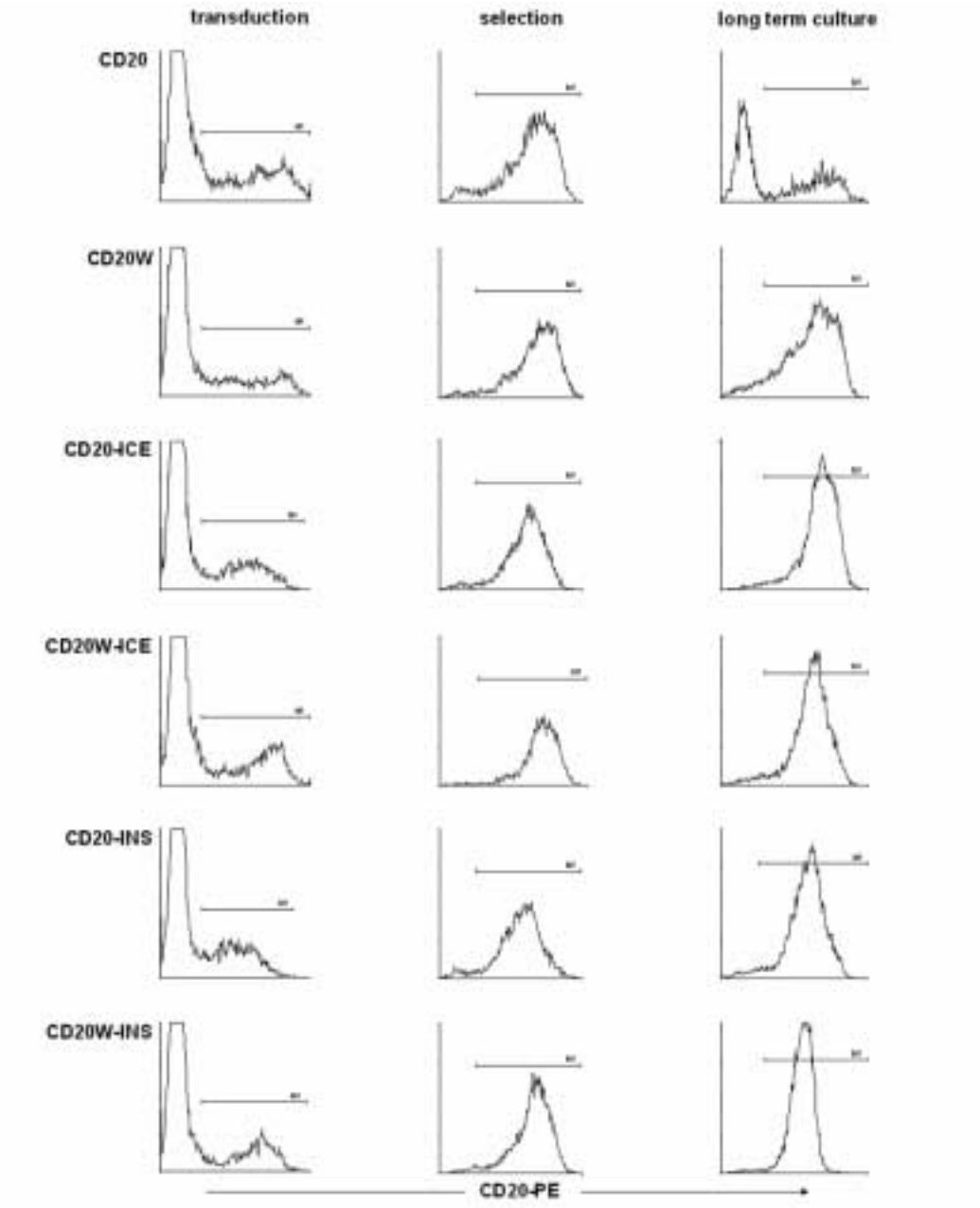

Figure 2. FACS-analysis of CD20-expression patterns of transduced CEM cells

The first column represents the transduction of CEM T cells at an MOI of 1 with the various CD20-encoding vectors. After 5 or 6 days cells were harvested and the percentage CD20 expression was analyzed by FACS. Transduced CEM-CD20 cells were selected with anti-CD20 monoclonal antibody conjugated with magnetic beads. The second column shows the FACS analysis of the CD20 selected cells. The transduced and selected cells were cultured for three months and again the MFI and the proportion of CD20-positive cells were determined as shown in the third column.

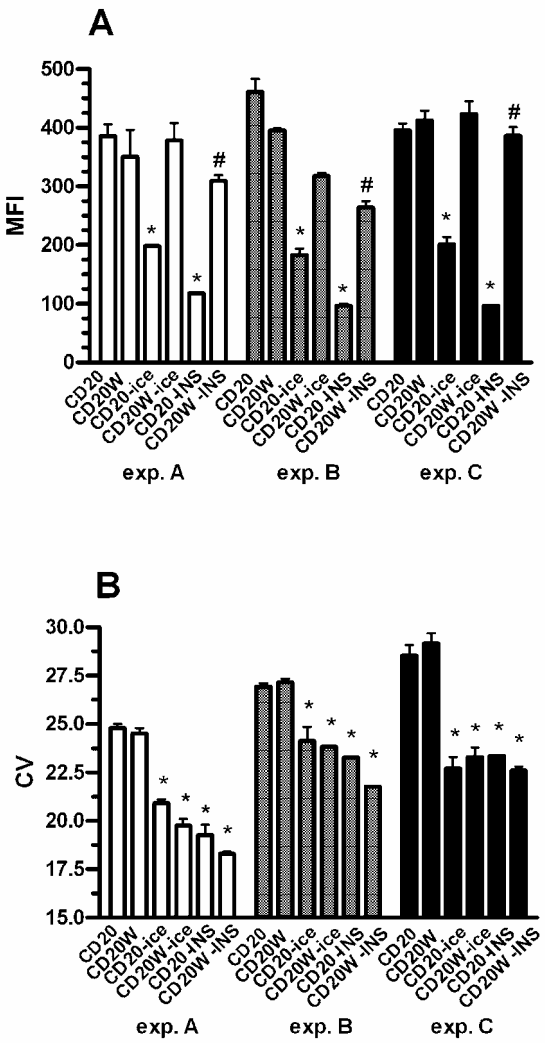

Figure 3. Analysis of CD20-expression pattern of three different independent transduction experiments in triplicate

(**A**) The MFI of CD20 expression. Cells transduced with the CD20-vector were used as the standard and compared with the cells transduced with the other vectors. A Dunnet's t test was used to test the significance. The bars represent the mean and s.d. of three independent transduction experiments performed in triplicate. (**B**) The homogeneity of CD20 expression, indicated by the CV value. The CV value of cells transduced with the CD20 vector was used as the standard and compared with the cells transduced with the other vectors. A Dunnet's t test was used to test the significance. The bars represent the mean ± SD of three independent transduction experiments performed in triplicate. * = $p < 0.001$ and # = $p < 0.037$.

The transduced cells were purified with immunomagnetic beads and after purification more than 95% of the cells expressed CD20. Shortly after selection the MFI and CV-value was

analyzed. The second column in figure 2 displays a typical example of the purification of transduced cells. The highest CD20 expression pattern was obtained with vectors containing the WPRE element compared with vectors lacking the WPRE element. The CV-value of cells transduced with insulator-containing vectors was significantly lower compared to CD20 and CD20W.

Next, we investigated the stability of CD20-expression. The purified cells were cultured for a period of 3 months after which the fraction of CD20-positive cells, MFI and the CV values were determined again (Figure 2, third column). CD20 expression was gradually lost in cells transduced with the CD20 vector. Only 42% of the cells displayed CD20 expression after continued culture for 3 months. A loss in CD20 expression, albeit low (5%), was observed in cells transduced with CD20W as well. In contrast, cells transduced with vectors containing the insulator elements maintained CD20 expression after culture, indicating that insulators prevent a temporal loss in CD20 expression in T cell lines. No difference in homogeneity of CD20 expression was observed in cells transduced with CD20 or CD20W. However, cells transduced with vectors containing the insulators provided an even more homogeneous CD20 expression. The most homogeneous CD20 expression was obtained with cells containing CD20W-INS (CV = 12.5). Together, these data demonstrate that the WPRE fragment increases expression of the transgene, i.c. CD20, in T cells. Moreover, the inclusion of insulator elements alone has a profound effect on the homogeneity and stability of expression.

Evaluation of the sensitivity of rituximab-mediated killing of CD20-transgenic CEM cells.

To study whether CD20-transgenic T cells are sensitive to rituximab and its mechanisms we subjected the cells to human complement and effector cells. CEM cells were transduced with the CD20 vector, selected and used within two weeks of cultering. All cells express CD20 and for the CDC assays we used human serum as complement source and rabbit serum as positive control. As shown in figure 4 maximum lysis is achieved at a minimum of 10 µg/ml rituximab (Figure 4A), 25% of human serum (Figure 4B) and after 1 min at 37°C (Figure 4C).

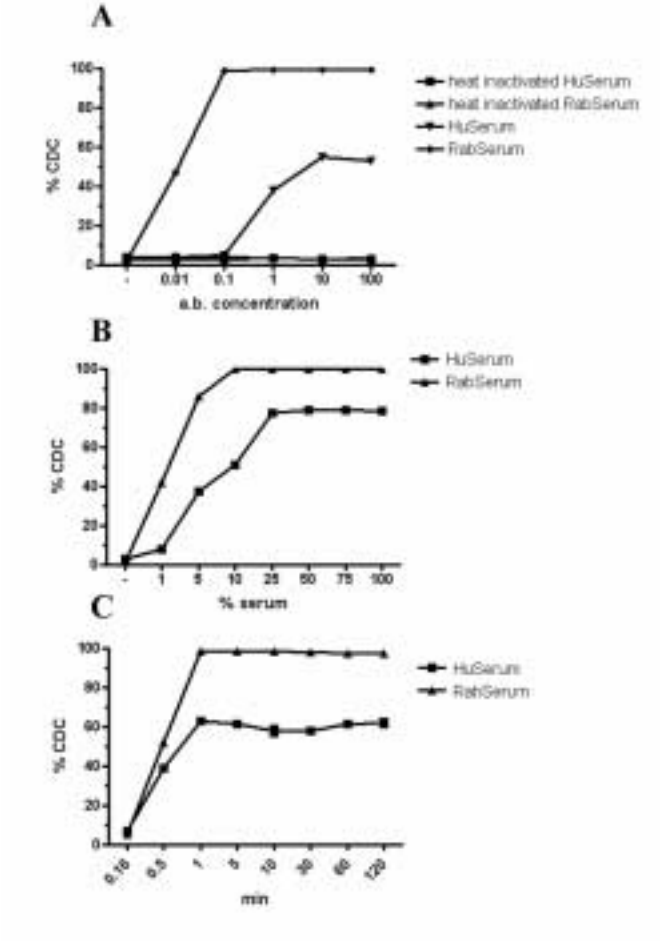

Figure 4. rituximab-mediated CDC

(**A**) Titration of the minimal concentration of rituximab needed for optimal lyses. $1.0x10^6$ CEM-CD20 cells were resuspended in 50% of serum, as a complement source, to a final concentration of 1 ml. The cells were kept at 37°C for 1 hr. Different concentrations of antibody were added. As a positive control rabbit serum was used and as negative control heat inactivated sera. (**B**) Titration of the minimal concentration of human serum needed for optimal killing of CEM-CD20 cells. $1.0x10^6$ CEM-CD20 cells were incubated with 10 µg/ml of rituximab and resuspended in different concentrations of serum to a final concentration of 1 ml. The cells were kept at 37°C for 1 hr. As a positive control rabbit serum was used (**C**) Time course of rituximab- induced CDC on CEM-CD20 cells. CEM-CD20 cells were incubated with 10 µg /ml and resuspended in 1 ml medium containing 25% serum. Cells were incubated at 37°C for various time points. Propidium iodide exclusion was used to determine the level of cell kill

To investigate the optimal condition for rituximab to activate effector cells we titrated the concentration of antibody, target-effector cell ratio and incubation time. The experiment was performed in triplicate and each bar represents the mean ± SD. Figure 5 displays the results of CEM-CD20 cells with or without rituximab and IL-2 stimulated PBMCs as

effector source. The surviving cells were counted after 4 hrs and results are expressed as % of the rituximab untreated cells. Increasing the concentration of rituximab (0.1 μg/ml – 100 μg/ml) had little effect on the effector function of the PBMCs towards CEM-CD20 cells. However, using more effector cells resulted in more rituximab-mediated cell kill. A maximum of 22% cell survival was obtained with 100 μg/ml of rituximab with a target to effector ratio of 1:30. Increasing the incubation time did not result in more rituximab-mediated killing of CEM-CD20 cells (Figure 5B). No direct effect of rituximab in proliferation or induction of apoptosis was observed in our experiments (data not shown).

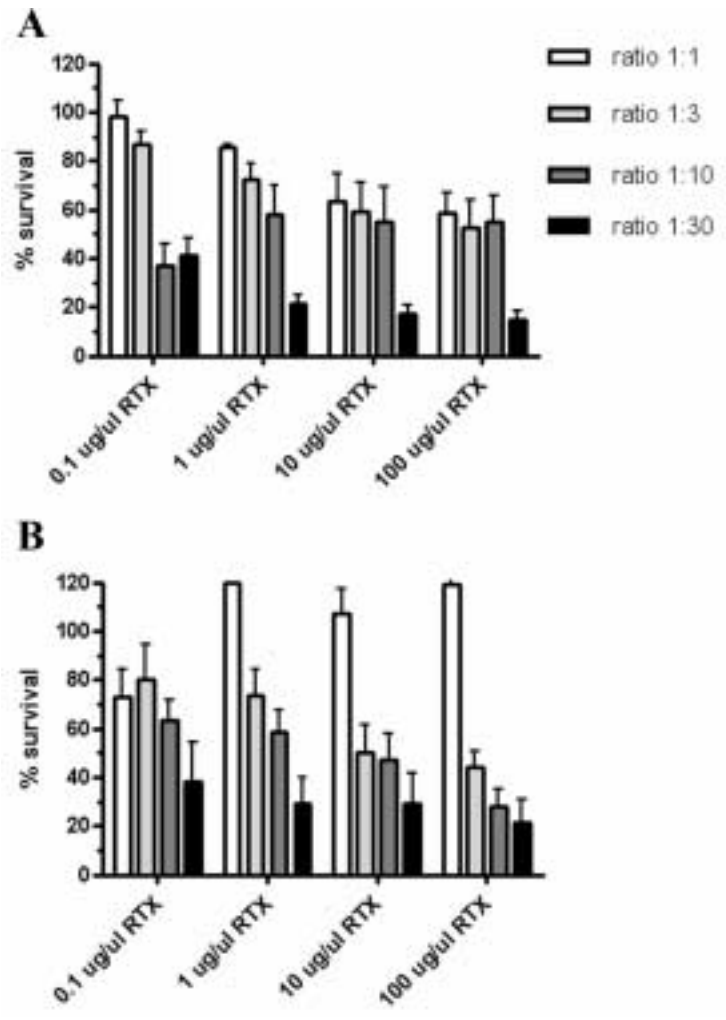

Figure 5. rituximab-mediated ADCC (**A**) CEM-CD20 cells were incubated with increasing concentrations of rituximab and increasing concentrations of target:effector cell ratios. Absolute numbers of surviving cells were counted and plotted against the proportion of the untreated CEM-CD20 cells (no rituximab). Cells were incubated for 4 (**A**) or 24 hrs (**B**) at 37°C. Each experiment was performed in triplicate and the bars represent the mean ± SD

Complete elimination of CEM-CD20 cells was not obtained with either CDC or ADCC alone. To obtain maximum kill we combined the two effector mechanisms of rituximab. In

this assay 10 µg/ml rituximab was used, 50% human serum and 1:10 target-effector cell ratio. After incubation of the cells for 4 hr at 37°C the surviving cells were counted and results were expressed as % of the rituximab untreated cells. Figure 6 shows a representative example of the direct effect of rituximab, activation of effector cells, CDC activity and the combined activity of both mechanisms. Again, no decrease in cell numbers was observed with rituximab alone. With ADCC alone, 67.8 ± 13.7% of the cells were killed and with CDC this was 69.5 ± 3.3%. Combining both effector mechanisms did lead to an increase in cell kill ($p < 0.001$), 28.6 ± 3.9% of the cells survived in this assay. These data suggest that the effector mechanisms act complementary.

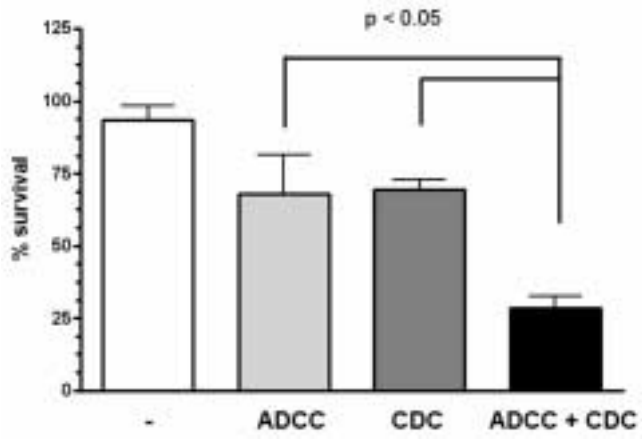

Figure 6. rituximab-mediated killing

CEM cells transduced with the CD20 vector were incubated with (10 µg/ml) or without rituximab. Absolute numbers of surviving cells were counted and plotted against the fraction of the untreated CEM-CD20 cells (no rituximab). The open bar shows the influence of rituximab alone on cell kill. The light-gray bar displays the ADCC activity of rituximab and the dark-gray bar the CDC activity of rituximab. The black bar displays the combined CDC and ADCC activity of rituximab on CEM-CD20 cells. Experiments were repeated three times with effector cells from different donors and different human sera as a source of complement. Here a representative experiment is displayed. All experiment s were performed in triplicate and the bars represent the mean ± SD A student's t test was used to test the significance of the CDC + ADCC activity versus CDC or ADCC alone.

To study whether the bulk transduced cells with the different constructs (CD20, CD20W, CD20-ICE and CD20W-ICE) were sensitive to rituximab and its effector mechanisms or whether one of the vectors resulted in better cell kill we employed these cells in the same killing assay (Figure 7). For the ADCC activity (1:20 target:effector ratio) of rituximab no

major differences were observed in cell survival that ranged from 13.2 ± 1.1 % to 25.5 ± 1.4%. In contrast, for CDC differences were observed in rituximab-induced cell kill between the cells transduced with the different constructs, only cells transduced with CD20-ICE vector were significantly less sensitive to rituximab and complement compared to CD20 alone. The cell survival of CEM-CD20 was 43.1 ± 1.2%, CEM-CD20W 47.5 ± 3.8%, CEM-CD20-ICE 62.4 ± 3.5% and for CEM-CD20W-ICE the cell survival was 36.1 ± 2.0%. Finally, we combined the effector mechanisms and again the cell survival was significantly decreased to 6.3 ± 1.4%, 11.3± 3.1%, 9.9 ± 1.8% and 8.7 ± 2.0% for CEM-CD20, CEM-CD20W, CEM-CD20-ICE and CEM-CD20W-ICE, respectively. There was no significant difference in the killing efficiency of the CD20 transduced cells compared to the improved CD20-encoding retroviral vectors. These data clearly show for the first time that CD20-transgenic T cells can be efficiently eliminated by rituximab via activation of different effector mechanisms of human origin.

Discussion

The success of any suicide system depends on the effectiveness of selective elimination of the desired cell type. Selectivity is achieved by the enforced, unique expression of a particular protein that renders the cell sensitive toward the eliminating reagent. In the case of the CD20/αCD20 system, selectivity is achieved through *ex vivo* transduction of T cells with a recombinant retrovirus encoding the CD20 transgene that is normally not expressed on T cells, but only on B cells. Thus, unmanipulated T cells will remain insensitive toward CD20-targeting reagents, such as the human-mouse chimeric mAb rituximab. As a side effect, patient's B cells will be eliminated at the same time. However, plasma cells, which are CD20-negative, will be preserved ensuring continuous production of serum immunoglobulin.

Retroviruses are still the favorite tools for genetic modification of T cells, despite their involvement in oncogenesis when used under certain circumstances to modify hematopoietic progenitor cells (10, 26). Nevertheless, also for modification of T cells caution is still warranted and safer vector configurations are desirable. We therefore evaluated the impact of the WPRE and insulator elements on transgene-expression in human T cells. We also explored the efficacy of rituximab-induced cell kill of CD20-expressing T cells with effector mechanisms of human origin.

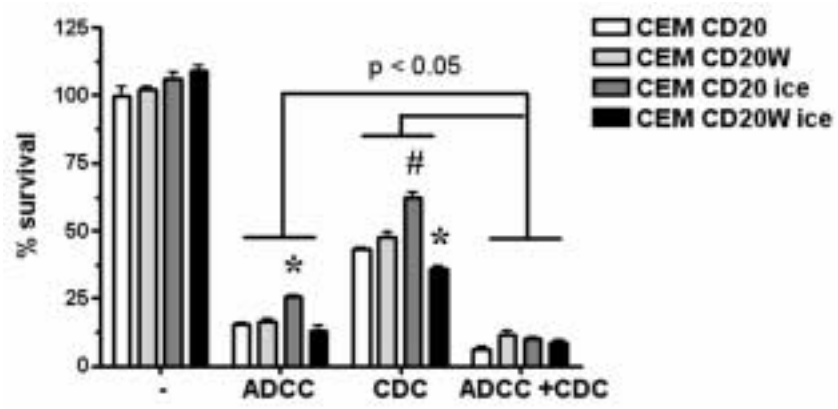

Figure 7. rituximab-mediated killing of CEM-CD20 cells transduced with the different constructs
Cell survival was determined as described in the legend of figure 6. The open bar represents CEM-CD20, light-gray bar CEM-CD20W, dark-gray CEM-CD20-ICE, and the black bar CEM-CD20W-ICE. (**A**) Induced cell kill of rituximab alone, (**B**) induced ADCC by rituximab, (**C**) induced CDC by rituximab, and (**D**) induced combined activity of CDC and ADCC by rituximab. Experiments were repeated three times with different effector cells from different donors and different human sera as a source of complement. Here a representative experiment is displayed. A student t test was used to test the significance of the CDC + ADCC activity versus CDC or ADCC alone, and to test the significance of rituximab-mediated cell kill of the cells transduced with the CD20 vector versus the cells transduced with the CD20W, CD20-ICE and CD20W-ICE vectors. The bars represent the mean ± s.d. of three independent transduction experiments performed in triplicate. * = $p < 0.001$ and # = $p = 0.0039$.

Rituximab has been shown to activate different effector mechanism for the elimination of CD20-positive cells leading to death of the cell. Rituximab alone or crosslinking of rituximab did not directly kill the CEM-CD20 transgenic T cells nor was their proliferation inhibited. *In vitro* and *in vivo* data have clearly demonstrated that rituximab recruits FcγR-expressing effector cells (27, 28). Consistent with these observations, CEM-CD20 cells were lysed efficiently by activated PBMCs in the presence of rituximab. It remains to be seen to what extent these *in vitro* findings correlate with the contribution of effector cells in *in vivo* clearance of CD20-expressing cells. Nevertheless, these ADCC experiments demonstrate that CD20-positive T cells can be eliminated efficiently by effector cells via rituximab.

As with ADCC, CEM-CD20 transgenic T cells were also sensitive to human serum. Rabbit serum was used as a positive control and was much stronger in inducing cell kill than human serum. However, we show that human serum can kill significant numbers of cells. Importantly, CEM-CD20 cells with higher CD20 expression level were more sensitive to

rituximab compared to cells with lower MFI, confirming previous observation with fresh tumor material of B cell origin (11). Combining CDC and ADCC did lead to almost complete elimination of CD20-positve T cells. An additive effect of both CDC and ADCC has been described previously in rituximab-mediated killing experiments on fresh B-cell leukemia samples (29).

In an attempt to positively influence CD20 expression the WPRE element was inserted downstream of the CD20 cDNA. The WPRE element has been widely proven to enhance transgene expression under control of several different promoters and in different cell lineages (13, 14). Its exact mode of action is unclear, but it has been suggested that the WPRE element acts on both the transcriptional and translational level. It promotes mRNA transport from the nucleus to the cytoplasm, and may facilitate protein expression from mRNAs that normally would be degraded within the nucleus (12-14). In our hands the WPRE element had only a minor positive effect on the level of CD20 expression. Surprisingly, a profound effect of the WPRE element was observed on the stability of CD20 expression in CEM cells after selection and continued culture for 3 months. These findings indicate that the WPRE element is a useful component in CD20-encoding retroviral vectors. Recently, suspicions have been raised with regard to the potential oncogenicity of the truncated X protein that is encoded within the WPRE (30). Although our construct encode the non-carcinogenic full-length X protein, we can not exclude the possibility at this stage that mutant X protein-expressing cells may arise *in vivo* and exhibit a selective growth advantage. Since the effects of truncated X proteins have been studied in liver cells only the relevance of these findings for other cell types is presently unknown (31, 32). Of note, the recently published MP71 vector did not give a higher CD20 expression in our hands (data not shown) (33). Apparently, for expression of heterologous genes in human T cells, pMX is already a much better vector than LGSN, which seems to give a relatively low GFP expression, compared to pMX-EGFP vectors (data not shown). The WPRE element has often been coupled to EGFP expression for enhancement of expression in T cells and others cell types (14, 33). CD20 expression has also been shown with vectors containing CD20 and the WPRE element in T cells (34). However, in this report two lentiviral constructs with two different promoters were compared, both vectors containing the WPRE element. So, the effect on CD20 expression with or without the WPRE element was not compared in this report.

The recent development of leukemia in three patients who had received retrovirally-modified hematopoietic progenitor cells proved the, till then theoretical, risk of insertional

mutagenesis to be real (10, 35, 36). Insertional mutagenesis by a recombinant retrovirus has also been observed after retroviral modification of murine hematopoietic progenitor cells (26). In response to these observations the inclusion of insulators has been postulated to increase the safety of retroviral constructs (37). Insulators are currently used in retroviral vectors for the treatment of γ-globin deficiencies and have been shown to reduce position effects and protect transgene expression from silencing. Insulators also have shielding capacity and block transgene expression by interfering with promoter-enhancer activity. As a consequence, insulators reduce the influence of neighboring promoters/enhancers on transgene expression and provide a more homogeneous transgene expression (12, 13, 17, 18, 23, 24, 38). Theoretically, insulators will therefore also protect from oncogene-activation by chromosomal insertion of the retrovirus, though this assumption still awaits formal proof. Our experiments clearly demonstrate that both the full-length 1.2 kb insulator as well as the double copy of the insulator core element had a profound effect on the homogeneity of the CD20 expression level. These observations indicate that both elements are fully functional in T cells and support their incorporation in future vectors. Obviously, their capacity to truly prevent insertional mutagenesis needs to be assessed empirically in *in vivo* experiments. To our knowledge, this is the first time that insulators have been employed successfully in T lymphocytes. We expect that the use of insulators in combination with low MOI will minimize the chance of insertional mutagenisis (25). Though the insulators resulted in an increased homogeneity of CD20 expression they also led to a decrease in viral titer and CD20 expression level. This is consistent with published data (23, 24). This phenomenon may be explained by prevention of the effect of endogenous enhancers on the internal promoters. In our experiments, the simultaneous inclusion of WPRE compensated this effect. The enhanced RNA processing of the WPRE element could explain the compensated loss of CD20 expression. The loss may be caused by the blocking of endogenous promoters and/or enhancers. Of course, this is speculative and needs requires further demonstration. If future experiments confirm the risks associated with the use of the WPRE fragment an interferon-β scaffold attachment region (IFN-SAR), which reportedly increased the transgene expression, may be included instead in insulator-containing constructs (23, 39).

In summary, we have generated safe CD20-encoding retroviral vectors that permit the generation of CD20-positive T cells at an MOI of 1 that can be killed efficiently via rituximab in the presence of human complement and effector cells. Previously, it has been shown that the complete insulator element and a double copy of the insulator core element

function equally well in erythroid cells. Our data now extend these findings to human T cells.

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Chapter 3

Transgenic expression of the CD20 suicide gene does not alter the function of human T lymphocytes

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Abstract

The introduction of a safety switch into alloreactive donor T cells is an elegant approach to control Graft-versus-Host Disease (GvHD) after allogeneic stem cell transplantation (allo-SCT) for the treatment of leukemia. Human CD20 can be used as a safety switch since CD20-positive cells can be efficiently eliminated by the anti-CD20 monoclonal antibody rituximab.

In this report, the impact of transgenic CD20 expression on T cell function was investigated by retroviral transduction of primary human T cells. Results showed that CD20 expression did not impair growth characteristics of transgenic T cells *in vitro*. Although on T cells CD20 was efficiently translocated into lipid rafts upon rituximab ligation, no apoptosis was induced, even after hyper cross-linking of rituximab.
The antigenic response of CD20-positive T cells to a specific stimulus was similar to the CD20-negative T cells. In addition, in mice we demonstrated that CD20-positive T cells distributed into similar organs as non-transduced cells and were able to induce xenogeneic-GvHD. Remarkably, whereas CD20-negative T cells were scattered throughout to whole organ, CD20-transgenic T cells were found only in the perioarteriolar lymphoid sheath. These data may suggest that CD20 affects the homing abilities of cells.

These data demonstrate that transgenic CD20 expression does not impair the functionality of human T cells, and suggest that CD20 should be further explored as a safety switch for prevention of GvHD after allo-SCT.

Introduction

Adoptive T cell therapy is a promising treatment for a variety of malignancies and is currently employed in many clinical trials (1). For example, in allogeneic stem cell transplantation (allo-SCT), donor T cells are transfused together with the stem cell graft into the patient to prevent graft rejection, reconstitute the immune system, and eliminate remaining leukemic cells through the Graft-versus-Leukemia (GvL) reaction (2-4).

Despite these valuable contributions, the donor T cells may respond to the patient's own tissue, resulting in the severe complication of Graft versus Host Disease (GvHD) (2). Treatment of GvHD with immune suppressive agents will make the already immunocompromised patients prone to life-threatening infections (5). An elegant way to control the T cells that circulate in the patient after the adoptive transfer is the insertion of a suicide gene into the T cell genome. Expression of a suicide gene, or safety switch, will allow the specific elimination of the transferred T cells by the administration of a selective and otherwise non-toxic pro-drug. Suicide genes may thus provide a safety switch in adoptive therapy with autologous or allogeneic T cells (6-9).

To date, the only clinically applied suicide gene is the Herpes Simplex Virus Thymidine Kinase (HSVtk) gene used in allo-SCT for the treatment of leukemia (6-9). Expression of the HSVtk gene in donor T cells makes them sensitive to elimination by the pro-drug ganciclovir (GCV), a thymidine analogue, as soon as GvHD develops. However, eliminating the alloreactive T cells will also result in loss of the GvL reaction.

Although successful results were obtained using the HSVtk suicide gene, the system also has limitations. In the first place, only dividing cells are sensitive to GCV, so that not all transfused T cells are eliminated (9). Secondly, HSVtk was shown to be immunogenic in immunocompetent patients, leading to premature elimination of the transgenic T cells (8, 10). Thirdly, GCV is used for the treatment of CMV reactivations that occur frequently after allo-SCT, and this might also result in the premature elimination of the HSVtk-positive cells (11, 12).

To overcome these limitations, the human CD20 gene was proposed as an attractive alternative (13-15). Human CD20 is non-immunogenic and the clinical-grade chimeric antibody rituximab (Mabthera®, Rituxan®) efficiently eliminates dividing as well as non-dividing cells (15, 16). Importantly, since CD20 is expressed on the cell surface it may be

used for immuno-magnetic cell sorting, so that no co-expression of a selection marker is needed.

Although CD20 is well-known as the target of anti-CD20 antibody therapy, the role of the CD20 molecule is not completely understood. Normally, CD20 is expressed on immature and mature B cells. Three isoforms (33, 35 and 37 kDa) can be immunoprecipitated from B cells, and the relative occurrence of these isoforms depends on the activation status of the B cells (17-19). Human and murine CD20 are well conserved in amino acid sequences, showing a 73% amino acid homology. However, CD20-knockout mice exhibit a normal B cell development and B cell function (20).

CD20 belongs to the membrane-spanning 4A (MS4A) gene family. The MS4A family components are oligomeric cell surface complexes involved in signal transduction in diverse cell lineages (21, 22). The CD20 molecule is located almost entirely within the plasma membrane and has 4 membrane spanning domains with only 2 small extracellular loops that are recognized by a diversity of antibodies (23). Both the amino and carboxy termini are positioned within the cytoplasm (24, 25).

No natural CD20 ligand has been described but the use of anti-CD20 monoclonal antibodies (mAbs) demonstrated that CD20 can exhibit different functions (25). Artificial CD20 ligation with mAbs showed that anti-CD20 regulates the cell cycle and induces a wide variety of other biologic responses (25-28), such as major histocompatibility complex (MHC) II up-regulation (29), shedding of CD23 (30, 31) and down-regulation of the B-cell receptor (32).
Some anti-mAbs induce apoptosis (25, 33-35), whereas others rescue the cells from apoptosis (36, 37). An important role for CD20 has been attributed to calcium regulation across the plasma membrane and maintenance of high intracellular Ca^{2+} levels for the activation of B cells (38).

Antibody ligation to CD20 leads to the translocation of CD20 molecules into cholesterol-rich microdomains (25, 39, 40). Within these lipid rafts clustered CD20 molecules (trans)activate tyrosine based pathways. One is the C-terminal Src kinase-binding protein (Cbp) (also called PAG, phosphoprotein associated with glycophospholipid-enriched membrane microdomain), a highly tyrosine phosphorylated adaptor protein, which is selectively localized in lipid rafts. After antibody binding, the C-terminal Src kinases (Csk) such as Lyn, Fyn, or Lck, phosphorylate Cbp and thereby induce a kinase activity (41),

which causes the release of calcium stores, up-regulation of Fas expression, phosphorylation of phospholipase C and activation of caspase-3 to induce apoptotic cell death (25, 34, 41-43).

In this report, we investigated the impact of transgenic expression of human CD20 on the function and efficacy of human T cells *in vitro* as well as *in vivo*. Our *in vitro* studies showed that the growth of CD20 positive T cells was not altered and after ligation and cross-linking of rituximab, apoptosis was not induced. Moreover, CD20 positive T cells could efficiently mount an antigenic response, which was comparable to non-transduced T cells. In addition, in mice, CD20 positive T cells were able to invade internal organs like skin, gut, liver, lung, BM and spleen to the same degree as non-transduced or unmanipulated T cells and induce a xenogeneic(X)-Graft-versus-Host reaction. However, despite this similar functionality, CD20 markedly altered the migration pattern in the spleen. Whereas CD20-negative T cells are scattered throughout to whole organ, CD20-transgenic T cells were found only in the perioarteriolar lymphoid sheath. These data may suggest that CD20 affects the homing abilities of cells.

Materials and Methods

Construction of CD20 retroviral vectors and transduction of primary human T cells

Construction of the CD20-encoding Moloney-Murine Leukemia Virus (MLV) based retroviral vector and the generation of CD20 viral particles was previously described (15, 16).

Primary human T cells were obtained from buffy coats of healthy donors (Bloodbank, University Medical Center Utrecht). Peripheral blood mononuclear cells (PBMCs) were centrifuged through Ficoll Hypaque (Amersham Pharmacia, Uppsala, Sweden) and stimulated with 300 U/ml human recombinant interleukin 2 (hurIL-2) (Proleukin, Chiron, Amsterdam, the Netherlands) and anti-CD3/anti-CD28 coated magnetic beads (XcyteTM Dynabeads®, Xcyte Therapies, Inc, Seattle, WA) for 48 hrs. Transductions were performed in non-treated flasks (Becton Dickinson, Kranlin Lakes, NJ) coated with 12.5 µg/ml retronectin (Takara, Otsu, Shiga, Japan). Next, after removing the beads from the cells, the cells were concentrated to 1.0 x 10^6 cells/ml and supplemented with 300 U/ml hurIl-2 and transduced with CD20 viral supernatant. After 24 hrs the virus supernatant was removed and the cells were resuspended in fresh culture medium supplemented with 300U/ml hurIL-

2. The cells were cultured for 3-4 days and CD20 expression was determined by flow cytometry.

The cow milk peptide (CMP) CD4-positive clone (BT55) was stimulated with different concentration of CMP loaded onto autologous or allogeneic EBV cells in the presence of 50 U/ml IL-2 and 50 U/ml IL-4 (Proleukin). After 48 hrs cells were transduced in the retronectin coated flasks with fresh viral supernatant. After 24 hrs the viral supernatant was removed and cells were cultured in culture medium, supplemented with 50 U/ml IL-2 and 50 U/ml IL-4. Cells were cultured for 3-4 days and CD20 expression was determined by flow cytometry.

Cell culture

Primary T cells, BT55 cells, Raji cells and EBV cells were cultured in RPMI (Gibco-BRL, Paisly, Scotland), 10% fetal calf serum (FCS, Integro, Zaandam, the Netherlands), penicillin (100 U/ml), streptomycin (100 µg/ml) (Gibco-BRL), 5 x 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany). The culture medium of the primary T cells was supplemented with 300 U/ml hurIL-2. The BT55-T cell clone was restimulated once a week with irradiated autologous EBV cells loaded with 10 µg of CMP in a 24-wells plate. Amphotropic Phoenix cells were cultured in DMEM (Gibco-BRL), 10% FCS, penicillin, streptomycin and 2-mercaptoethanol. All cells were cultured at 37°C in a 5% CO_2 atmosphere.

Antibodies and flow cytometry

CD20 expression was determined by mAbs specific for the human CD20 molecule conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (clone L27, BD Biosciences, San Jose, CA). For FACS analyses, T cells were stained with the following antibodies: CD3-PE, CD4-PE, CD8-PE, CD25-PE, CD69-PE and HLA-DR-PE (BD Biosciences). Analyses were performed on a FACS Calibur (Becton Dickinson). For histology, cells were stained with the following antibodies: huCD3 (Dako cytomation: A0453) and huCD20 (Beckman Coulter, clone L26). WinMDI 2.8 software was used to analyze CD20 expression level.

Western blot analysis

Transduced and non-transduced T cells were lysed with Laemli Sample Buffer and protein quantitation was performed with Bradford reagent (Bio-Rad, Hecules, CA, US). A 15% SDS polyacrylamide gel was used to separate 50 µg of protein extracts. The proteins were

electrophoretically transferred to Hypond-P membrane (Amersham Pharmacia, Freiburg, Germany), blocked with TBS/0.1% Tween containing 5% non-fat dry milk and incubated with L27 anti-CD20 monoclonal antibody at 1:1,000 dilutions. An anti-actin mAb (Sigma-Aldrich) was used as an internal control at a dilution of 1:10,000). Antibodies were detected by incubation with HRP-conjugated goat anti-murine IgG (DakoCytomation, Glostrup, Denmark) at 1:1,000 dilution. Excess of the second antibody was washed away and membranes were developed using ECL (Amersham Pharmacia).

Measurement of raft-associated antigens by Triton X-100 insolubility.
Cells were washed in PBS and resuspended at 2.5 x 10^6 cells/ml. Next, the cells were incubated with 10 µg/ml of rituximab or control anti-CD4-FITC monoclonal antibody (Becton Dickinson, Mountain View, CA) for 15 minutes at 37°C. The samples were washed in cold PBS and then divided in half. One half was maintained on ice and stained later with rituximab to calculate the 100% surface antigen expression. The other half was treated with 0.5% Triton X-100 for 15 minutes on ice to determine the proportion of antigens remaining in the Triton X-100 (Riedel-deHaen, Seelze, Germany) insoluble fraction. Next, the rituximab ligated pellets of the Triton X-100 treated and the non-treated cells were stained with anti-human IgG1-FITC antibody. The mean fluorescence intensity (MFI) was determined by FACS as described above.

CD20- induced apoptosis assay
Transduced and non-transduced cells were cultured in the absence or presence of rituximab with or without cross-linking with goat anti human IgG. Antibody-mediated induction of apoptosis was measured after 24, 48 and 72 hrs Annexin V-FITC and propidium iodide (PI) staining according to the manufacturer's protocol (BD Pharmingen, San Diego, Ca, US). Cell death caused by transgenic expression of CD20 itself was determined after 7-12 days of culture. Viable cells were also counted by staining with trypan blue.

IL-13 ELISA
The CMP-specific CD4-positive T cell clone (BT55) was stimulated with different concentrations of CMP (100 µg – 0.001 µg) presented on autologous or allogeneic EBV cells for 5 hours at 37 ºC as previously described (44). Briefly, 3 x 10^4 EBV cells were co-cultured with 3 x 10^4 BT55 cells or BT55-CD20 cells in RPMI supplemented with 10 % human serum overnight at 37 ºC. The supernatant was harvested and the concentration of IL-13 was determined with the Pelikine Compact human IL-13 ELISA kit according to the manufacturer's instructions.

Mice and conditioning regimen

$RAG2^{-/-}\gamma c^{-/-}$ mice were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands) (45). The mice were bred and kept in the specified pathogen-free (SPF) breeding unit of the Central Animal Facility of the University of Utrecht. The animals were supplied with autoclaved sterilized food pellets and distilled water ad libitum. All animal experiments were conducted according to Institutional Guidelines after acquiring permission from the local Ethical Committee for Animal Experimentation and in accordance with current Dutch laws on Animal Experiments.

Mice were used at 8-12 weeks of age. On day 0 mice were irradiated with a single dose of 350 cGy (TBI, 3.0 Gy X-rays) and received 0.2 ml clodronate-containing liposomes intravenously as previously described (46, 47).

Injection of human T cells into the $RAG2^{-/-}\gamma c^{-/-}$ mice

Isolation of PBMCs and culture with or without transduction of T cells with CD20 is described above. At day 6, the cells were harvested and 40 x 10^6 CD20 transduced/cultured or only cultured T cells were intravenously injected into the lateral tail vein of the mice (n=10 for both groups). Five mice received 15 x 10^6 freshly isolated human PBMCs.

Collection of plasma and histology of the organs

Mice were weekly bled from the retro-orbital vein under anesthesia. The peripheral blood (PB) was collected in ethylenediaminetetraacetic (EDTA)-coated cups. Erythrocytes were lysed with lysis buffer (0.17 M NH_4Cl/0.1mM EDTA/0.1% $KHCO_3$) and subsequently the mononuclear cells were washed with PBS and prepared for flow cytometry analysis. Mice were sacrificed when there was weight loss of more than 20% or other signs of GvHD, such as impaired movement and ruffled fur. The X-GvHD score was defined as previously described (47). The mice were killed by cervical dislocation under anesthesia. Organs (spleen, liver, lung, colon, skin, and bone marrow) were isolated and prepared for histology. The organs were stored in formaldehyde and embedded in paraffin. Sections were put on coated slides. Next, the sections were stained with huCD3 and huCD20 and counterstained with Hematoxylin (Klinipath). Sections were analyzed by microscopy.

Statistical analysis

Where indicated, the mean values and standard deviation (SD) were calculated. The significance of differences between rituximab and HuMab-7D8 was determined by

Student's *t*-test. A p-value of < 0.05 was considered significant. All statistics were analyzed using GraphPad Prism software (version 4.0).

Results

Expression of the CD20 molecule on T cells.

To study the influence of CD20 expression on the functionality of T cells, human PBMCs from healthy donors were stimulated with antiCD3/antiCD28 coated magnetic beads and 300 U/ml IL-2. Subsequently, the cells were transduced with the complete human CD20 cDNA by a MLV-based recombinant retrovirus. CD20 expressing cells were purified based on CD20 expression by immunomagnetic bead selection. More than 95% of the cells expressed the CD20 protein on the cell surface together with CD3 (figure 1A). Three isoforms (33, 35 and 37 kDa) of the CD20 molecule can be immunoprecipitated from B cells and CD20 transfected cells (17-19). A Western blot analysis of CD20-transduced T cell lysates demonstrated that the isoform in the transduced T cells is 37 kDa (figure 1B), indicating that the CD20 molecule is strongly phosphorylated. Non-transduced T cells and Raji cells were used as controls. The Raji cells displayed two isoforms: the 35 kDa and the 37 kDa isoform.

CD20-transgenic T cells have normal growth properties

The CD20 molecule displays properties of a calcium channel (25, 38) and increased levels of intracellular Ca^{2+} have been associated with apoptosis-induced cell death (34, 48). Moreover, high levels of CD20 expression after transfection of Balb/C 3T3 cells has been proposed to be toxic and G_1 progression was accelerated in CD20 transfected cells by different stimuli (49). To study whether CD20 expression by itself induces cell death, transduced cells were cultured for 6-12 days and stained with Annexin-V and PI. Transduction with the clinical retroviral vector SFCMM-3 (6, 9) was used as a control. Transductions with this vector allowed expression of the Nerve Growth Factor Receptor (NGFR) together with HSVtk. CD20-positive and NGFR-positive cells were gated to discriminate between the transduced and the non-transduced cells. Figure 1C shows the cell death of transduced and non-transduced cells 7 days post transduction of 3 different transduction experiments. Early apoptotic stained Annexin-V+ / PI- and late apoptotic / necrotic cells stained Annexin-V+ / PI+. For early apoptotic cell death, NGFR-negative cells and NGFR-positive cells were 7.3 ± 0.8% and 6.8 ± 1.9% Annexin-V+ / PI-, respectively. The early apoptotic cells (Annexin-V+ / PI-) for the CD20 transductions was

6.9 ± 0.9% for the CD20-negative cells and 11.9 ± 3.2% for the CD20-positive cells. As with the early apoptotic cells, no differences were found for the late apoptotic cells. For NGFR-negative and NGFR-positive cells 9.5 ± 1.1% and 10.0 ± 0.7% stained positive for, Annexin-V+ / PI+, respectively. For the CD20-negative cells 6.9 ± 2.2% and for the CD20-positive cells 6.3 ± 1.7% stained Annexin-V+ / PI-. Similar results were obtained 9-14 days post transduction (data not shown).

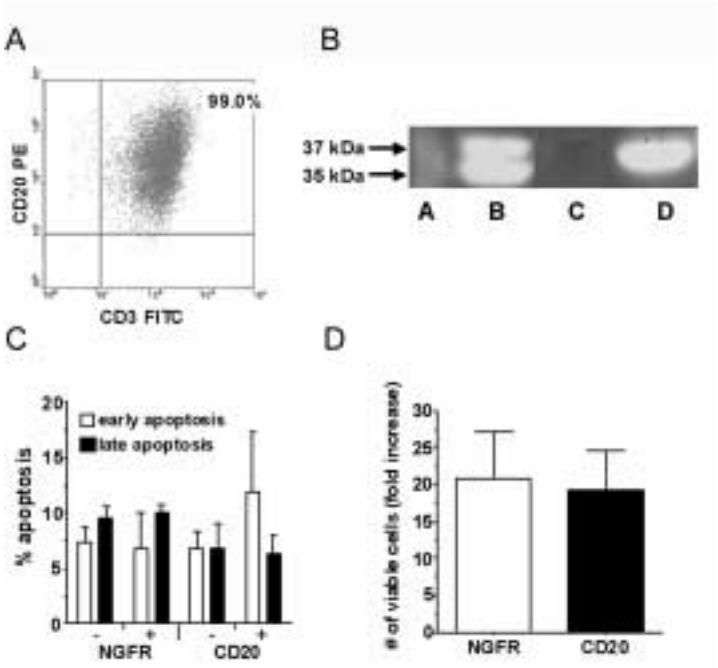

Figure 1. CD20 expression on primary T cells

A FACS-analysis of CD20-positive T cells. Primary T cells were isolated from peripheral blood and transduced with the CD20-encoding retroviral vector. The transduced cells were purified by immunomagnetic cell sorting based on CD20 expression. **B** CD20 expression was evaluated by Western blot analysis of lysates from CD20-transduced T cells with anti-CD20 mAb clone L27. Lane A: marker, lane B: Raji cells, lane C: CD20-negative T cells and lane D CD20-positive T cells. **C** 1.0 x 10^6 primary T cells were transduced with the CD20 or SFCMM-3 retroviral vector (mean of 50% CD20-positive or 50% NGFR-positive) and the influence of CD20 expression on the viability of cells was investigated by Annexin-V and PI staining at 7 days post transduction. Early apoptotic cells were Annexin-V+ / PI- and late apoptotic / necrotic cells were both Annexin-V+ / PI+. **D** At day 7 post transduction the absolute numbers of viable cells were determined. The bars represent the mean ± SD of three independent transductions.

In addition, both the SFCMM-3- and the CD20-transduced T cells had a 20-fold increase in viable cells after 9 days post transduction (figure 1D).
Of note, stimulating cells with only anti-CD3 mAb led to more cell death than co-stimulation with CD28 in the transduced and the non-transduced cells for both the CD20- and the HSVtk-encoding retroviral vectors (data not shown), confirming previous data (50, 51).

CD20, expressed on T cells, is efficiently translocated into lipid raft upon antibody ligation
One of the key features of CD20 signaling is the translocation of the molecule into lipid rich microdomains upon antibody binding (25, 39, 40). To investigate whether the anti-CD20 mAb rituximab induces lipid raft formation of CD20 molecules on CD20-transgenic T cells, a flow cytometry based assay was performed. Figure 2A demonstrates that rituximab binding protects against Triton X-100 insolubility, indicating the formation of lipid rafts. As a control a CD4 mAb that does not induce lipid raft formation was used.

No induction of antibody-mediated apoptosis of transgenic expression of CD20 T cells
One of the final stages of antibody-mediated CD20 signaling is the induction of apoptosis (25, 34, 40, 43). Cross-linking (XL) of the CD20 ligated antibody generally induces more apoptotic cell death compared to a single antibody (25). To that end, we studied whether rituximab binding and cross-linking of rituximab resulted in the induction of apoptosis. Primary T cells of three different donors were transduced and a mean of 50% transduction efficiency was obtained (data not shown). Cells were ligated with rituximab with or without cross-linking. Induction of apoptosis by Annexin-V and PI staining was measured on the CD20-positive and CD20-negative cells after 48 hrs. Figure 2B shows for the CD20-negative cells $6.1 \pm 1.1\%$, $5.5 \pm 0.8\%$ and $6.4 \pm 0.9\%$ cell death with no antibody, rituximab and rituximab + XL, respectively. No significant differences were found for the CD20-positive cells, $5.4 \pm 1.0\%$, $6.4 \pm 1.2\%$ and $6.7 \pm 1.2\%$ with no antibody, rituximab and rituximab + XL respectively. In addition, no differences were found between the early and late apoptotic cells of the CD20-negative cells compared to the CD20-positive cells (data not shown). To conclude, ligation of rituximab to CD20-transgenic T cells with or without XL does not lead to apoptotic cell death.

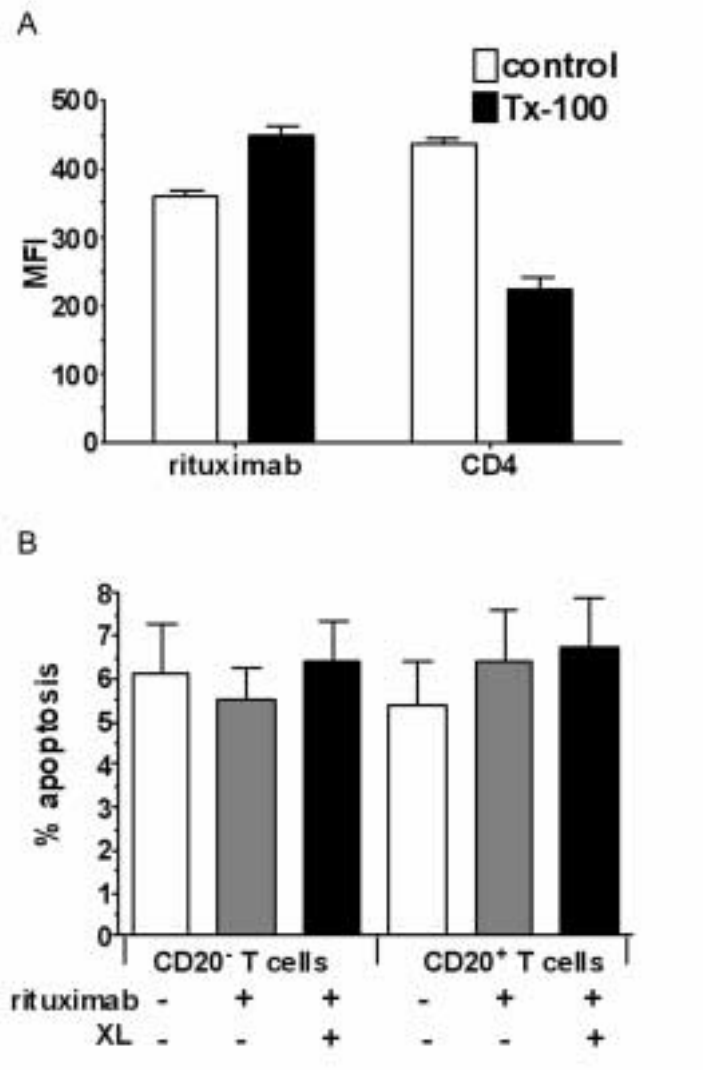

Figure 2. Induction of antibody-mediated apoptosis

A Transgenic CD20-positive T cells were incubated with 10 µg/ml of rituximab or anti-CD4 and the samples were divided in half. The white bars represent the directly stained cells with anti-IgG1-FITC and the black bars represent the cells that were first treated with Triton X-100 and subsequently stained with anti-IgG1-FITC. The bars represent the mean ± SD of three independent experiments. **B** CD20-negative and CD20-positive cells were incubated with or without 10 µg of rituximab and with or without cross-linking (XL). The bars represent the mean ± SD of three independent experiments.

Transgenic CD20 expression does not alter the phenotype of T cells

To investigate whether CD20 transduction alters the T cell phenotype, we studied the expression of a variety of relevant activation markers. Within the transduced population the cells were gated for CD20 expression. Figure 3A shows no differences in expression of activation markers between the CD20-negative and the CD20-positive population. Both were positive for CD3 and CD25. Only a small portion of the cells was positive for CD69. For CD20-negative cells this was 10.3 ± 0.6% and for CD20-positive cells this was 13.3 ± 1.1%. Not all cells expressed HLA-DR on their cell surface, 55.4 ± 8.5% and 60.2 ± 11.1%

for CD20-negative and CD20-positive cells, respectively (Figure 3A). The expression levels (mean fluorescence intensity, MFI) of CD3, CD25, CD69 and HLA-DR did also not differ between the CD20-nagative and CD20-positive cells (Figure 3B). The CD4:CD8 ratio was inversed upon CD3/CD28 co-stimulation (data not shown), confirming previous results (14).

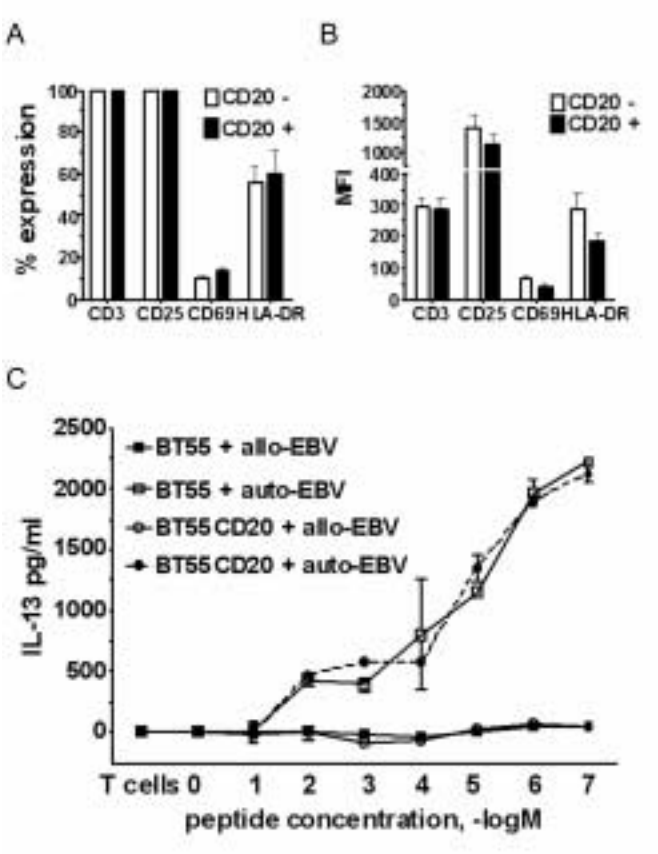

Figure 3. Determination of activation markers on CD20-positive cells and antigenic response to CMP loaded on antigen presenting cells

Cells were transduced with the CD20-encoding retroviral vectors (mean transduction efficiency of 50%). CD20-negative (white bars) and CD20-positive (black bars) cells were gated and the percentage CD3, CD25, CD69 and HLA-DR (**A**) and the mean fluorescence intensity (MFI) (**B**) were determined. The bars represent the mean ± SD of three independent experiments. (**C**) The cow milk peptide (CMP) $CD4^+$ T cell clone (BT55) was transduced with the CD20-encoding retroviral vector and subsequently purified. Different concentrations of CMP (100 µg – 0.001 µg) were loaded on autologous and allogeneic EBV cells and presented to the transduced and the non-transduced cells. IL-13 production was measured upon stimulation of the BT55 clone with increasing concentrations of CMP. The mean ± SD of three independent experiments are shown.

CD20 expression on T cells does not alter the specific antigenic response

To evaluate whether CD20 expression alters the specific antigenic response a CD4-positive human T cell clone (BT55) was transduced with the CD20-vector. Different concentration of CMP (100 µg – 0.001 µg) were loaded onto autologous or allogeneic antigen presenting B cells (44). This CD4-positive T cell clone produces IL-13 upon CMP stimulation. As shown in figure 3C non-transduced BT55 cells do not produce IL-13 upon stimulation with CMP loaded on allogeneic EBV cells. In contrast, CMP loaded on autologous EBV cells did lead to IL-13 production by BT55 cells. Increasing the CMP concentration leads to increased IL-13 production. Similar results were obtained with BT55-CD20-positive cells. No significant difference between BT55 and BT55-CD20-positive cells was observed when stimulated with CMP-loaded autologous EBV cells (figure 3B).

CD20-positive T lymphocytes cause X-GvHD in RAG2$^{-/-}\gamma c^{-/-}$ mice.

Next, we evaluated the ability of the CD20 transduced T cells to cause a X-GvHD reaction. The huPBMC-RAG2$^{-/-}\gamma c^{-/-}$ mouse model is to date the most sensitive model for engraftment of human T cells and development of X-GvHD (46, 47). CD3/CD28 stimulated human T cells were transduced with the CD20-encoding retroviral vector, leading to 20% transduction efficiency (data not shown). Cells were cultured for 6 days and 40 x 10^6 cells were intravenously injected into the tail vein (n = 10). As a positive control 5 mice received 15 x 10^6 fresh human PBMCs (huPBMCs), and 10 mice received 40 x 10^6 non-transduced cultured T cells derived from the huPBMCs. Engraftment of human cells was monitored weekly by FACS analysis of the PB. For the transduced and non-transduced cultured cells a mean of 28% and 32% were human CD45 positive already at day 7 post injection, respectively. In the control group (15 x 10^6 PBMCs) the engraftment of human cells was delayed, probably due to the lower number of cells (46) (figure 4A).

Figure 4B shows the analyses of CD20 expressing cells collected from the PB. Within the transduced population an average of 20% of the cells were CD3 and CD20 positive, resembling the population that was injected. No CD20 expression was found on the cells collected from the control mice (fresh PBMCs and the cultured T cells). Both CD4 and CD8 subsets were found on the CD3 and CD20 double-positive cells with a CD4:CD8 ratio of 4:1 (data not shown).

From day 7, the increase in human cells was associated with an acute X-GvHD. This X-GvHD reaction was scored by characterizing weight loss, hunched posture, ruffled fur and mobility (47) (figure 4C). Due to X-GvHD the mice were sacrified. Figure 4D shows the Kaplan-Meier survival estimates of mice that received the T cells. No significant differences were found between the transduced and the control cells.

Cell suspensions of the spleen and bone marrow were made of the sacrificed mice and analyzed by FACS. Figure 4E demonstrates the fraction of CD45-positive cells in the splenic cell suspensions. For the fresh huPBMC group this was 33.8 ± 14.6%, for the cultured cells group this was 28.3 ± 12.0% and for the transduced group 28.1 ± 24.6% were CD45-positive cells. Only the transduced cells were CD20 positive (18.7 ± 4.5%). In the bone marrow only few human cells were found at the time of death (3.8 ± 1.3%, 2.1 ± 0.7% and 1.5 ± 0.5% were human CD45 positive in the PBMC, transduced and cultured group, respectively). Again, only for the transduced group CD20 positive cells were found (13.7 ± 1.9%) (figure 4F).

Distribution of CD20-positive human T cells in the organs of the mice.

Organs were collected from the sacrificed mice and histochemical analysis was performed to study the distribution of the transduced cells. Histology was performed on spleen, lung, liver, skin, colon and bone marrow. Figure 5 shows the histology of the spleen (figure 5A), long (figure 5B), liver (figure 5C), and the colon (figure 5D) of the transduced group. Figure 5A demonstrates the distribution of cells in the spleen. Macroscopic evaluation showed splenomegaly in all the mice and microscopic evaluation showed lymphocytic infiltrates. Figure 5A-i shows that in the spleen CD3 positive cells were fully dispersed and figure 5A-ii shows that about 20% was CD20 positive. Interestingly, almost all CD20-positive cells were located in the periarteriolar lymphoid sheath. These data suggest that CD20 expression may affect the homing abilities of the cells. In the lung, liver and colon, the CD3- and CD20-positive cells were more diffusely spread (figure 5B-D, see arrows). Hardly any cells were found in the skin and bone marrow (see also figure 4G). These data show that CD20-positive human T cells are able to engraft and infiltrate into various organs of the $RAG2^{-/-}\gamma c^{-/-}$ mouse.

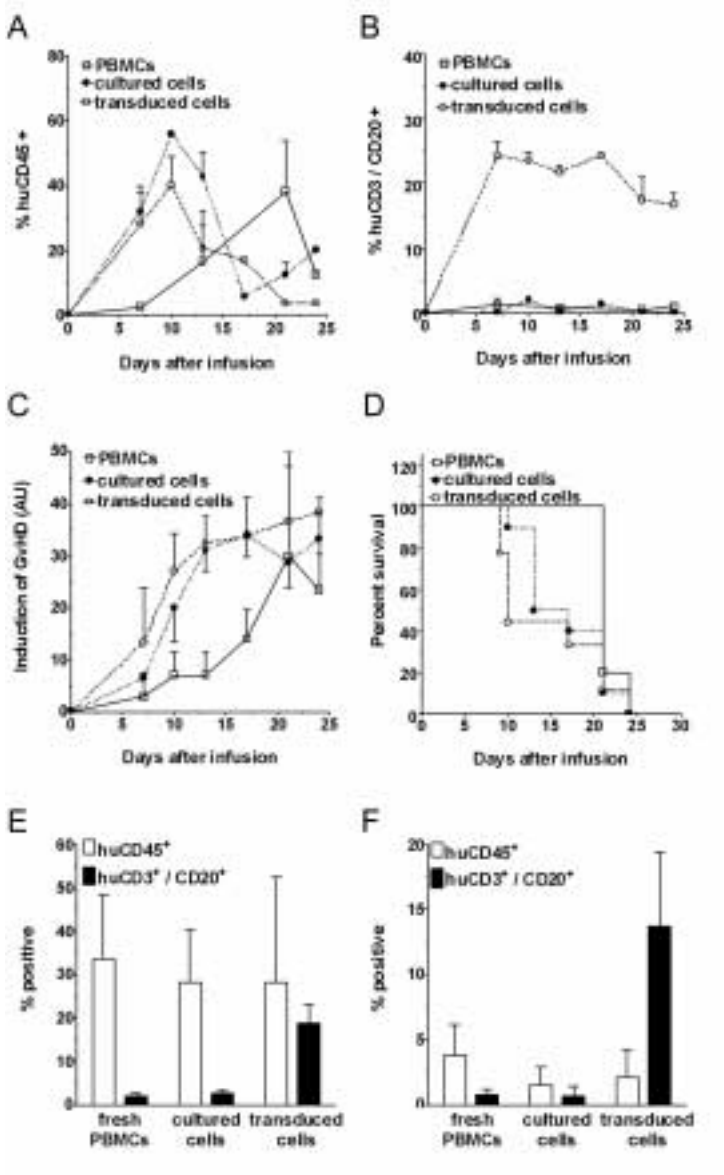

Figure 4. GvHD inducing capacities of CD20-transgenic T cells.

15 x 10^6 fresh huPBMCs (n=5), 40 x 10^6 CD3/CD28 stimulated cultured primary T cells (n=10) and 40 x 10^6 CD3/CD28 stimulated CD20-transduced primary T cells (n=10) from the same donor were injected into irradiated and macrophage-depleted mice. The non-transduced and transduced cells were cultured for 6 days. Peripheral blood was collected once a week and the percentage of human CD45-positive cells (**A)** and the percentage of human $CD3^+$ / $CD20^+$ cells (**B**) were determined. (**C**) The capability of the T cells to induce GvHD was determined by weight loss, reduced mobility of the mice and development of ruffled fur. These criteria were scored as arbitrary units (AU) and set against time. (**D**) Kaplan-Meier survival estimates of mice that received the T cells. (**E**) Spleens and (**F**) both femora were isolated from the mice at time of death and cell suspensions were made. The figures E and F show the percentages of human CD45-positive and CD3-positive / CD20-positive cells determined. The mean ± SD are shown.

Discussion

In this study, we investigated *in vitro* and *in vivo* whether the retrovirally-mediated expression of the human CD20 molecule on human T cells would alter the growth and the immune response of the cells.

Transduction of T cells with a MLV-based vector encoding the human CD20 cDNA leads to efficient expression of the complete CD20 molecule. Western blot analysis showed that after transduction and stable expression of the CD20 gene the strongly phosphorylated isoform was precipitated. This is in concordance with the status of the CD20 molecule on transiently transfected cells (19).

Cell division is required for efficient retroviral transduction. This means that cells need to be stimulated and cultured prior to transduction. Prolonged *in vitro* culture leads to impaired T cell function *in vivo* (47). We used an optimized transduction/culture protocol by stimulating the cells with anti-CD3/anti-CD28 coated magnetic beads and kept the culture time as short as possible to optimally preserve the reactivity of the T cells (47, 50, 51). Cells expressing the CD20 molecule can be purified from the CD20-negative cells by immunomagnetic-based selection and can be efficiently eliminated by human effector mechanisms (15, 16).

The structure of the CD20 molecule suggests that it could act as an ion conducting pathway. Indeed, Li et at demonstrated that CD20 is a component of the store-operated Ca^{2+} (SOC) entry pathway and that Ca^{2+} movement depends on the lipid raft formation (38). In addition, ectopic expression of the CD20 molecule by transfection of T cell lines showed that CD20 positive cells had increased Ca^{2+} conductance, which was not found in CD20 negative cells (19, 49).

Intracellular calcium is required for a diversity of cellular functions. In lymphocytes, receptor-mediated increase of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) levels has been associated with various lymphocyte responses, including changes in morphology, long-term gene expression patterns, enzyme activation, secretion and cell division (52-55).

In preliminary experiments in long-term culture assays of CD20-transgenic CEM T cells, we did not observe increased intracellular Ca^{2+} compared with the CD20 negative CEM T cells (data not shown). Increased intracellular Ca^{2+} by transient transfection of cell lines has been suggested to be toxic for cells and ectopic expression of CD20 has led to the acceleration of G_1 progression due to increased intracellular Ca^{2+} (49). In addition, depletion of intracellular Ca^{2+} stores prevents from otherwise induced apoptotic cell death (40, 43, 48).

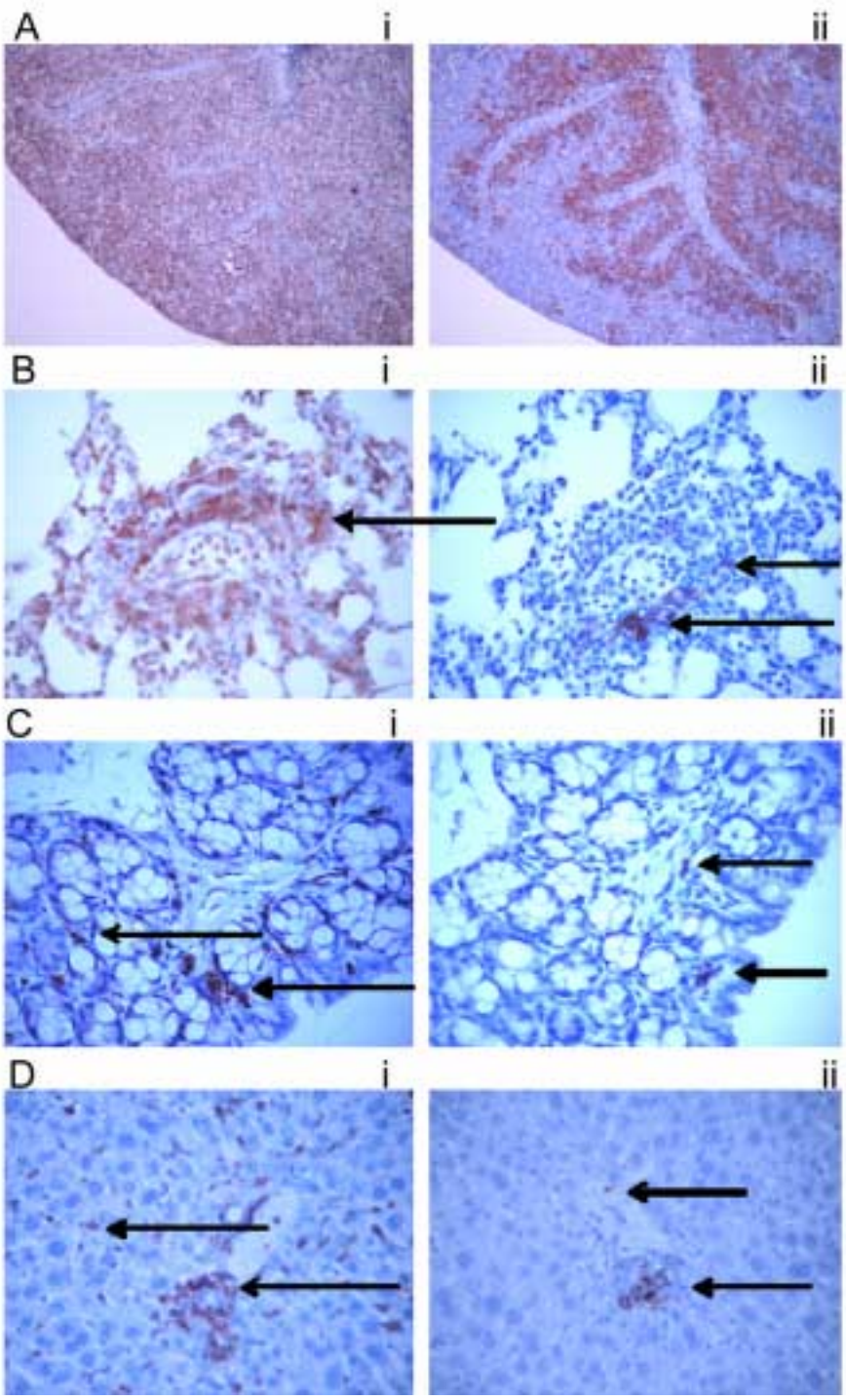

Figure 5. Infiltration of human T cells in the organs of the RAG2$^{-/-}$γc$^{-/-}$ chimeras
Immunohistochemical evaluation was performed in mice injected with the CD20-transgenic T cells. Infiltration of human CD3-positive cells is shown in the left column (**i**) and the infiltration of CD20-positive cells into the organs is shown in the right column (**ii**). These figures show the spleen (**A**), the lung (**B**), the colon (**C**) and the liver (**D**).

However, here we show for primary T cells that transgenic expression of CD20 does not interfere with the cell growth after retroviral transduction (Figure 1). One explanation could be that other channels or mechanisms are able to compensate for Ca^{2+} changes after CD20 expression. T cells express a specific Ca^{2+} channel (CRAC), which could be a candidate for this role (56). Also, CD20 belongs to the MS4A gene family (21, 22). In T cells, a CD20 homolog has been found recently, MS4A4b (57). MS4A4b behaves in a similar way to CD20 and is also translocated into lipid rafts upon activation of naïve primary T cells. Over-expression of MS4A4b leads to enhanced TCR-mediated secretion of IL-2 and INF-γ (57). Additional experiments will point out whether CD20 over-expression will also lead to enhanced IL-2 and INF-γ. Serafini et al. already showed that the cytokine secretion of CD20-transgenic T cells to various stimuli was not different from the non-transduced cells (14). In addition, we showed that the expression of activation markers was not enhanced on CD20-transgenic cells compared to CD20 negative cells, even after (hyper) crosslinking of the CD20 molecule (Figure 3).

In general, induction of apoptosis is thought to be one of the mechanisms of action of rituximab for eliminating B cells. However, contradictory results were published concerning the importance of apoptosis (reviewed by Deans et al) (25). It has been suggested that Ca^{2+} release is necessary to initiate caspases for the induction of apoptosis (40, 43, 48).
We showed that rituximab ligation and cross-linking of rituximab did not lead to the induction of apoptosis, although rituximab induces the translocation of the transgenic CD20 molecules into lipid rafts. As mentioned before, these lipid rafts are the platforms for signaling pathways.
On the other hand, activation of T cells *in vitro* by CD3 and CD28 induces lipid raft formation and increases and activates the lipid raft proteins Csk, Cbp and Fyn (58). This results in the phosphorylation and activation of PLCγ and ultimately in the release of Ca^{2+} from the endoplasmatic reticulum or Ca^{2+} entry from the extracellular space (58). Activation of the raft proteins are known to be negative regulators of TCR activation and re-stimulation of the TCR does not lead to increased intracellular free Ca^{2+} (59, 60). This insensitivity to re-releasing Ca^{2+} might explain the protection against rituximab-induced apoptosis in CD20 positive T cells. Future experiments must elucidate the exact role of human CD20 on T cells on a biochemical level.

In addition to previous *in vitro* work by Serafini and colleagues (14), we demonstrated that CD20-transgenic and non-transduced T cells respond similarly to an antigenic response.

The CD20-positive and the CD20-negative CD4 T cell clone showed an identical dose-response curve upon stimulation with the CMP peptide presented by autologous EBV cells. We extended these results to an *in vivo* mouse model. The RAG2$^{-/-}\gamma c^{-/-}$ mouse model can be considered the most sensitive mouse model to date for the study of genetically manipulated human T cells and the development of X-GvHD (46, 47, 61). By iv injection of transduced and non-transduced human T cells into RAG2$^{-/-}\gamma c^{-/-}$ mice, we demonstrated that CD20 positive T cells were able to induce X-GvHD (Figure 4 and 5). CD20-positive T cells distribute within the mice in the same fashion as non-transduced human T cells. Interestingly, in the spleen the CD3/CD20 double-positive cells were found mostly around the arterioles, whereas the CD3-positive/CD20-negative T cells were scattered throughout the organ. This may suggest that CD20 expression affects the homing of cells or plays a role in lodging of the cells within the murine spleen. Ongoing experiments will address these questions. These data demonstrate that CD20-positive human T cells are able to efficiently infiltrate various organs of the RAG2$^{-/-}\gamma c^{-/-}$ mouse suggesting that CD20 expression does not interfere with xenoreactivity of the human T cells.

In conclusion, we show that transgenic expression of CD20 does not alter the reactivity of the human T cells. These preclinical results add to the further development of a safe clinical application of the CD20 suicide gene in adoptive T cell therapy.

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Chapter 4

Evaluation of selection markers for suicide gene therapy

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Abstract

In allogeneic stem cell transplantation, the adoptive transfer of T lymphocytes is a well-established approach for the treatment of many hematological malignancies. Unfortunately, these alloreactive T lymphocytes may cause life-threatening Graft versus Host Disease (GvHD). Transgenic expression of the human CD20 molecule on the alloreactive T cells may allow the specific control of GvHD by the administration of rituximab, a human-mouse chimeric anti-CD20 monoclonal antibody. Since a uniform CD20-transgene expression is warranted for efficient therapy, purification of CD20-positive cells is needed prior to administration to the patients.

In this report we investigated different selection markers and selection methods. Different mono-and bicistronic CD20-encoding retroviral vectors were constructed to transduce the T cells and subsequently purify the transduced population. Immunomagnetic selection of transgenic CD20-positive cells purified the cells to more than 95%, with a recovery of 2.6-17.0%. With FACS sorting the recovery increased to 24%. Selection through the expression of the ΔLNGFR or the ouabain resistance gene together with CD20 increased the recovery to 34 and 52%, respectively, albeit at the expense of the purity of CD20-positve cells (65% and 73%, respectively).

In conclusion, the CD20 molecule can be combined with ΔLNGFR or ouabain resistance in the next generation vectors for suicide gene therapy.

Introduction

Adoptive immunotherapy of T lymphocytes is a promising approach for the treatment of many hematological malignancies (1, 2). However, clinical application carries significant risks. For example, in the setting of allogeneic stem cell transplantation (allo-SCT) and donor lymphocyte infusion (DLI) for the treatment of leukemia, alloreactive donor T cells can respond to malignant cells (Graft versus Leukemia), but also to the patients' normal tissues (Graft versus Host Disease; GvHD) (1). Therefore, elimination of the T cells by a specific safety switch may aid in controlling GvHD. Indeed, in two trials this approach has been successful (3-6). In these studies, donor T cells were harnessed with the Herpes Simplex Virus thymidime kinase (HSVtk) suicide gene through retroviral transduction prior to administration to the patient (3-7). Expression of HSVtk renders these cells sensitive to specific elimination with ganciclovir (8).

In order to purify retrovirally-modified T cells prior to administration, recombinant retroviruses need to encode a selection marker beside the suicide gene. In an ideal situation, the selection marker exhibits the following characteristics. Firstly, the selection gene has to be small to obtain reasonable viral titers and an efficient transcriptional and translational processing (9, 10). Secondly, the selection procedure must be fast to preserve the functionality of the T cells, since prolonged culture of T lymphocytes has been shown to reduce their reactivity *in vivo* (11-14). Thirdly, selection markers must not be immunogenic, or alter the cell function in any respect. Finally, for clinical application the selection procedure must be compatible with current requirements for good manufacturing practice (GMP).

The first selection marker used for clinical application to purify T cells containing the HSVtk suicide gene was the neomycin resistance gene (neoR) (3, 5, 15, 16). This gene is derived from the bacterial transposon Tn5 that encodes an enzyme conferring resistance to a variety of aminoglycoside antibiotics such as kanamycin, neomycin, and geneticin (G418). However, selection requires 7-14 days of culture in the presence of G418 that results in impaired T cell function *in vivo* (11-13, 17). Importantly, because of its bacterial origin the neoR has been shown to be immunogenic in human (18).

The most extensively used selection marker for clinical application is the truncated form of the low-affinity Nerve Growth Factor Receptor (ΔLNGFR) (6, 18-20). The nerve growth factor plays an important role in the survival and maintenance of sensory and sympathetic

neurons (21). The truncated form lacks a signaling peptide and ligation via antibodies does not lead to cell activation. The transgenic ΔLNGFR-expressing cells can be rapidly purified via immunomagnetic beads (6). Although exogenous expression of ΔLNGFR by mouse bone marrow cells has been suggested to promote malignant transformation of the transduced cells (22), extensive data from other research groups employing ΔLNGFR in T cells could not reproduce these unwanted effects (19).

In an elegant approach a fusion protein between the extracellular domain of CD34 and the HSVtk served as both selection and suicide gene (23). CD34 is only expressed at early lympho-hematopoietic stem and progenitor cells, small-vessel endothelial cells and embryonic fibroblasts and could therefore be used to select transduced T lymphocytes (24-26). The functions of naturally occurring CD34 are signal transduction (intracellular domain), mediating cell-cell adhesion (extracellular domain) and differentiation of hematopoietic progenitor cells. Transgenic expression of the full-length or truncated CD34 molecule led to impaired cellular trafficking to hematopoietic stroma. The suppressive migration effect of the full-length CD34 molecule was more pronounced than the truncated form (27). Selections can be performed by GMP-approved immunomagnetic beads and commercial cell separation devices and the culturing procedure can be shortened to only 5 days (23, 28).

The cardiac glycoside ouabain was described as a promising selection by Aints and co-workers (29). Ouabain is a Na^+K^+-ATPase enzyme, a membrane pump present on all mammalian cells. The Na^+ concentration in the cell increases, thereby activating other ion channels. The result of this Na^+ lowering action is that the Ca^{2+} concentration rises. Due to the osmotic pressure the cell swells and dies. Although ouabain is active on human cells, the Na^+K^+-ATPase of rat origin is resistant to ouabain, and hence can serve as a selection marker. Ouabain-induced cell death is rapid and transgene-expressing cells may be purified within 24 hrs.

HSVtk is the only clinically used suicide gene in T cells so far (3-7). In these studies, NGFR or neomycin was used as a selection marker and ganciclovir as an elimination agent. However, the T cells expressing HSVtk were shown to be immunogenic, leading to premature elimination of the transgenic T cells (18, 30). Moreover, ganciclovir is active only in dividing cells so that not all T cells can be eliminated (6). In addition, in the setting of allogeneic stem cell transplantation ganciclovir may be used for the treatment of CMV

reactivations (31, 32). Obviously, this leads to an unwanted, premature elimination of the transduced donor lymphocytes.

We have previously described the use of the human CD20 gene as a suicide gene for T cells. In contrast to HSVtk, the human CD20 molecule is expected not to be immunogenic and the anti-CD20 monoclonal antibody (mAb) rituximab can eliminate both dividing and non-dividing cells (33). An appealing idea is that CD20 may not only be used for elimination, but also for the required selection of the transgenic T cells (33-35). Previously, we and others have demonstrated that CD20-positive T cells could be purified to > 95% purity by magnetic activating cell sorting (33-35). However, no data are available on the recovery of the CD20-positive population. These selection markers are summarized in table 1.

In the present study, we have compared various systems to purify genetically-modified T cells. We show that selection by CD20 with magnetic cell sorting results in high purity but low recovery. We therefore investigated ways to improve efficiency and safety of clinical application of CD20 positive T cells. We report for the first time the effect of gene transfer of human CD20 in combination with different selection markers. The selection efficiency, recovery, as well as the influence on the CD20 expression of manipulated T cells were investigated.

Table 1. Summary of frequently used selection markers in T cells.

Selection marker	Type of selection	Selection agent	Origin	Suicide Gene	Reference
Neomycin	metabolic	Antibiotic (G418)	Bacteria	no	(3, 5, 16)
Ouabain Resistance	metabolic	Ouabain	Rat	no	(29)
ΔLNGFR	Cell surface	20.4 mAb	Human	no	(6, 12, 18-20)
CD20	Cell surface	Anti-CD20 mAb	Human	yes	(33-35)
CD34	Cell surface	Anti-CD34 mAb	Human	no	(23, 28)

Materials and Methods

Construction of the CD20 vectors

The CD20, CD20-IRES-β (CD20-I-β) and CD20-WPRE-INS (CDWINS) vectors were generated by inserting the human CD20 gene into the Moloney Murine Leukemia Virus (MLV) based vector pMX-α-IRES-β, as previously described (33).

Insertion of SV40-ΔLNGFR downstream of CD20

The SV40-ΔLNGFR fragment was amplified from the SFCMM-3 vector (6) with primers containing the NotI and SacII restriction sites and subsequently cloned into the pCR2.1-TOPO vector. Next, the SV40-ΔLNGFR fragment was isolated by digesting with NotI and SacII. The internal ribosome entry site (IRES) was released from the MX-CD20-IRES-β (33) by digesting with NotI and SacII and the SV40-ΔLNGFR fragment vector was ligated downstream of the CD20 molecule. The Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) was obtained as previously described (33). The WPRE fragment and the MX-CD20-SV40-ΔLNGFR-β were digested with SacII and SalI and the WPRE element was subsequently ligated downstream of the ΔLNGFR, resulting in the CSNW vector.

Insertion of OuaR downstream of CD20-IRES

The pGIO was kindly provided by dr. Dilber (Cell and Gene Therapy Group, Division of Hematology, Karolinska Intsitute, Stockholm, Sweden) and the Rat Na+K+ATPase gene (OuaR) was amplified from the pGIO vector with the following primers: OuaR-FW-GGGCCGCGGCGCCACCATGGGAAGGGGG and
OuaR-RV-GGGGTCGACAGGGCAGTGGGCTAGTAGTA, thereby creating a SacII restriction site and a SalI restriction site at the 3' end of the OuaR gene. The PCR-product was ligated in the pCR2.1-TOPO vector and subsequently digested with SacII and SalI for the ligation into the MX-CD20-I-β vector. For translation initiation the OuaR gene was ligated in frame with the authentic viral ATG (ATG 11), creating the CD20-I-OuaR vector.

Generation of viral particles and transduction protocol

CD20-encoding viral particles were produced by calcium phosphate transfection of amphotropic Phoenix packaging cells as previously described (33). Viral titers were determined with serially diluted virus supernatant on CEM T cells. CEM cells were transduced as previously described (33). Primary T cells, isolated from peripheral blood mononucleated cells (PBMC) by centrifugation through Ficoll (Amersham Pharmacia,

Uppsala, Sweden), were stimulated with 300 U/ml human recombinant interleukin 2 (hurIL-2) (Proleukin, Chiron, Amsterdam, the Netherlands) and anti-CD3 / anti-CD28 coated magnetic beads (XcyteTM Dynabeads®, Xcyte Therapies, Inc, Seattle, WA) for 48 hrs. Next, the beads were washed away and 1.0 x 10^6 cells were transduced with the different virus supernatants at a multiplicity of infection (MOI) of 1. After 24 hrs the virus supernatant was removed and the cells were resuspended in fresh culture medium supplemented with 300U/ml hurIL-2. The cells were cultured for 3-4 days and CD20 or ΔLNGFR expression was determined by flow cytometry.

Cell culture

The CEM T cell line and primary T were cultured in RPMI (Gibco-BRL, Paisly, Scotland), 10% fetal calf serum (FCS, Integro, Zaandam, the Netherlands), penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco-BRL), 5 x 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany). Amphotropic Phoenix cells were cultured in DMEM (Gibco-BRL), 10% FCS, penicillin, streptomycin and 2-mercaptoethanol. All cells were cultured at 37°C in a 5% CO_2 atmosphere.

Flow cytometry and analysis

CD20 expression was determined by mAbs specific for the human CD20 molecule conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (clone L27, BD Biosciences, San Jose, CA). ΔLNGFR was stained with mAb 20.4 (12). ΔLNGFR was then visualized by goat-anti-mouse IgG-PE. FACS analysis was performed on a FACS calibur (Becton Dickinson). WinMDI 2.8 software was used to analyze CD20 expression level.

Selection procedures

Magnetic Activated Cell Sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained with either CD20-PE antibody or 20.4 for 20 minutes on ice, washed in a PBS/2mM EDTA/0.5% BSA buffer and taken up in 80 μl of this buffer. 20μl Goat-Anti-Mouse beads (Miltenyi Biotec) were added and the cells were incubated again for 20 minutes on ice. This solution was resuspended in 400 μl cold buffer. The selection procedure was performed according to manufacturer's instructions. CD20 and ΔLNGFR expression was analyzed by flowcytometry.

Fluorescence Activated Cell Sorting (FACS) (FACS ARIA Cell Sorter) (Becton Dickinson). The cells were labeled with CD20-PE or with 20.4 conjugated with GAM-PE, and then sorted according to manufacture's instructions.

Ouabain selection. Ouabain (Sigma, St. Louis, USA) was dissolved in sterile water to a concentration of 10 mM. Selection experiments were performed with concentrations 0.01 µM; 0.1 µM; 1 µM; 10 µM and 100 µM. The transduced cells were selected by adding ouabain at day three post transduction. After 48 hours the cells were quantified by trypane blue staining and CD20 expression was analyzed by flow cytometry.
Recovery was calculated by dividing the number of cells positive for CD20 / ΔLNGFR after selection with the number of cells positive for CD20 / ΔLNGFR before selection.

Results

Construction of vectors
We set out to compare the selection markers ΔLNGFR, CD20 and OuaR for purification of retrovirally-modified T cells. To this end, several mono- and bicistronic CD20-encoding pMX-based retroviral vectors were constructed (Figure 1). In these vectors the CD20 gene is under transcriptional control of the MLV LTR. Expression of the second gene is directed by either an internal ribosomal entry site (IRES; CD20-I-β, CD20-I-GFP and CD20-I-OuaR) or the SV40 early promoter (CSNW). The latter vector additionally contains regulatory elements, which enhance and stabilize CD20 expression and ΔLNGFR expression (33). For comparison, we included the ΔLNGFR-encoding SFCMM-3 vector, which has already been used successfully in clinical protocols vector (6, 18, 20).

Transduction and selection of T cell lines
In a first series of experiments we analyzed the result of immunomagnetic bead selections with CD20-specific antibodies after transduction of the CEM T cell line. For these experiments two vectors were used for the transfer of the CD20 molecule, CD20-I-β and CWINS. For selection of CD20-positive cells, transduced cells were labeled with CD20-specific antibodies and immunomagnetic beads and purified on an MACS cell separation device. Figure 2A and B show representative examples of CD20-selection experiments of transduced CEM cells. As demonstrated previously, the CD20 expression level of CD20-positive cells after CWINS-transduction (figure 2A) was lower (MFI of 42) compared to that after CD20-I-β-transduction (MFI of 96) (Figure 2B) (33). After immunomagnetic bead selection cell populations of > 95% purity were consistently obtained for both vectors.

Figure 1. Schematic representation of the CD20-encoding retroviral vectors.
CD20 cDNA was placed into the pMX backbone under control of the Moloney-Murine Leukemia Virus (MLV)-Long Terminal Repeat (LTR). In the CNSW and SFCMM-3 vector, the expression of truncated nerve growth factor receptor (ΔLNGFR) is under control of the Simian Virus 40 (SV40) internal promoter. The Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (WPRE) was included to optimize transgene expression of both the CD20 and ΔLNGFR for the CSNW vector. The enhanced green fluorescence protein (eGFP), the ouabain resistant gene and the murine T cell receptor β chain gene (β) was inserted downstream of the Internal Ribosome Entry Site (IRES).

Although the purity of selected cells was excellent, analyses of the viability of selected cells revealed that a large fraction of the cells was dead after the MACS procedure. Prior to selection 100 ± 9.7% and 100 ± 4.5% of the CWINS- and CD20-I-β-transduced cells, respectively, were viable (Figure 2C). After selection only 0.9 ± 0.5% (CWINS) and 3.7 ± 1.3% (CD20-I-β) of the cells were still viable, indicating that the majority of the cells did not survive the selection procedure. As a result of this profound cell death the recoveries were low: 6.7 ± 2.1% for CWINS and 7.8 ± 0.9% for CD20-I-β (figure 2D). Similar results were obtained with transduced Molt and Jurkat T cell lines (data not shown). Importantly, despite the lower CD20 expression level of the CWINS vector, selection did not affect the purity. However, extremely low CD20 densities can affect the purity (see below).

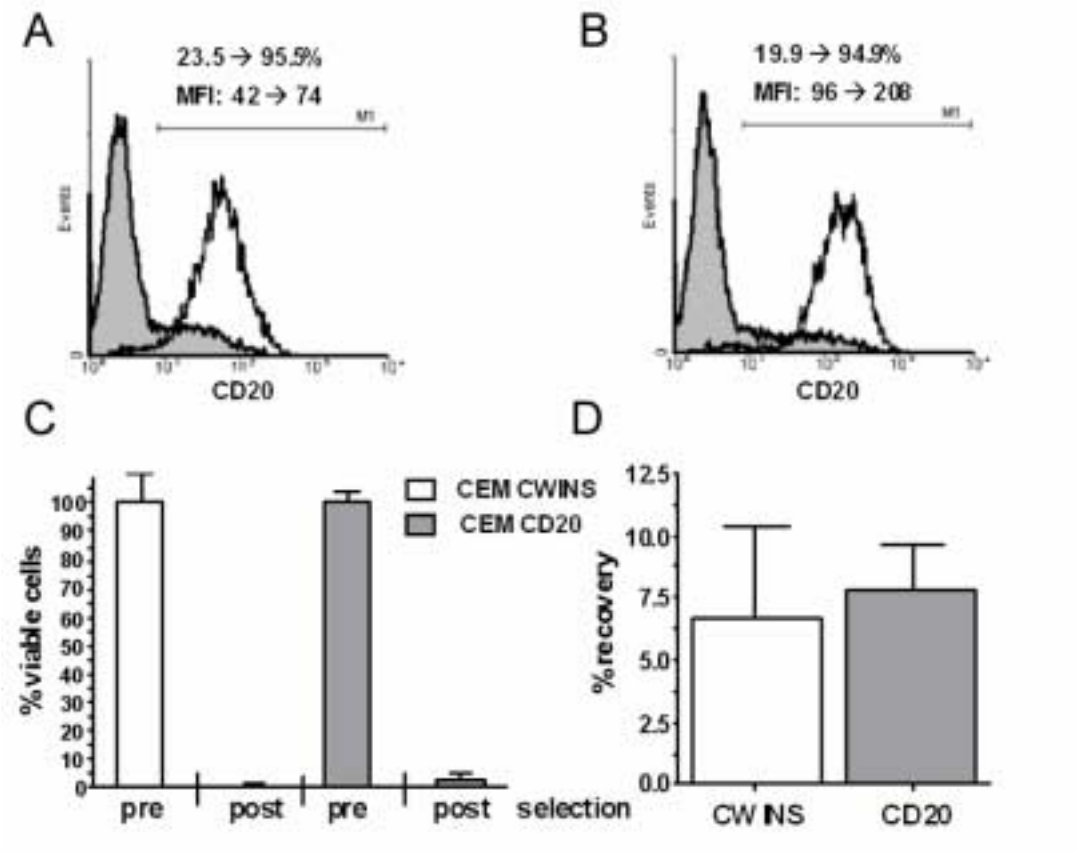

Figure 2. Transduction and selection of CEM cells

CEM cells were transduced with CD20-I-β (A) or CD20WINS (B) at an MOI of 1 (grey plots) and selected with anti-CD20 mAb clone L27, conjugated with magnetic beads (open plots). Shown are FACS plots of CD20-expression. (C) The fraction of viable (-) and dead (+) cells of four independent experiments before (pre) and after (post) MACS selection of CEM cells transduced with CD20-I-β or CD20WINS. (D) The recovery of CD20-positive cells after selection of CEM cells transduced with CD20-I-β or CD20WINS (n=4).

Transduction and selection of primary T cells

In a next series of experiments we investigated whether primary T cells could be transduced and selected in sufficient numbers for clinical application. Selection of CD20-positive cells resulted in a purity of 98.5% (Figure 3A). However, as with T cell lines, selection of primary T cells via CD20 resulted in major cell death (up to 80% of the population), and a low recovery (9.4 ± 4.1%) (figure 3D and E).

As mentioned above, the SFCMM-3 vector has been used in clinical protocols for the transfer of the HSVtk gene to T lymphocytes (6, 20). SFCMM-3 encodes ΔLNGFR that allows immunomagnetic bead selection and used this vector as a control for our selection methods. Parallel to CD20 transduction, primary T cells were transduced with SFCMM-3 vector and purified with the anti-NGFR antibody 20.4 in combination with immunomagnetic beads (Figure 3B). Purities of more 95% were obtained, but importantly, only few dead cells were found after selection (15%) resulting in a recovery of 53.0 ± 10.6% (Figure 3D and E).

To study whether some particular characteristic of the CD20 molecule was responsible for the loss of cells by MACS selection we tested whether selection via CD20 resulted in cell death in all circumstances. To this end, endogenous CD20-expressing B cells were purified from PBMC (Figure 3C). In this example, 14.6% of the PBMCs were CD20-positive and after purification by magnetic cell sorting > 95% of the cells were CD20-positive. Viability analyses revealed no dead cells and a recovery of 69 ± 0.7% was obtained (figure 3D and E).

In conclusion, CD20-based immunomagnetic selection of CD20-transgenic T cells results in purities of > 95%, but is associated with profound cell death, leading to a low recovery. Similar results were obtained when selections were performed with different anti-CD20 antibodies, a different immunomagnetic bead system (Dynal™), different MACS devices (miniMACS, VarioMACS, autoMACS), and varying the time period between transduction and selection. Just culturing CD20-transgenic T cells after conjugation with anti-CD20 antibodies and immunomagnetic beads did not lead to cell death. Neither did magnetic cell sorting of these T cells via the CD4 or CD8 antigens lead to cell death (data not shown). Therefore, we conclude that it is neither the CD20-ligation *per se* nor the magnetic-based sorting *per se* that induces cell death during CD20-based MACS-purification. Rather, we speculate that cell death is induced by the combined ligation of CD20 and the strong magnetic forces pulling on the cells during purification.

Combining CD20 with other selection markers

Since CD20 selection by MACS results in a low recovery bicistronic, vectors were constructed to identify selection markers that could be combined with CD20: OuaR and ΔLNGFR (Figure 1). In separate experiments CEM cells were transduced with the CD20-Iβ, CSNW and CD20-I-OuaR vectors. After transduction the CD20 expression level of the CEM cells transduced with the CSNW vector and the CD20-I-OuaR vector was extremely

low compared to the CD20-Iβ vector (data no shown). Cells were selected by MACS based on CD20-expression and purities of 95.1% ± 5.0, 55.0 ± 9.7% and 4.8 ± 0.12%, respectively, were obtained. Again, recoveries were low (3.3 ± 0.7%, 4.1 ± 3.7%, and 0.01 ± 0.0%, respectively; Figure 4A).

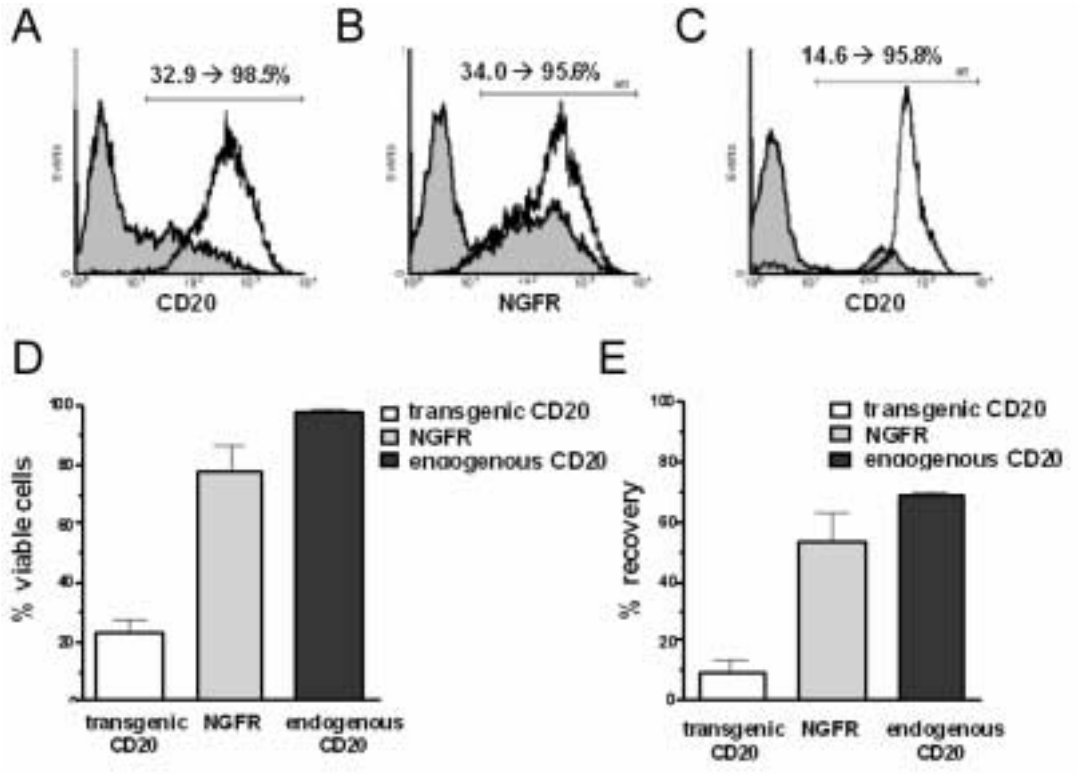

Figure 3. Transduction and selection of primary T cells and selection of B cells via CD20.
(A) T cells were activated by anti-CD3 and anti-CD28 monoclonal antibodies in the presence of 300 IU interleukin-2. On day 2 primary T cells were transduced with the CD20 vector (MOI of 1, gray plot). On day 7 the transduced cell were purified by immunomagnetic selection with anti-CD20 mAb clone L27 (open plots). (B) Transduction of primary T cells with the SFCMM-3 vector (MOI of 1, gray plot) and the selection with anti-NGFR 20.4 conjugated with magnetic beads. (C) CD20 expression in a PBMC pool (grey plot) and MACS selection of B cells with anti-CD20 mAb clone L27, conjugated with magnetic beads (open plots). (D) The percentage living cells after MACS selection. (E) The recovery of marker-expressing cells after the indicated immunomagnetic selections.

FACS-sorting is less stressful for cells than the magnetic forces pulling on the cell within a MACS column. The same cells used for CD20-based MACS purification were also used to purify the cells by CD20-based FACS-sorting. FACS-sorting on CD20-I-β-transduced cells resulted in a purity of 96.1 ± 2.9%. For the CSNW-transduced cells 85.3 ± 12.6% of the cells expressed CD20 after FACS sorting. However, FACS-sorting on CD20-I-OuaR-transduced cells resulted to a somewhat lower purity, 69.5 ± 3.3% (Figure 4B). This lower purity is probably due to the low CD20 expression level. The recovery rates with FACS-sorting were significantly higher compared to MACS selection. For the CD20-I-β-transduced cells the recovery was 23.9 ± 2.6%, for the CSNW-transduced cells this was 22.8 ± 5.0% and for the CD20-I-OuaR-transduced cells the recovery was 51.6 ± 3.9. The higher purity of FACS sorting compared to MACS purification is due to the fact that >95% of the cellswere viable after FACS sorting, whereas MACS sorting induced cell death (see above).

CSNW-transduced cells were also subjected to ΔLNGFR-based MACS-selection. The selection was performed on the ΔLNGFR-positive cells and ΔLNGFR and CD20 expression was analyzed (Figure 4C). After selection 95.2 ± 0.8% expressed ΔLNGFR and 78.7 ± 3.6% expressed CD20. The recovery rate based on ΔLNGFR expression was 34.2 ± 11.1%, while it was 13.4 ± 4.9% based on CD20 expression.

CD20-I-OuaR-transduced cells were also subjected to ouabain selections. In the presence of 10 μM ouabain cells were selected within 24 hrs (Figure 4D). Selections were very rapid and efficient with a recovery of 52.2 ± 4.3%. The majority of the selected cells, 72.8 ± 2.5%, still expressed CD20.

Taken together, these data show that CD20 purification is feasible and that FACS sorting gives the best results if CD20 density is high enough. In our hands, immunomagnetic bead selection of ΔLNGFR-positive cells is a comparable alternative.

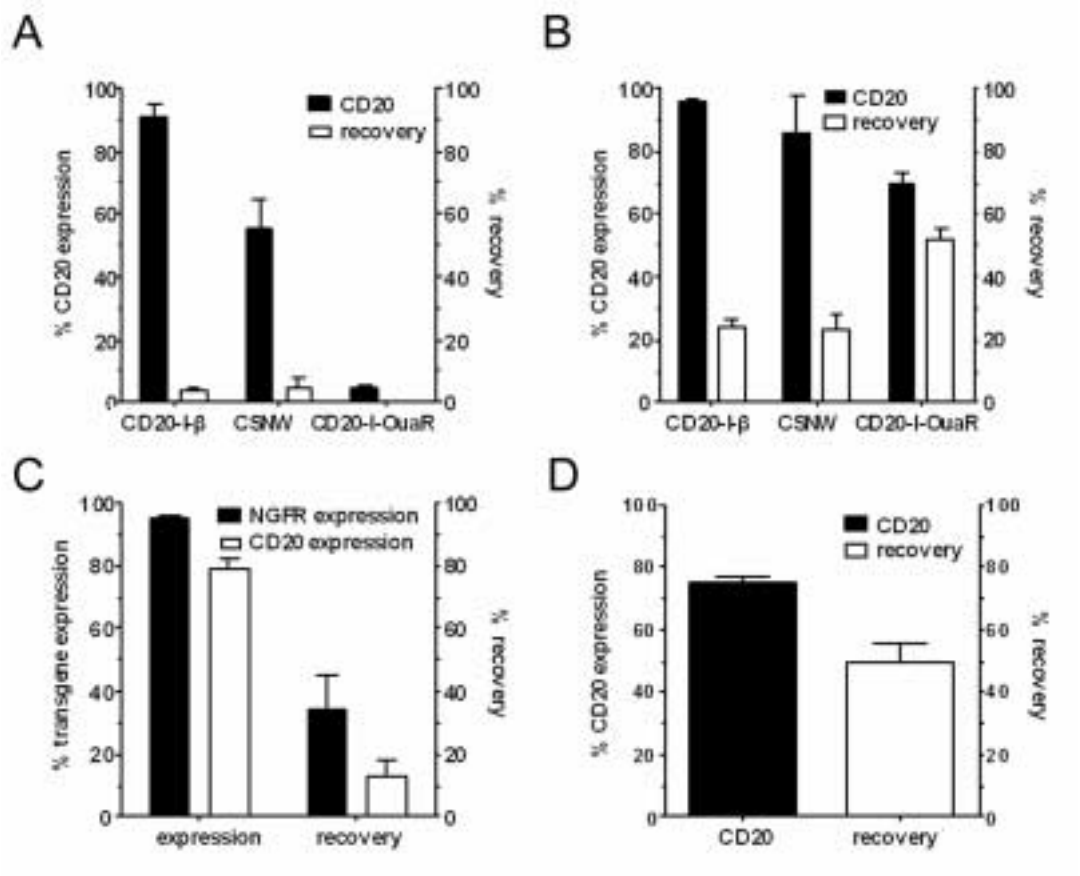

Figure 4. CD20 expression after MACS sorting, FACS sorting and ouabain selection.
(A) CEM cells were transduced with the three indicated CD20-encoding retroviral vectors and subjected to CD20-based MACS-sorting. After sorting the fraction (left y-axis, black bars) and recovery (right y-axis, open bars) of CD20-positive cells within the selected population were determined. (B) CEM cells were transduced with the three indicated CD20-encoding retroviral vectors and subjected to CD20-based FACS sorting using the PE-conjugated CD20 mAb L27. After sorting the fraction (left y-axis, black bars) and recovery (right y-axis, open bars) of CD20-positive cells within the selected population were determined. (C) CEM cells were transduced with the CNSW vector and subjected to ΔLNGFR-based MACS-selection. After sorting the fraction (left y-axis and set of bars) and recovery (right y-axis, right set of bars) of ΔLNGFR-positive cells (black bars) and CD20-positive cells (open bars) within the selected population were determined. (D) CEM cells were transduced with CD20-I-OuaR vector and cells were purified based on ouabain selection by adding 10 μM ouabain to the culture medium. After 48 hours CD20 expression (left y-axis, black bar) and recovery of CD20-positive cells (right y-axis, open bar) were determined. At least three independent experiments are shown.

Discussion

GvHD is a life-threatening complication that is caused by transfer of immunocompetent donor T cells contained in the allogeneic graft. These T cells may exhibit alloreactivity against recipient cells because of major or minor histocompatibility antigens disparities between the donor and the immunosuppressed recipient. GvHD can occur in the acute or chronic form and is based on the time of onset before or after 100 days post SCT. Acute GvHD is observed in 30-60% of the patients after allo-SCT and resulting mortality can reach 50% (36). Chronic GvHD occurs in almost 50% of the long-term transplant surviving patients and can be life-threatening in 20-40% of the patients (37).

T cell depletion of the graft reduces the incidence of GvHD, but this has been associated with increased graft rejection and leukemia relapse (38, 39). Many efforts have been made to separate the beneficial effects of the T cells from the detrimental ones. A specific way to eliminate the alloreactive T cells in case of GvHD is through the prior introduction of a so-called suicide gene. The only suicide gene applied in clinical protocols so far is the HSVtk gene (3, 5, 6, 15, 17, 18, 20, 30). HSVtk positive cells are eliminated by ganciclovir. Ganciclovir sensitivity is limited to dividing cells only (6). Another disadvantage is the fact that HSVtk is immunogenic (18, 30) and that a truncated non-functional HSVtk variant has been reported (40, 41).

Because of these limitations other suicide genes have been proposed. Transgenic expression of the Fas molecule is an example of a developed suicide gene (42). Cross-linking of the Fas molecule results in the recruitment of a death-inducing signaling cascade leading to apoptosis (43). The HSVtk and Fas genes are excellent suicide genes but cannot be used as tools to purify the genetically manipulated cell population prior to administration to the patient. Co-expression of a so-called selection marker is therefore needed. However, this leads to larger retroviral vectors, which are more difficult to handle (44) and competition between the two transgenes may occur (28, 45-48). The human CD20 molecule has the characteristics of a suicide gene and selection marker in one so potentially by-passing the limitations of the dual transgene expressing vectors (33-35).

In this report we show that selection of CD20-transgenic T cells leads to cell death within the MACS column. This is in contrast to a report of others, in which a recovery of 50% was obtained (35). It is not clear what the mechanism for this cell death is. Cells surviving the selection procedure do not have detrimental growth patterns compared to non-selected or

non-transduced cells and the CD20 molecule is expressed stably for long term culture, especially with CWINS vector (33).

The CD20 molecule is normally expressed on B cells and is a transmembrane molecule that spans the membrane four times. The cytoplasmic regions contain consensus sites for serine or threonine phosphophorylation. The function of CD20 is not fully understood yet, but it conducts calcium in cells and is probably involved in B cell activation and differentiation (49-51). Cross-linking of transgenic CD20 molecules in T cells leads to lipid raft formation, but does not induce apoptosis (52). Even hyper-crosslinking with magnetic beads did not lead to cell kill. In contrast to MACS-based selection of CD20-positive cells, purifying the cells by FACS sorting did not lead to profound cell death in the current study. Nor did selection of B cells by CD20 mAbs and MACS lead to cell death within the MACS column. We therefore speculate that the Ca^{2+}-conducting properties of the CD20 molecule may make the membrane of T cells more sensitive to the strong magnetic forces. Current research addresses the influence of the CD20 molecule on the function of T cells.

To study whether CD20 expressing cells can be purified to > 95% by including other selection markers we constructed different bicistronic vectors. In the past it has been demonstrated that these vectors cannot express both genes in all cells or expression levels of both genes may differ (28, 53). We also show that cells transduced and selected with vectors containing two promoters, expressed the gene that was used for selection for > 95% (ΔLNGFR). But not all cells express the second gene (CD20). This phenomenon can be explained by the fact of promoter interference (54). Efficient transgene expression from large constructs has been shown to be difficult. Expression of transgenes depends on the place of chromosomal integration and the availability of cellular control elements (reviewed in (55)).

Of these other selection methods, immunomagnetic bead selection of ΔLNGFR-positive cells yielded cell populations of high purity. Importantly, this method is rapid and can be performed under conditions compatible with GMP-guidelines. Similarly, ouabain-selection is rapid, within 24-48 hours, and easily adaptable to GMP-requirements. Moreover, ouabain selection is based on a readily-available chemical, compared to the tailor-made antibodies required for ΔLNGFR-selection. In addition, costs of ouabain selection are much lower. In the configuration of our current bicistronic vectors CD20-expression was diminished compared to the monocistronic CD20-encoding vectors, confirming previous reports on expression interference if more than one gene is to be expressed (28, 53).

Genetic correction of single genetic disorders has been successful by the delivery of the defective gene. For some diseases there is no need to purify the manipulated population of cells prior to administration (56). However, for these patients there is an increased risk of developing malignant transformation after gene transfer (57). To that end, in addition to the therapeutic gene, inclusion of a safety switch may be considered. The safety switch may not be of viral origin because of the premature elimination of these cells (18, 30). The inclusion of the CD20 molecule seems to be a promising candidate to act as suicide gene in case of an adverse event in repairing single genetic disorders.

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Chapter 5

Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cell-mediated cytotoxicity

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Abstract

The use of the CD20-specific antibody rituximab has greatly improved the response to treatment of CD20-positive follicular lymphoma. Despite the success of rituximab, resistance has been reported and prognostic markers to predict individual response are lacking. The level of CD20 expression on tumors has been related to response, but results of several studies are contradictory and no clear relationship could be established. Complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) are thought to be important effector mechanisms, but the exact mechanism of rituximab-mediated cell kill is still unknown. Importantly, no data have been reported on the combined contribution of CDC and ADCC.

We have developed a system of clonally-related CEM-CD20 cells by retroviral transfer of the human CD20 cDNA (n=90). This set of cells, with the CD20 molecule as the only variable parameter, was used to study the importance of CD20 expression level on rituximab mediated CDC, ADCC and the combination of both.

We demonstrate a sigmoidal correlation of CD20 expression level and rituximab-mediated killing via CDC, but not ADCC. Both on high and low CD20-expressing cells all CD20 molecules were translocated into lipid rafts after rituximab-binding. Furthermore, CDC and ADCC act simultaneously and CDC-resistant cells are sensitive to ADCC, and vice versa.

These findings suggest that CDC depends on CD20 expression level and that both CDC and ADCC act complementary. These data give new insights into novel strategies to improve the efficacy of CD20-specific antibodies for the treatment of CD20-positive tumors.

Introduction

The CD20-specific monoclonal antibody (mAb) rituximab has been widely proven to be a successful treatment of a variety of B-cell malignancies and B-cell related diseases (1, 2). However, resistance against rituximab occurs and there is no prognostic marker to predict individual response (3-5).

Various *in vitro* and *in vivo* experiments have demonstrated that elimination of CD20-positive cells is effected by the IgG1 chain of rituximab, which triggers complement-dependent cytotoxicity (CDC), and induces the recruitment of effector cells, leading to antibody-dependent cellular cytotoxicity (ADCC) (6-13). On the other hand, the complement regulatory proteins (CRPs) CD46, CD55 and CD59 have been shown to inhibit rituximab-mediated cell kill by interfering with complement activation (10, 11, 13). However, despite these current understandings, the exact mechanism of rituximab-mediated cell kill is still unknown. Importantly, no data have been reported on the combined contribution of CDC and ADCC. As a consequence, the differential susceptibility of CD20-expressing tumors to rituximab is incompletely understood.

In general, follicular lymphoma (FL) cells can be killed effectively by rituximab, while B-cell chronic lymphocytic leukemia (B-CLL) cells show a poor response. A major difference between these cell types is the higher level of CD20 expression on FL cells compared to B-CLL cells (7, 11, 14)**.** This suggests that rituximab-sensitivity depends on CD20 expression. Therefore, several studies have addressed the question whether the CD20 expression level may be used to predict progression of disease and response to treatment (5, 10-13). The results are conflicting. *In vitro* analysis of FL cells revealed no correlation between CD20 expression level and CDC-sensitivity (10, 12). In another study, rituximab-mediated CDC *in vitro* did not correlate with clinical response (13). In a comparison of B-CLL samples no significant correlation was found between CD20 expression level and rituximab-response *in vivo* (5). In contrast, a marked correlation was reported in two other studies involving a variety of B cell malignancies (7, 11). This significant variability in the reported sensitivity to rituximab-mediated CDC among B cell lines and primary tumor samples may have been affected by biological parameters other than CD20 expression level. Moreover, within primary tumor samples not all cells may express CD20 (11).
To unequivocally define the role of CD20 expression level in rituximab-mediated killing, a controlled experimental setting is required, in which CD20 expression level is the only

variable parameter. Importantly, the relationship between CD20 expression level and the combined activity of CDC and ADCC require investigation.

In this study, we present a unique experimental model consisting of a set of clonally-related CD20-positive transgenic cells that collectively cover a wide spectrum of CD20 expression levels. Using this model, we investigated the relationship between CD20 expression level and rituximab-mediated cell kill and the separate and combined contribution of CDC and ADCC. Our results demonstrate that rituximab-induced CDC, but not ADCC, clearly depends on the CD20 expression level. Moreover, we show that the activity of rituximab significantly increases when CDC and ADCC act simultaneously. Importantly, we found that CDC-resistant cells are still susceptible to ADCC and vice versa, which leads us to conclude that these effector mechanisms act complementary.

Material and Methods

Generation of CD20-positive CEM cells

The human CD20 cDNA was amplified by PCR from the pCMV-CD20-expression vector by using the following primers: CD20-FW: 5'-GGG CCG CGG CCG CCG CCA TGA CAA CAC CCA GAA ATT CAG TA-3' and CD20-SalI RV: 5'-GGG GTC GAC AAA TCA CTT AAG GAG AGC TGT CAT-3' (15). The amplified CD20 fragment was ligated into a pCRII TOPO cloning vector (Invitrogen, Paisley, UK) according to manufacturer's instructions, resulting in the pCRII-CD20 vector. The CD20 cDNA was released from the pCRII-CD20 vector with BamHI and SalI endonuclease restriction enzymes. The CD20 cDNA was then inserted into the BamHI and SalI sites of the pMX-retroviral vector (16). Generation of virus particles, transduction of the CEM and Jurkat CD20-negative T cell lines and purification of CD20-positive cells with paramagnetic beads was done as previously described (17, 18). Clonal CEM-CD20 and Jurkat-CD20 cells were generated by limiting dilution culture of the selected cells in a 96-wells flat bottom tissue culture plate (Nunc, Roskilde, Denmark).

Cells and cell culture

The CEM T cell line, Jurkat T cell line and Raji lymphoma cells (18) were cultured in culture medium consisting of RPMI (Gibco-BRL, Paisly, Scotland), 10% fetal calf serum (FCS, Integro, Zaandam, the Netherlands), penicillin (100 U/ml), streptomycin (100 μg/ml) (Gibco-BRL), 5 x 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany). PBMCs were

isolated by density centrifugation through Ficoll (Amersham Pharmacia, Uppsala, Sweden) and stimulated with 300 U/ml human recombinant interleukin 2 (IL-2) (Proleukin, Chiron, Amsterdam, the Netherlands) and interleukin 12 (IL-12) (Peprotech, London, United Kingdom) for 24 hrs. All cells were cultured at 37°C in a 5% CO_2 atmosphere. Chronic lymphatic leukemia's (CLL, 6x) and lymphoma's (6x) were stored in liquid nitrogen. Upon thawing they were used directly to determine the absolute number of CD20 molecules per cell and compared with the clonal CD20-transgenic cells.

Flowcytometric analysis

Expression of CD20 and CRPs was determined by FACS (Becton Dickinson, Mountain View, CA). Antibodies used were anti-CD20-phycoerythrin (PE) (BD Biosciences, San Jose, CA), anti-CD46-PE (Immunotech, Marseille, France), anti-CD55-PE and anti-CD59-PE (CLB, Amsterdam, Netherlands). Rituximab was obtained from Roche (Basel, Switzerland). The absolute numbers of CD20 molecules per cell were determined with QuantiBRITE CD20 PE (Becton Dickinson, San Jose, Ca) kit, according to manufacturer's instructions. The antibodies-bound-per-cell (ABC) represents the absolute number of CD20 molecules per cell.

Measurement of raft-associated antigen by Triton X-100 insolubility

To study the presence of CD20 in cholesterol-rich microdomains before and after rituximab ligation we used a rapid flow cytometry method based on Triton X-100 insolubility at low temperatures, as described previously (19). Briefly, cells were washed in PBS and resuspended at 2.5 x 10^6 cells/ml. Cells were incubated with 10 µg/ml of rituximab or control anti-CD7-Fluorescein isothiocyanate (FITC) monoclonal antibody (Becton Dickinson, Mountain View, CA) for 15 minutes at 37°C. Next, the samples were washed in cold PBS and then divided in half. One half was maintained on ice and stained later with rituximab to calculate the 100% surface antigen expression. The other half was treated with 0.5% Triton X-100 for 15 minutes on ice to determine the proportion of antigens remaining in the Triton X-100 (Riedel-deHaen, Germany) insoluble fraction. Cell fragments were spun down and the pellet, containing the lipid rafts, was stained with rituximab. Next, the rituximab ligated pellet of the Triton X-100 treated and the non-treated cells were stained with anti-human IgG1-FITC antibody. The mean fluorescence intensity (MFI) was determined by FACS as described above.

rituximab-mediated cytotoxicity assays

For CDC-assays, 1 x 10^6 cells were resuspended in 500 µl human serum and 500 µl culture medium with or without 10 µg/ml of rituximab at 37°C for 30 min. Dead and viable cells were discriminated by addition of 1 µg/ml propidium iodide (PI). Measurement of ADCC and the combination of ADCC and CDC was based on a recently described FACS-based assay (20). In brief, CD20-positive cells were washed in PBS and resuspended at 1 x 10^6 cells/ml. Next, the cells were stained with 5 µM of carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Europe BV, Leiden, The Netherlands) for 10 min at 37°C. The reaction was stopped by adding an equal volume of FCS. Cells were washed twice with PBS and 2,500 cells/well were plated in a 96 round bottom microtiter plate (Nunc) in the absence or presence of 10 µg/ml rituximab. Stimulated PBMCs were added at 1:10 target/effector ratio without (ADCC) or with (ADCC + CDC) human serum (50%). The optimal conditions for rituximab-mediated ADCC to obtain maximum cell kill have been determined previously (21). After incubating the plates for 4 hours at 37°C the cells were harvested and 1 µg/ml PI and 5,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, Florida) were added. To determine the absolute number of surviving cells data acquisition was stopped after measurement of 2,500 Fluorospheres and the CFSE-positive and PI-negative cells were counted. The fraction of rituximab-mediated cell kill was calculated as follows:

$$\%\ \text{rituximab survival} = \frac{\text{with rituximab absolute viable CFSE+ PI- cells}}{\text{without rituximab absolute viable CFSE+ PI- cells}}$$

Statistical analysis

Sigmoidal dose-response equation or linear regression was used to compare the rituximab-mediated CDC and ADCC versus CD20 expression level by using SPSS 11.5 or GraphPad Prism 4.0 software. A two-way ANOVA test was used to demonstrate the significance of CDC and ADCC compared with the combined activity. Three or 4 replicate killing assays per clone were averaged, and the mean and standard deviation (SD) were calculated and used for statistical comparison.

Results

To define the impact of CD20 expression level on rituximab-mediated cell kill a system is required in which the CD20 molecule is the only variable parameter. To obtain such a system we chose to generate CD20-positive clones of the CD20-negative T cell lines CEM and Jurkat. This collection of clones, expressing variable levels of the CD20 molecule, was then used to evaluate rituximab-induced cytotoxicity. For stable expression of the CD20 molecule on CD20-negative cells we constructed a CD20-encoding retroviral vector (Figure 1A). After transduction, CD20-positive cells were selected with CD20 antibody-conjugated paramagnetic beads and were purified to more than 95% homogeneity (Figure 1B). To obtain a panel of CD20-positive cells with different intensities of CD20-expression we generated CD20-transgenic clones by limiting dilution culture of the purified cells. Figure 1C shows an example of the diversity in CD20 expression level of the CEM-CD20 transgenic clones with low, low-intermediate, high-intermediate and high CD20-expression profiles. Figure 2A shows the mean fluorescence intensity (MFI) of the CD20 expression level of all clones. The MFI of individual CD20-positive clones ranged from 256 to 693 with a mean MFI of 505 (n = 90). Twelve clones were selected that cover a wide range of CD20 expression levels and the level of expression of CRPs was determined. As shown in figure 2B, a similar expression level of CD46, CD55 and CD59 was observed in all clones (MFI of 421 ± 17, 201 ± 22 and 441 ± 24, respectively). The same results were obtained with transduced CD20-positive Jurkat clones (data not shown). This collection of clones provided a unique set up to analyze the impact of the CD20 expression level on rituximab-mediated killing by both CDC and ADCC.

First, 50 CEM-CD20-clones with low, intermediate and high CD20 expression level and the non-transduced CEM cells were subjected to rituximab-induced CDC. Figure 2C displays the correlation between CD20 expression level, indicated by the MFI of CD20 expression, and rituximab-mediated CDC. A minimum CD20-MFI of approximately 450 was required to induce rituximab-specific CDC. Maximum CDC was obtained with an MFI of > 600. These data show a significant correlation between the CD20 expression level and rituximab-induced CDC (r^2=0.83, n=51). Similar results were obtained with CD20-transgenic Jurkat clones as shown in figure 2D (r^2=0.88, n=42).

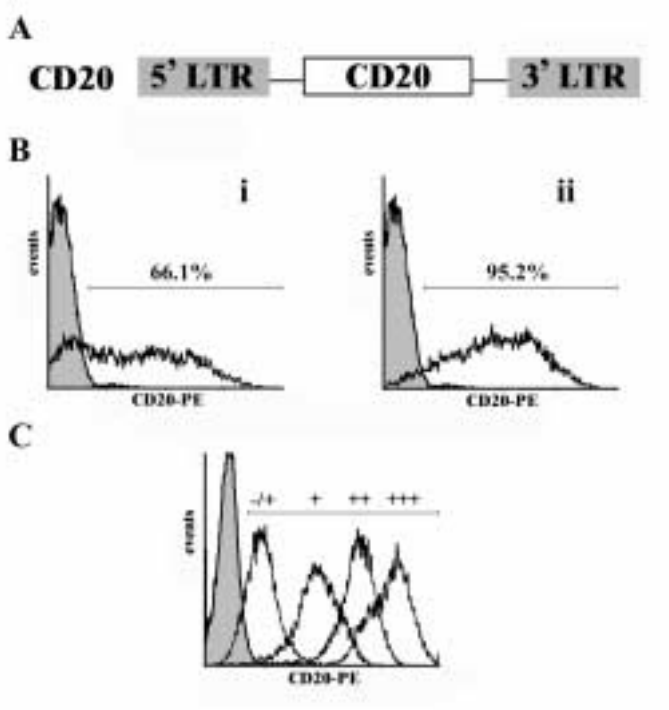

Figure 1. CD20-transduction of CEM T cells.
(**A**) The CD20-encoding retroviral vector. LTR: long terminal repeat. (**B**) FACS-analysis of transduced (i) and purified CD20-positive CEM cells (ii). (**C**) Examples of the CEM-CD20 transgenic clones, with low (-/+), low-intermediate (+), high-intermediate (++) and high (+++) CD20-expression profiles.

Induction of CDC by rituximab has been shown to be dependent on the translocation of CD20 into Triton X-100-insoluble cholesterol-rich microdomains (lipid rafts) (19). Therefore we addressed the question whether the low level of CDC at low CD20-expression was associated with incomplete translocation of CD20 into lipid rafts after rituximab-ligation.
Figure 2E shows the redistribution of CD20 into lipid rafts after rituximab-binding of five different clones with different CD20 expression levels. In all clones, CD20 was completely reorganized in the plasma membrane after binding with rituximab, independent of the number of CD20 molecules expressed. No significant amount of CD20 antigen was detected in rafts before rituximab binding and hyper-crosslinking of rituximab by anti-human IgG $F(ab')_2$ did not increase raft formation (data not shown). The percentage CDC of the clones to rituximab and complement is also shown in this figure. Again, high CD20 expressors are very sensitive to rituximab and the low expressors are not, despite the lipid

raft formation. As a negative control, CD7, expressed on CEM cells, is not completely Triton X-100-insoluble after antibody binding.

We next investigated the relationship between CD20 expression level and ADCC by incubation of CEM-CD20 clones with rituximab and effector cells. Again, clones with different CD20 expression levels were used. We found an average cell kill of 49.3 ± 14.2% (Figure 2F). In contrast to CDC, there was no significant correlation between the number of CD20 molecules per cell and rituximab-mediated cell kill by effector cells (r^2=0.19, n=13). Maximum ADCC was already achieved at the lowest MFI tested (300).

In several studies tumor samples have been used in which the absolute number of CD20 molecules per cells (CD20-ABC) was determined (7, 11). To quantitatively compare our CD20-transgenic cells with those tumor samples we also determined the CD20-ABC of our CD20-transgenic cells and of 6 CLL and 6 lymphoma samples. Figure 3A shows again the correlation between the absolute number of CD20 molecules per cell and the rituximab-mediated CDC (r^2=0.87, n=21). In contrast, maximum ADCC was already achieved at a CD20-ABC of 15,889 (Figure 3B). In both figures the range of CD20-ABC of the CLL and lymphoma samples is plotted.

To obtain additional proof of the impact of the CD20 expression level on CDC-sensitivity we transduced the CD20-positive Raji Burkitt's lymphoma cell line with the CD20 retroviral vector (Figure 4A). Figure 4B shows that the additional CD20 molecules on the cell surface made the Raji cells more sensitive to rituximab-mediated CDC, since 15% more cells were killed in the presence of rituximab and human serum as compared to non-transduced cells. This was also observed for CD20-transduced EBV-transformed B cells (data not shown). Thus, the CD20 expression level is of critical importance for rituximab-mediated CDC.

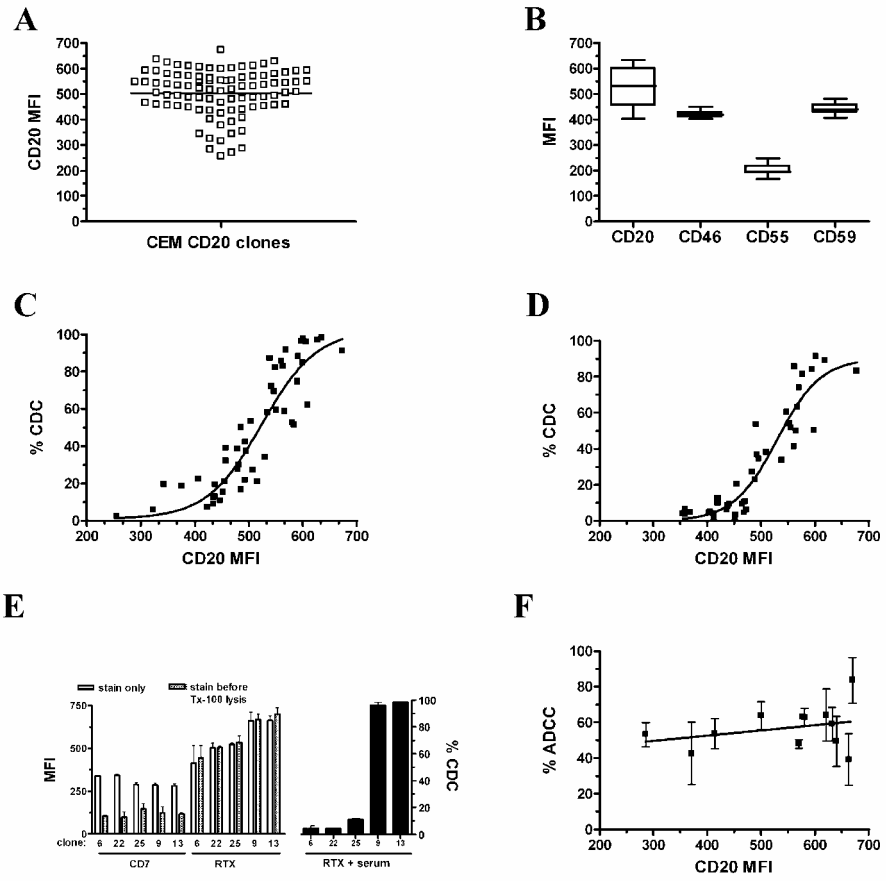

Figure 2. rituximab-mediated CDC and ADCC assays on CEM-CD20 clones.

(**A**) MFI of individual CD20-expressing CEM clones (n=90). (**B**) The range of CD20 and CRP (CD46, CD55, and CD59) expression of CEM-CD20 clones (n=12). Clones were selected that covered a wide range of CD20-expression. (**C**) The relationship between the level of CD20-expression and CDC-mediated cell kill in the presence of rituximab and human serum. The MFI of CD20 expression level of individual CEM-CD20 clones were determined and plotted against the fraction of cell death induced by rituximab and human serum. The experiments were performed in duplicate. (**D**) The relationship between the level of CD20-expression and CDC-mediated cell kill in the presence of rituximab and human serum. The MFI of CD20 expression level of individual Jurkat-CD20 clones were determined and plotted against the fraction of cell death induced by rituximab and human serum. The experiments were performed in duplicate. (**E**) CEM-CD20 cells were incubated with 10 µg/ml of rituximab or anti-CD7 and the samples were divided in half. ☐ were directly stained with anti-IgG1-FITC and ■ were first treated with Triton X-100 and than stained with anti-IgG1-FITC. The black bars,■, represent the percentage CDC of the clones in the presence of rituximab and serum, as described previously. All experiments are performed in duplicate. (**F**) The relationship between the CD20 expression level and ADCC-mediated cell death in the presence of rituximab. The CD20-ABC was plotted against the % ADCC after incubation with effector cells and rituximab. The experiments are performed in triplicate and represent the mean and SD.

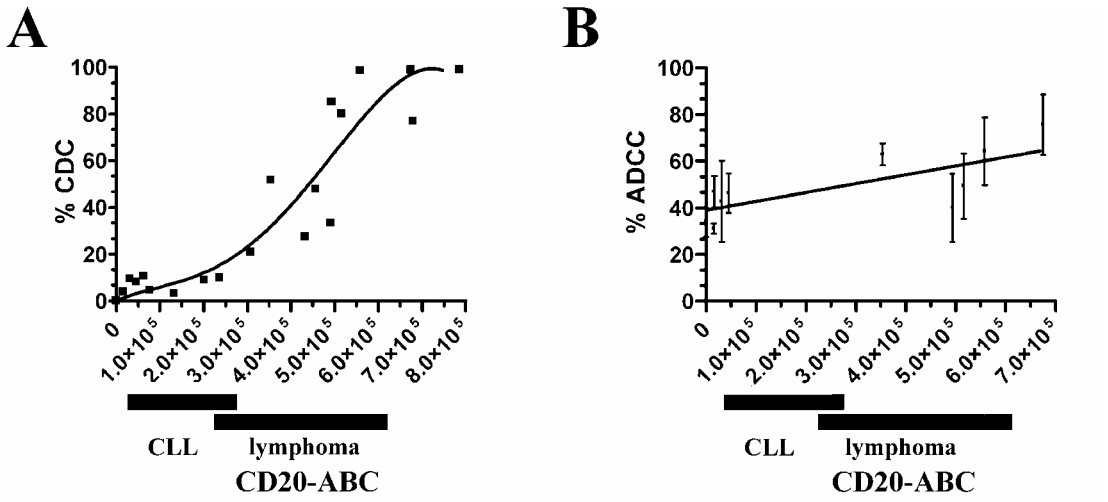

Figure 3. Correlation of CD20-ABC and rituximab-mediated CDC and ADCC assays on CEM-CD20 clones.

The absolute numbers (ABC) of CD20 molecules of individual CEM-CD20 clones were determined and plotted against the extent of CDC **(A)** or ADCC **(B)**. The bars under the x-axis represent the CD20-ABC of CLL and lymphoma samples.

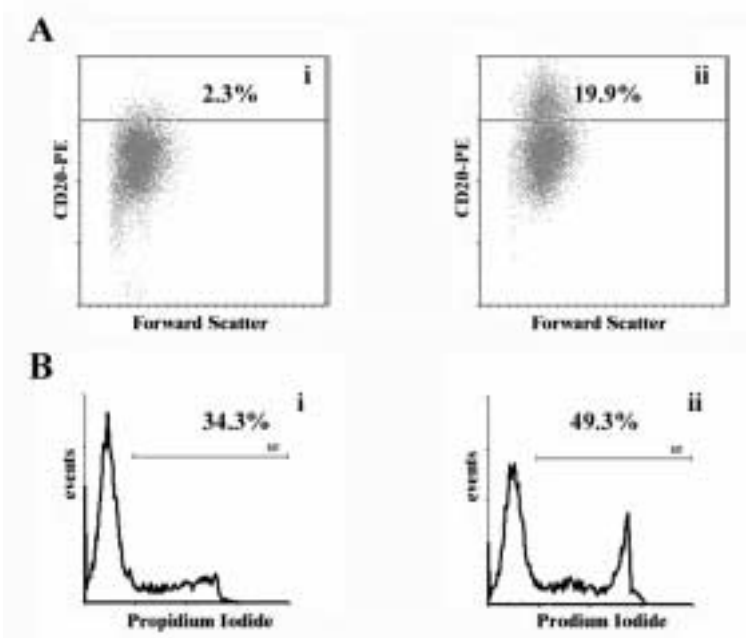

Figure 4. rituximab-mediated CDC assay on Raji cells.

(**A**) CD20-positive (**i**) non-transduced and (**ii**) transduced Raji cells. (**B**) CDC-assay on (**i**) non-transduced and (**ii**) CD20-transduced Raji cells. Dead cells were stained with propidium iodide.

Next, we investigated the impact of both CDC and ADCC on rituximab-mediated cell kill on eight different CEM-CD20 clones in three independent experiments (Figures 5A, B, C). These clones were selected because their CD20-MFI was within the linear range of the rituximab-mediated CDC. Figure 5D summarizes these results. Again, there was a significant correlation between the CD20 expression level and cell kill by CDC ($p=0.002$, $r^2=0.82$). These results also show that the correlation of CD20 expression and rituximab-induced kill is donor (serum) independent. Again, no correlation was observed between the level of CD20 expression and rituximab-induced ADCC ($p=0.74$; $r^2= 0.02$).

The combined activity of CDC and ADCC correlated with the level of CD20-expression ($p<0.02$, $r^2=0.62$) and was significantly higher than the activity of ADCC alone, except for two clones with a very low CD20 expression (clone 4, MFI 423 and clone 17, MFI 462). Hence, no activation of complement was induced by rituximab with these clones. Also, the combined activity of CDC and ADCC was significantly stronger than CDC alone, except for the clones with very high CD20-expression (clone 19, MFI 662 and clone 21, MFI 683), since virtually all cells were killed by CDC alone. Apoptosis is a third mechanism of action described for rituximab, but we did not observe any apoptosis in this 4hr assay, nor after 24 or 48 hrs. In addition, binding of rituximab did also not result in growth arrest (data not shown).

These data suggested that cells not sensitive to CDC could be sensitive to ADCC and vice versa. To test this hypothesis, we first subjected a CEM-CD20 clone with high CD20-expression to CDC alone and observed 5.3 ± 0.5 % cell survival (Figure 6A). These surviving, CDC-resistant, cells were still rituximab-positive as measured with an antihuman IgG1 goat antibody after 48 hr of culture (data not shown). Subsequently, the CDC-resistant cells were cultured for another 4 hr in the presence of effector cells. Only 1.0 ± 0.7 of the CDC-resistant cells survived this ADCC (Figure 6B), while addition of serum had no effect. In the reciprocal experiment, the same CEM-CD20 clone was first subjected to ADCC (35.8 ± 8.5 % cell survival). Human serum was then added to the remaining ADCC-resistant cells. Only 0.4 ± 0.1 % of the ADCC-resistant cells survived this treatment. Together, these data show that CDC and ADCC act complementary in mediating rituximab-induced cell death. Moreover, this suggests that resistance to CDC and ADCC is mediated by separate mechanisms.

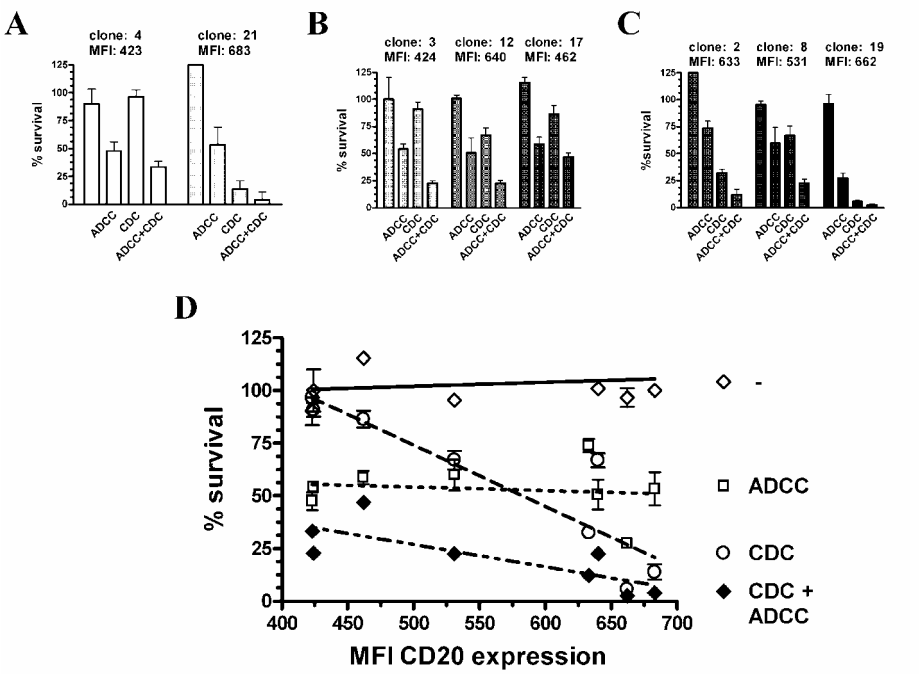

Figure 5. rituximab-mediated killing assays.

(**A-C**) Eight individual CEM-CD20 clones with different CD20 expression levels were used and their sensitivity to rituximab-mediated CDC and ADCC was compared. These graphs display the results of 3 different experiments with effector cells and serum from three different donors. Each experiment was performed in triplicate, and each bar shows the mean survival ± SD. (**D**) Correlation between CD20 expression level on CEM-CD20 clones and sensitivity to rituximab-induced cell kill. ◇: rituximab alone without effector cells or serum (control); □: ADCC induced by rituximab and effector cells; ○: CDC induced by rituximab and complement; ◆: combination of ADCC and CDC induced by rituximab

Discussion

Clinical data with respect to the efficacy of rituximab have shown variable response rates of different CD20-positive malignancies (5, 22-24). The *in vivo* mechanisms of action of rituximab are not completely understood. Moreover, the mechanisms underlying the resistance to rituximab-treatment are unresolved (25). The most important effector mechanisms of rituximab are activation of the complement system and the recruitment of FcR-bearing effector cells. In addition, some reports showed that cross-linking of the CD20 molecule could lead to apoptosis and even a vaccinal effect of rituximab has been proposed (3, 26-29). It probably depends on the cellular microenvironment and the phenotype of the tumor cells which of these effector mechanisms is/are dominant *in vivo*. At this stage, there

is insufficient understanding with respect to the interaction of the multiple mechanisms of action of rituximab.

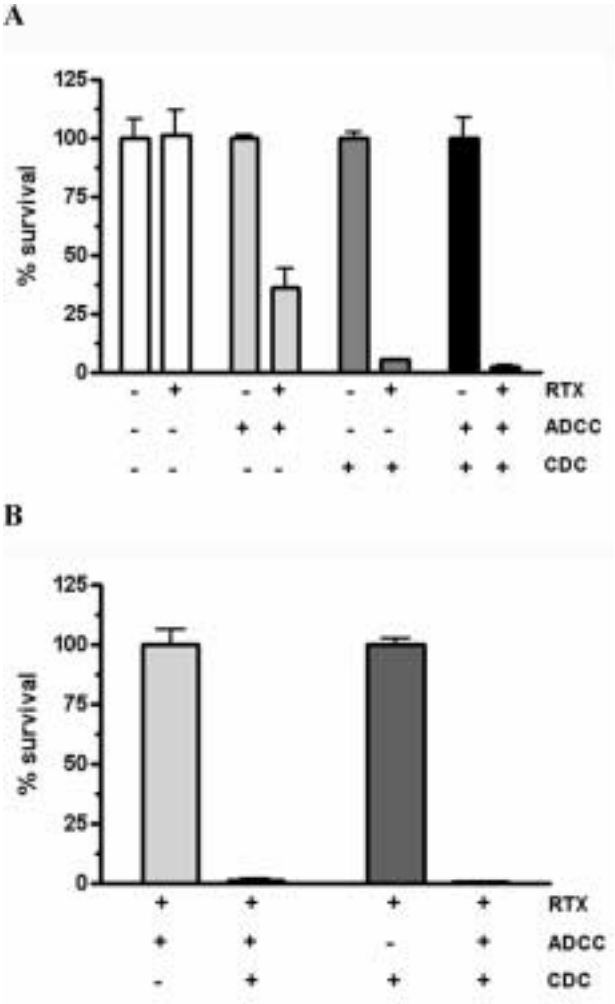

Figure 6. Complementary activity of CDC and ADCC in rituximab-induced cell kill. (**A**) rituximab-mediated killing of a CEM-CD20 clone with high CD20-expression by CDC, ADCC and the combination of both. The bars indicate the mean ± SD of experiments performed in triplicate. (**B**) In a second experiment, the ADCC-resistant cells from the first experiment were subjected, after 48 hr of culturing, to CDC through addition of human serum (light gray bars). Conversely, the CDC-surviving cells from the first experiment were subjected to ADCC through addition of effector cells (dark gray bars).

The efficacy of rituximab has often been correlated with CD20 expression. In contrast to a wide-spread belief this issue is still open, since the results of the various studies addressing this question are conflicting (5, 7, 10-12). Moreover, these studies only concern CDC but not ADCC, nor the combined contribution of CDC and ADCC to effectuate cell kill. In our experimental model the level of CD20 expression is the only variable parameter. The clones collectively cover a wider range of CD20 expression than the samples in other studies, which mainly involved low expressors (10-13). Our data clearly demonstrate that

the sensitivity to rituximab-induced CDC correlates well with the level of CD20 expression, confirming several studies (7, 11). However, in contrast to these studies, our data indicate that the correlation is characterized by a sigmoidal (S) rather than a linear curve. Of note, those studies mainly involved low-CD20 expressing samples and the lack of a sigmoidal relationship may have been caused by the paucity of high expressors. As a consequence, the curves did not reach a maximum plateau level. The S-shape of the curve implicates a minimum threshold number of CD20 molecules for induction of CDC. This observation may well explain the poor response of B-CLL that express only low levels of CD20 (14). The impact of CD20 expression level was further strengthened by the observation that an enforced increase in CD20 expression level on Raji cells resulted in an increase in CDC-susceptibility.

The activation of the complement system by CD20 antibodies has been correlated to the ability of these antibodies to translocate CD20 into lipid rafts (19). In our hands, rituximab ligation also translocates all CD20 molecules into lipid rafts, independent of the number of CD20 antigens present on the cells. This creates a complex for the binding of C1q and initiates the classical pathway of the complement system. These data indicate that low CDC-sensitivity of low CD20-expressors does not result from incomplete lipid raft formation. Rather, low CDC-sensitivity likely results from insufficient complement-fixation.
The clearly established relationship between a high CD20 expression level and high rituximab-mediated CDC in our model remains in contrast to some *in vitro* studies involving primary lymphomas (10, 12). Apparently, other biological characteristics beside CD20 expression critically affect rituximab-susceptibility of these tumors, even if they highly express CD20. An obvious candidate is CRP-expression. Although CRP-blocking antibodies could enhance rituximab-efficacy *in vitro*, CRP-expression did not influence rituximab-induced CDC (7, 11, 13). In addition**,** clinical outcome nor *in vitro* CDC-sensitivity correlated with CD20 expression level in a study involving rituximab-sensitivity of primary FL cells (13). Both in responding and non-responding patient's high CD20 expression was found. However, in relation to clinical outcome several factors, like the type of tumor and/or localization, tumor load or tumor vascularization, play an additional role.

Although ADCC has often been implicated as an rituximab-effector mechanism, only few studies have addressed the extent of cell death by ADCC so far. These studies confirm our findings of partial ADCC-induced cell kill by rituximab *in vitro*, irrespective of CD20 expression level (11, 12, 30). Various *in vivo* models have shown the significance of

immune activation through Fc receptors (FcγR) for IgG (8, 31). Uchida et al. (ref 31) demonstrated that only the innate immune system was responsible for depleting B cells in mice. They showed that anti-CD20 mouse monoclonal antibodies (mAbs) primarily deplete B cells through FcγR-dependent and C3-, C4-, and C1q-independent mechanisms. B cells were also cleared in NK- or T cell deficient mice by the different types of anti-CD20 mAbs. In contrast, B cells were not significantly eradicated in mice treated with liposome-encapsulated clodronate or in mice with CFS-1 deficiency that completely lack macrophages. Altogether, these results favor the depletion of B cells through macrophages in this model. Of note, in this study mouse anti-CD20 mAbs were used, including the IgG1 subtype. Mouse IgG1 does not activate mouse complement (32). In contrast, rituximab, which contains a human IgG1 chain, does trigger the human complement system *in vitro* and *in vivo* (9, 33). Moreover, others have reported the essential role of complement and rituximab in a mouse model (9). Nevertheless, a pivotal role of cells expressing FcγR for the efficacy of rituximab was proven in studies with patients who differed in expression of distinct FcγRIIa and FcγRIIIa polymorphisms. FcγRIIa (only expressed by macrophages) and FcγRIIIa polymorphisms correlated with the efficiency of tumor- or B cell-depletion during rituximab treatment (34-36). In addition, *in vitro* experiments confirmed the importance of polymorphisms rituximab-induced ADCC (37). In our experiments we used total PBMC from healthy donors stimulated for 24 hrs with IL-2 and IL-12, resulting in a very strong NK-activity (38). Although we did not check for FcγR polymorphisms no significant differences between the different donors were found (data not shown and ref 21).

The relative contribution of both CDC and ADCC on rituximab-induced cell kill has often been questioned. We here demonstrate that CDC and ADCC can act both independently and simultaneously and significantly enhance cell kill when combined. As with CDC alone, the efficiency of the combined activity of CDC and ADCC also depends on CD20 expression level. The C1q binding site of the complement system is located within the C_H2 domain of the IgG1 chain of rituximab (39). Mutations within the C_H2 domain of rituximab resulted in impaired CDC and ADCC activity. This can be explained by the close proximity of the C1q binding site of IgG1 to the binding site involved in binding to most FcR subtypes (39-42). We show that CDC and ADCC act cooperatively. Thus, binding of C1q does not fully inhibit FcγR-binding and vice versa. Alternatively, complement components deposited on target cells can act as ligands for their specific receptors on effector cells (complement-dependent cellular cytotoxicity) (43). The relative contribution of CDC and

ADCC *in vivo* is unclear, but complement activation has been shown to trigger the release of inflammatory cytokines and therefore the activation of effector cells (44, 45). Importantly, we show that cells resistant to CDC are still sensitive to ADCC and vice versa. The observation that (tumor-associated) CDC- or (host-associated) ADCC-resistance can be overcome by the complementary effector mechanism indicates that different mechanisms underlie CDC- and ADCC-resistance, which is most likely a reflection of the different molecular mechanisms of ADCC and CDC (induction of apoptosis and lysis, respectively).

In these studies CD20-transgenic T cells were used and no apoptosis or growth inhibition was observed. In contrast, binding of rituximab to endogenous CD20 on B cells could lead to direct killing of the target cells and cross-linking of rituximab with secondary antibodies even increased apoptosis. rituximab translocates the CD20 molecules into lipid rafts and reportedly activates protein tyrosine kinases of the CD20 molecule, increases intracellular Ca^{2+} concentrations, caspase activation, and subsequent cleavage of caspase substrates (26, 27, 46). At this moment we do not know whether rituximab could induce these caspases in our CD20-transgenic T cells but studies addressing this issue are underway.

Within this *in vitro* model we have studied the impact of CD20 expression as the only variable parameter and have shown a clear correlation between CD20 expression and CDC-sensitivity. Since high CD20 expressing lymphoma cells are not always sensitive to rituximab *in vivo* other parameters likely impact rituximab-efficacy. Nevertheless, this model demonstrates that reduced CD20 expression leads to impaired CDC and that both CDC and ADCC can act simultaneously. To further understand the relative *in vivo* contribution of both effector mechanisms related to the CD20 expression level these clones are currently employed in *in vivo* mouse studies. These experiments give us useful information of the importance of CD20 expression level and the efficacy of different anti-CD20 monoclonal antibodies (30).

These studies add ADCC-resistance to the list of potential mechanisms of rituximab-resistance. Enhancing ADCC activity with, for example, IL-2 or IL-12 could enhance the susceptibility of CD20-positive cells to FcR-bearing effector cells (47). Alternatively, ADCC could indirectly be enhanced by changing the microenvironment of tumor cells, e.g. with CpG DNA sequences. These immunostimulatory sequences induce a.o. secretion of numerous cytokines (IL-12, IL-18, IFN-α and IFN-β) by macrophages and dendritic cells (25, 48-50). On the other hand, strategies that enhance complement-fixation of anti-CD20 antibodies or that induce an upregulation of CD20-expression, such as cytokine-treatment,

or the use of immune modulator agents such as bryostatin-1, may improve CD20-targeted cell kill of low-CD20-expressing cells (30, 51, 52). In addition, blocking of the complement regulatory proteins CD55 or CD59 may increase the sensitivity to complement (7, 11).

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Chapter 6

The CD20 Expression Level Related Resistance of Rituximab Can Be Overcome by HuMab-7D8 anti-CD20 monoclonal antibody

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In submission

Abstract

Incorporation of the chimeric anti-CD20 monoclonal antibody (mAb) rituximab in the treatment schedule of patients with Non-Hodgkin's lymphoma has significantly improved the outcome. Despite this success, about half of the patients do not respond to treatment or relapse from the original disease, and additional therapy is often required. We recently showed that a low CD20 expression level may be in part responsible for resistance towards rituximab.

In this report, we investigated whether CD20 expression-level related rituximab resistance could be overcome by other CD20 mAbs. Recently, a group of anti-CD20 mAbs (a.o. HuMab-7D8 and ofatumumab), have been developed that bind to a novel CD20 epitope and induce exceptionally potent complement-mediated tumor cell lysis.

Here, we show in a unique *in vitro* system, in which the CD20 expression level is the only variable parameter that, in the presence of complement, HuMab-7D8 was able to kill low CD20 expressing cells whereas rituximab was not. Moreover, HuMab-7D8 was able to kill rituximab resistant cells. Using a mouse model, we further demonstrated that HuMab-7D8 killed all CD20-expressing cells, while rituximab killed only the high CD20-expressing cells.

In conclusion, HuMab-7D8 or antibodies that recognize a similar epitope, such as ofatumumab, can be used as a second generation anti-CD20 mAb that may overcome CD20 expression level related resistance of rituximab.

Introduction

The Non-Hodgkin's Lymphomas (NHL) represent a heterogeneous group of lymphoid neoplasms. The prevalence has been increasing over the years and the NHLs have now become fifth in cancer incidence and mortality (1, 2). Diffuse Large B-Cell lymphoma (DLBCL) is the most common NHL, followed by Follicular Lymphoma (FL) (2-4). Since the 1970s, the best treatment option for patients with B-cell NHL consisted of various combinations of chemotherapy with or without radiotherapy (5-7). During the last decade, inclusion of the monoclonal anti-CD20 antibody rituximab (Mabthera, Rituxan, IDEC-C2B8) in the chemotherapy regimens, has significantly improved response rates, progression free survival (PFS) and overall survival (OS) rates (2, 8).

Addition of rituximab to the chemotherapy regimens in DLBCL improved the seven-year OS from 36% to 53% (9, 10). For FL, with a medium follow-up of 53 months, the OS improved from 71% to 81% by the addition of rituximab (11, 12). Moreover, PFS and OS is substantially prolonged with scheduled rituximab maintenance treatment (13, 14).
Next to hematologic cancers, rituximab has shown promising activity in autoimmune indications such as rheumatoid arthritis (RA) (15, 16).

Despite the success of rituximab, resistance towards rituximab occurs in patients who do not respond to treatment or relapse from the original disease. The mechanism of rituximab resistance may be host and/or tumor related but is still largely unknown (17, 18). Therefore, the need to study rituximab-resistance as well as the development of more potent CD20-directed immunotherapy is imperative.

Rituximab is a chimeric human-mouse monoclonal antibody (mAb) targeting the CD20 molecule and activating different effector mechanisms. Complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) are considered to be the most important effector mechanisms (18-22). In addition, induction of apoptosis and growth arrest have been observed, especially after hyper-crosslinking of CD20 (18, 19, 23). In previous experiments, we demonstrated that the CDC activity of rituximab significantly correlates with the number of CD20 molecules on the cell surface and that CDC and ADCC show an additive effect. Importantly, we also showed that low CD20 expressing cells could not be killed by rituximab (22). This may explain the poor response to rituximab of B-cell malignancies expressing low CD20 levels, such as B-cell chronic lymphocytic leukemia B-CLL (24, 25).

Recently, a panel of fully human antibodies including ofatumumab (HuMab-2F2), HuMab-2C6 and HuMab-7D8, were generated in human Ig transgenic mice. This group of human antibodies represents a group of CD20 mAbs that bind to a novel CD20 epitope including the small extracellular loop and induce exceptionally potent complement-mediated tumor cell lysis (26). Ofatumumab is currently in clinical evaluation in a phase III clinical trial for B-CLL and NHL, and in a phase II clinical trial for RA. HuMab-7D8 differs from rituximab in two ways. Firstly, HuMab-7D8 binds to this alternative and unique epitope which is located N-terminally from the rituximab binding site. Secondly, HuMab-7D8 has a slower off-rate (27). These differences may explain the superior activity of HuMab-7D8 in inducing CDC.

In this report, we investigated whether HuMab-7D8 could overcome the rituximab CD20 expression level-related resistance by comparing the activity of rituximab and HuMab-7D8 *in vitro* and *in vivo* using clonally related CD20 transgenic T cells. HuMab-7D8 shows a higher capacity to kill the low CD20 expressing cells than rituximab. In addition, we observed that rituximab-resistant cells are still sensitive to HuMab-7D8. We further demonstrate in a mouse model that rituximab did not kill the low CD20-expressing cells, while HuMab-7D8 was able to kill all CD20-expressing cells.

Materials and Methods

Generation of CD20-positive CEM cells and CD20-positive + lucR-IRES-eGFP-positive CEM cells.

Clonally-related CEM-CD20 cells were generated as previously described (22). The Moloney Murine-Leukemia virus based vector (pMX) was constructed by digesting the pCBR-Control vector (Promega Corporation, Madison Wi, USA) with *Bgl* II and *Xba* I endonuclease restriction enzymes, releasing the red luciferase (LucR) fragment. Subsequently, the pMX-IRES-eGFP vector was digested with *BamH* I and *Not* I to create the insertion space for the LucR fragment. Next, the *Xba* I site of the LucR fragment and the *Not* I site of the viral backbone were blunted and subsequently the LucR fragment was ligated into the retroviral backbone.

Generation of viral supernatant and transduction of CEM-CD20 cells was performed as previously described (22). CEM-CD20-LucR-IRES-eGFP cells were purified with a fluorescence-activated cell sorter (FACS) (FACSAria, Becton Dickinson, Mountain View,

CA) based on eGFP expression. *In vitro* luciferase expression was determined with a luminometer (EG&G Berthold, Lumat LB 5507) by lysing 0.1 x 10^6 cells with 100 µl lysing solution and adding 100 µl of luciferase substrate according to the manufacturer's protocol (Promega Corporation.)

Cell culture and isolation of PBMCs

The CEM T cells were cultured in culture medium consisting of RPMI (Gibco-BRL, Paisly, Scotland) supplemented with 10% fetal calf serum (FCS, Integro, Zaandam, the Netherlands), penicillin (100 U/ml), streptomycin (100 µg/ml) (Gibco-BRL), and 5 x 10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany).

PBMCs were isolated by density centrifugation through Ficoll (Amersham Pharmacia, Uppsala, Sweden) and stimulated with 300 U/ml human recombinant interleukin 2 (rehIL-2) (Proleukin, Chiron, Amsterdam, the Netherlands) and interleukin 12 (IL-12) (Peprotech, London, United Kingdom) for 24 hrs. All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Flowcytometric analysis and anti-CD20 mediated cytotoxicity assays

Expression of CD20 and eGFP was determined by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA). Antibodies used for staining were anti-CD20-phycoerythrin (PE) / allophycocyanin (APC), anti-CD7-fluorescein isothiocyanate (FITC) (BD Biosciences, San Jose, CA) (BD Biosciences, San Jose, CA), rituximab was obtained from Roche (Basel, Switzerland) and HuMab-7D8 was a gift from Genmab B.V. (Utrecht, The Netherlands). Goat F(ab')2 anti-human-IgG-FITC/PE was obtained from Southern Biotech (Birmingham, Al, USA). All anti-CD20 mediated cytotoxicity assays were performed as previously described (22, 28). Based on optimization assays 10 µg/ml of anti-CD20 mAb and 20% human serum was used. Cells were incubated for 30 minutes at 37°C.

To study CDC-mediated antibody resistance CD20-positive cells were treated with rituximab or HuMab-7D8 in the presence of human complement for 1 day at 37°C. The next day human serum was washed away and the cells were cultured for 7-14 days. Every 2 days antibody ligation was checked by staining the cells with Goat-anti-Human-FITC. After 14 days, when no antibody was detected, cells were subjected to a second round of antibody-induced cell kill, either by rituximab or by HuMab-7D8. Cell kill was analyzed by propidium iodide staining as previously described (22, 28).

Measurement of raft-associated antigen by Triton X-100 insolubility.

To study the presence of CD20 in cholesterol-rich microdomains before and after antibody ligation a rapid flow cytometry method based on Triton X-100 insolubility was performed at low temperatures, as described previously (26). Briefly, cells were washed in PBS and resuspended at 2.5 x 10^6 cells/ml. Cells were incubated with 10 μg/ml of anti-CD20 antibody (rituximab or HuMab-7D8) or control anti-CD7-FITC mAb (Becton Dickinson, Mountain View, CA) for 15 minutes at 37°C. Next, the samples were washed in cold PBS and then divided in half. One half was kept on ice and was employed to determine the 100% surface CD20 expression. The other half was treated with 0.5% Triton X-100 (Riedel-de Haen, Seelze, Germany) for 15 minutes on ice to determine the proportion of antigens remaining in the Triton X-100 insoluble fraction. Cell fragments were spun down to obtain the pellet containing the lipid rafts. Both samples were simultaneously stained with anti-human IgG-FITC. The mean fluorescence intensity (MFI) for both samples was determined by flow cytometry as described above.

Mice, conditioning regimen and transplantation

$RAG2^{-/-}\gamma c^{-/-}$ mice were obtained from the Netherlands Cancer Institute (Amsterdam, The Netherlands). Mice were bred and housed in the specified pathogen-free (SPF) breeding unit of the Central Animal Facility of the University of Utrecht. The animals were supplied with autoclaved sterilized food pellets and distilled water ad libitum. All animal experiments were conducted according to Institutional Guidelines after acquiring permission from the local Ethical Committee for Animal Experimentation and in accordance with current Dutch laws on Animal Experiments.

Mice were used at 8 to 20 weeks of age. On day 0 all mice received total body irradiation with a single dose of 300 cGy (TBI, 3.0 Gy X-rays). On day 1, cell suspensions containing 4.0 x 10^6 CEM-CD20-LucR-IRES-eGFP cells in 0.25 ml of PBS/0.1%BSA (GIBCO-BRL) were intravenously (iv) injected into the lateral tail vein. Within one hour, PBS (control) or different doses of anti-CD20 antibody were administrated intraperitoneally (ip).

Bioluminescent Imaging (BLI)

Mice were monitored for luciferase expression twice a week using a cooled charge-coupled device (CCCD) camera (Roper Scientific, Princeton instrument, Trenton, NJ, USA). Mice were anaesthetized by intramuscular injection of Ketamine/Xylazin/Atropine (ratio 8:7:1, 35 μl). Subsequently, 100 μl of D-luciferin (7.5 mM) (Synchem, Kessel, Germany) was injected ip and the ventral side of the mice was imaged for 10 minutes inside a light-tight chamber. Light emission was quantified by using MetaVue and MetaMorph software

(Universal Imaging Corporation, Downingtown, PA, USA). Blood was collected once a week to determine the serum levels of human IgG1. Diseased and paralyzed mice were sacrificed and bone marrow (BM) was collected. Cells were harvested from the BM and the CD20 expression level was detected after culture for at least 14 days by flow cytometry.

Determination of human IgG concentration

Human IgG concentrations in mouse plasma were determined using a sandwich ELISA. Mouse mAb anti-human IgG clone MH16-1 (#M1268, CLB Sanquin, The Netherlands) was coated onto 96-well Microlon ELISA plates (Greiner, Germany) at a density of 200 ng/well. After blocking plates with PBS supplemented with 2% chicken serum (Invitrogen, Groningen, The Netherlands) and subsequent washing, samples were added, serially diluted in ELISA buffer (PBS supplemented with 0.05% Tween 20 (Sigma Aldrich, Zwijndrecht, The Netherlands) and 2% chicken serum), and incubated on a plate shaker for 1 h at room temperature. Serial dilutions of injected mAb preparations were used as reference. After washing, the plates were incubated with peroxidase-labeled F(ab')2 fragments of goat anti-human IgG immunoglobulin, Fcγ fragment specific (#109-035-098, Jackson, West Grace, PA), 1:5000 diluted in PBS. Plates were subsequently developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Roche, Mannheim, Germany). Absorbance was measured in a microplate reader (Biotek, Winooski, VT) at 405 nm.

Statistical analysis

Where indicated, the mean values and standard deviation (SD) were calculated. The significance of differences between rituximab and HuMab-7D8 was determined by Student's *t*-test. A p-value of < 0.05 was considered significant. All statistics were analyzed using GraphPad Prism software (version 4.0).

Results

Rituximab- and HuMab-7D8 binding to CEM-CD20 cells and induction of CDC

Recently, fully human anti-CD20 mAbs (ofatumumab, HuMab-7D8 and HuMab-2C6) were generated in human Ig-transgenic mice. These mAbs were shown to bind to a novel CD20 epitope including the small extracellular loop, and to induce an exceptionally potent CDC (26, 27). To compare the *in vitro* effectiveness of rituximab and HuMab-7D8 we first tested the binding pattern of both antibodies on transgenic CEM-CD20 cells. Figure 1A shows

that, after staining with a FITC-conjugated anti-human IgG1 antibody, both antibodies bound with the same specificity to the transgenic CEM-CD20 cells.
CEM-CD20 cells were lysed by incubating the cells with antibody in the presence of normal human serum as complement source. For rituximab, maximum cell lysis occurred at a minimum concentration of 10 μg/ml, while for HuMab-7D8 maximum lysis was observed already at a concentration of 500 ng/ml (data not shown). Both antibodies induce CDC very rapidly, within 5 minutes at 37°C. Twenty percent normal human serum was sufficient to obtain maximum CDC (data not shown). Based on these results we used 10 μg/ml of antibody, 20% human serum and incubation periods of 30 minutes at 37°C to standardize the experiments.

Next, the influence of the CD20 expression level on CDC was studied. For this we used clonally-related transgenic CEM-CD20 clones. These CEM-CD20 clones are not complicated by the different surface densities of complement regulatory proteins (CD46, CD55, CD59 (22)). Thus, the only variable parameter between these clones is the CD20 expression level. For 20 clones the absolute number of CD20 molecules per cell was determined and subsequently the clones were subjected to rituximab- and HuMab-7D8-induced CDC in separate experiments (figure 1B). For HuMab-7D8 only 150,000 molecules/cell were required to induce maximum cell lysis. In contrast, rituximab required at least 500,000 CD20 molecules/cell. A paired t-test demonstrated significant more efficient cell kill for each of the clones with HuMab-7D8 in comparison to rituximab ($p = 0.0013$).

Efficient lipid raft formation for both rituximab and HuMab-7D8
Both antibodies are able to translocate the CD20 antigen into cholesterol rich microdomains, the so-called lipid rafts (26). Efficient lipid raft formation is required for induction of CDC (29). We addressed the question whether the higher capacity to mediate CDC of low CD20-expressing cells was related to a superior capacity of HuMab-7D8 to induce lipid raft formation compared to rituximab. To this end, we determined the induction of lipid raft formation by both antibodies using different clones showing different CD20 expression levels. Figure 1C shows that both antibodies are capable of efficiently translocating the CD20 molecules into lipid microdomains, independent of the CD20 expression level. At the same time, HuMab-7D8 is more effective than rituximab in mediating CDC of CEM clones with a low surface density of CD20 (figure 1D). These data suggest that factors other than efficient translocation of CD20 molecules into lipid rich microdomains are responsible for the poor lysis of low CD20-expressing cells by rituximab.

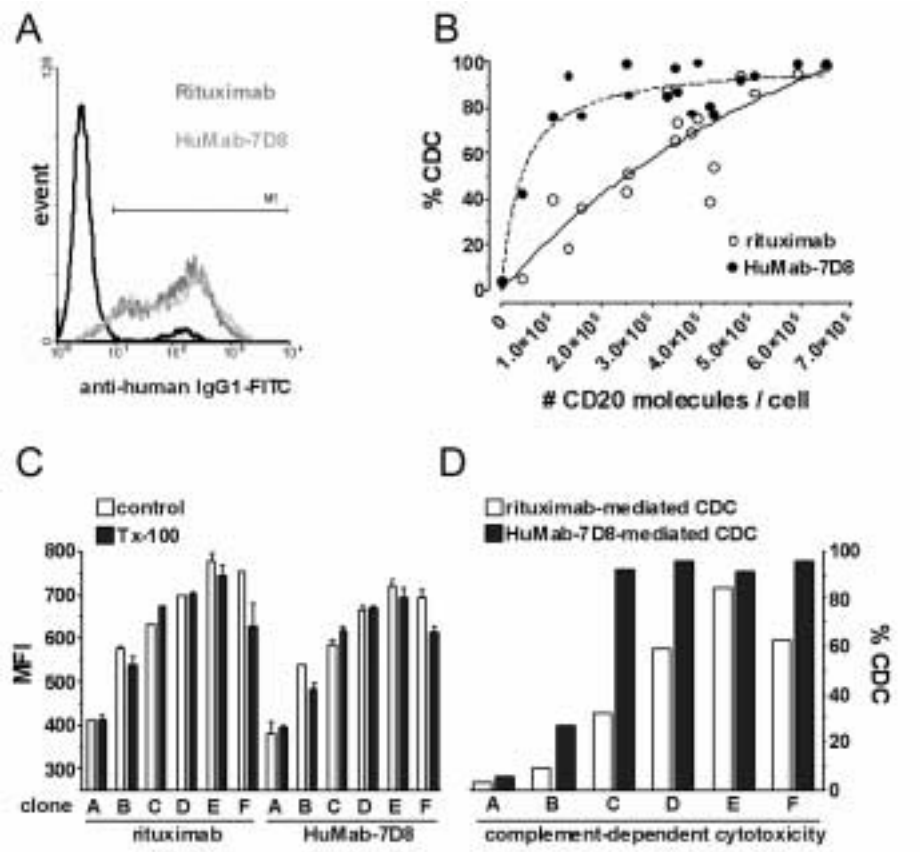

Figure 1. Antibody-mediated CDC

(A) FACS-analysis of the binding capacity of HuMab-7D8 (light gray) and rituximab (dark gray) on transgenic CEM-CD20 cells. (B) Individual CEM-CD20 clones were subjected to rituximab- and HuMab-7D8-induced CDC in separate experiments. The absolute numbers of CD20 molecules of those clones were determined as well and plotted against the extent of CDC. The open dots represent rituximab-mediated CDC and the closed dots HuMab-7D8. (C) CEM-CD20 clones (A-F) with increasing MFI were incubated with 10 μg/ml of CD20 antibody and the samples were divided in half. The cells were directly stained with anti-IgG1-FITC (open bars) or cells were first treated with Triton X-100 (black bars), spun down and the pellets were then stained with anti-IgG1-FITC. (D) The open bars show the percentage CDC of the clones in the presence of rituximab and serum, as described previously. The black bars show the HuMab-7D8 induced CDC. All experiments are performed in duplicate

Rituximab- and HuMab-7D8-induced CDC and ADCC

We recently demonstrated that rituximab induced CDC and ADCC act complementary in the lysis of tumor cells (22). This is very important because tumor-related resistance (CDC)

may be overcome by host-related effector mechanisms (ADCC). Therefore, we tested the capacity of both antibodies to mediate ADCC and the combination of ADCC and CDC. In figure 2, three different sets of CEM-CD20 clones, each expressing distinct CD20 levels, were used to investigate the contribution of CDC and ADCC in CD20 mAb-induced cell lysis. Four independent experiments were performed in which different donor effector cells and different sources of human complement were used. No effect of HuMab-7D8 or rituximab on cell lysis of CEM-CD20 cells after 4, 24, 48 or 72 hours in the absence of complement was observed, indicating that neither one of the antibodies induced apoptosis (data not shown). Both antibodies were equally effective in inducing ADCC of cells showing low CD20-expression levels ($p = 0.4901$). No correlation between ADCC and the CD20 expression level was observed for both antibodies (rituximab; $p = 0.5986$, $r = 0.1694$, HuMab-7D8; $p = 0.2424$, $r^2 = 0.3657$) (figure 2A). Thus, for rituximab and HuMab-7D8 maximum ADCC was already achieved at the lowest CD20-expression levels analyzed, corroborating previous evidence (22).

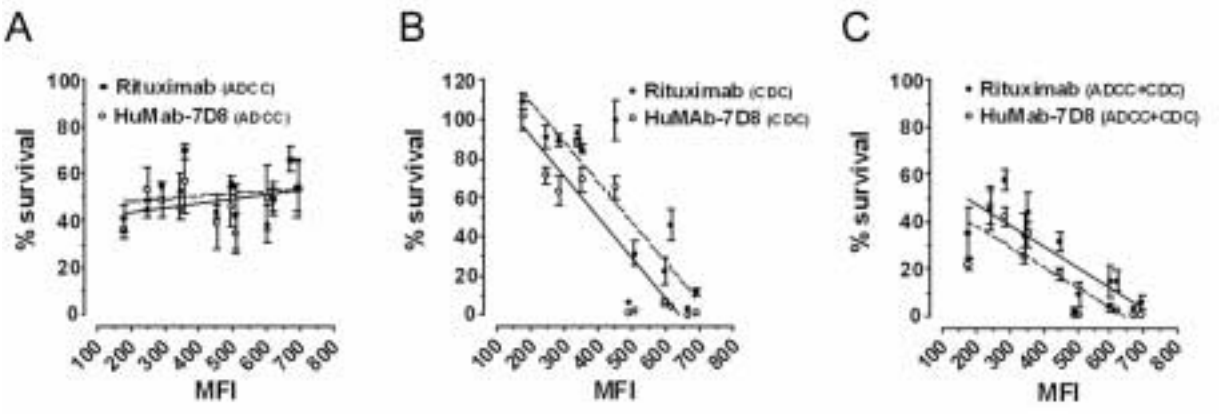

Figure 2. Antibody-mediated CDC and ADCC.

12 CEM-CD20 clones were used that together covered cells with a wide spectrum of CD20 expression levels. 10 μg of rituximab was used in a 4 hr assay as previously described (22, 28). All experiments were performed in triplicate and the mean values ± SD are shown. (A) For anti-CD20-mediated ADCC, PBMC were isolated from healthy donors and stimulated over night with IL-2 and IL-12. Next, these cells were added to the clones at an effector:target ratio 10:1 in the presence of 10 μg/ml antibody. (B) Clones were incubated in 20% normal human serum as complement source and 10 μg/ml antibody to induce CDC. (C) Effector cells, complement and antibody were added to the clones as described above.

However, this was not the case for induction of CDC. Figure 2B shows that in comparison to rituximab HuMab-7D8 is again significantly more efficient in lysis of cells with low CD20 expression levels ($p = 0.0005$). For HuMab-7D8 as well as for rituximab, the CDC-activity depends on the CD20 expression level (rituximab; $p = 0.0003$, $r = 0.8601$, HuMab-7D8; $p = < 0.0001$, $r = 0.8999$).

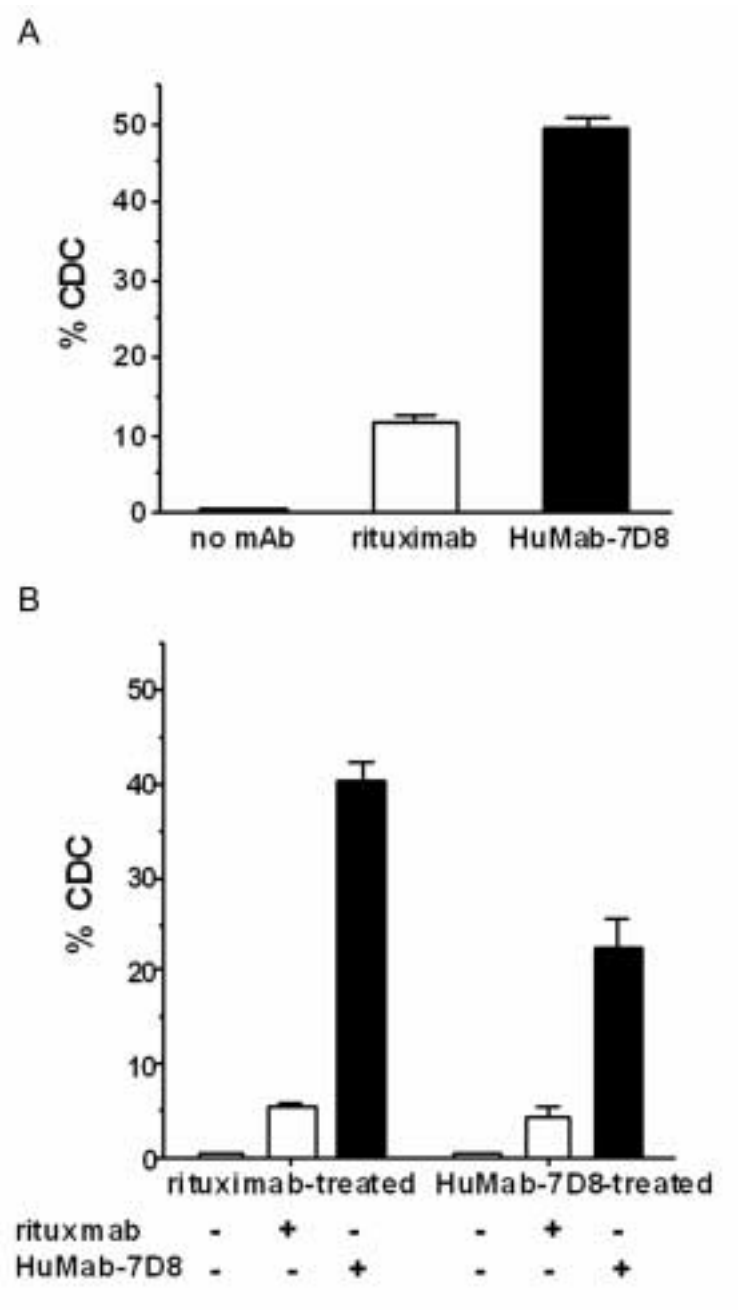

Figure 3. Rituximab- and HuMab-7D8-mediated CDC of rituximab- and HuMab-7D8-resistant cells.
(A) Anti-CD20-induced CDC of a CEM-CD20 clone with low CD20-expression by rituximab and HuMab-7D8 as described in the materials and methods. The bars indicate the mean ± SD of experiments performed in triplicate. (B) After two weeks of culture, the rituximab-resistant cells from the first experiment were subjected to CDC induction using either rituximab (open bars) or HuMab-7D8 (black bars). Conversely, the HuMab-7D8-surviving cells from the first experiment were subjected to CDC induction using either rituximab (open bars) or HuMab-7D8 (black bars).

Importantly, for both anti-CD20 antibodies, combining both effector mechanisms (CDC and ADCC) led to an enhanced cell kill, especially for CEM cells expressing low CD20 levels (Figure 2C). Rituximab- and HuMab-7D8-induced cell kill was significantly increased when using both CDC and ADCC compared to cell kill induced by either CDC or ADCC alone ($p < 0.0001$). Combining CDC and ADCC, HuMab-7D8 is more potent in inducing cell kill compared to rituximab ($p = 0.007$), resulting in an overall better killing of CEM-CD20 cells using HuMab-7D8 (Figure 2C). As for CDC, the combined effectiveness of CDC and ADCC significantly depends on CD20-expression level (rituximab; $p = 0.0008$, $r = 0.8307$, HuMab-7D8; $p = 0.0006$, $r = 0.8389$).

Overcoming antibody-resistance

We next addressed the question whether rituximab-resistant cells were still sensitive to HuMab-7D8 and vice versa. Since the difference in cytotoxic capacity between rituximab and HuMab-7D8 is most prominent in CDC, we used this assay for read out. In parallel experiments, low CD20-expressing CEM-CD20 cells were subjected to either rituximab- or HuMab-7D8-induced CDC, respectively. The extent of cell death was determined (10% for rituximab versus 50% for HuMab-7D8, figure 3A) and the surviving cells were kept in culture for 14 days. To check for remaining antibody on the cell surface cells were regularly stained with anti-human IgG1-PE antibody. After 14 days no antibody was detected on the cells any more (data not shown). Of note, the HuMab-7D8-surviving cells showed lower CD20 expression profiles compared to rituximab-surviving cells (data not shown), indicating that rituximab could not lyse cells expressing CD20 beneath a certain amount. The rituximab- and HuMab-7D8-resistant cells were then subjected to a second round of CDC by incubating the cells with either one of the CD20 antibodies in the presence of complement. A second incubation with rituximab resulted in only 5% cell lysis of rituximab-resistant cells. Interestingly, incubation of the rituximab-resistant cells with HuMab-7D8 resulted in 40% cell death, indicating that rituximab-resistant cells remained sensitive to lysis by the fully human antibody. A second incubation of HuMab-7D8-resistant cells with HuMab-7D8 still led to 25% cell death. In contrast, hardly any cell kill was found (3%) upon incubation of the HuMab-7D8-resistant cells with rituximab. These data indicate a variable sensitivity or resistance of the CEM-CD20 cells to the anti-CD20 antibodies. As a control experiment, no CDC was observed with heat-inactivated serum (data not shown), indicating that culture in the continuous presence of antibodies does not induce apoptosis. Combining both HuMab-7D8 and rituximab at the same time led to a decreased cell killing as compared to HuMab-7D8 alone. This is probably due to the

competition of HuMab-7D8 and rituximab binding to CD20 due to steric hinderance in trying to bind to the antigen (data not shown). Similar results were obtained using other CEM-CD20 clones with different CD20 expression levels using different normal human serum concentrations (data not shown).

Comparison of the in vivo effectiveness of rituximab and HuMab-7D8

To study the *in vivo* effectiveness of both antibodies, we developed a mouse model in which we can trace intravenously (iv) injected luciferase-tagged CEM-CD20 cells by monitoring with bioluminescence imaging. For this we used the RAG2$^{-/-}\gamma c^{-/-}$ mouse model, in which human cells can efficiently engraft (30-33). To determine whether CEM T cells could survive in these mice, mice were subjected to total body irradiation with a sublethal dose of 350 cGy, and cells were titrated (1.0 x 10^6 – 15.0 x 10^6 cells) by iv injection into the tail vein. A dose of 4.0 x 10^6 cells was found to paralyze the hind legs by compression of the cervical spine after two to three weeks. Collection of blood samples revealed the presence of CEM T cells (data not shown).

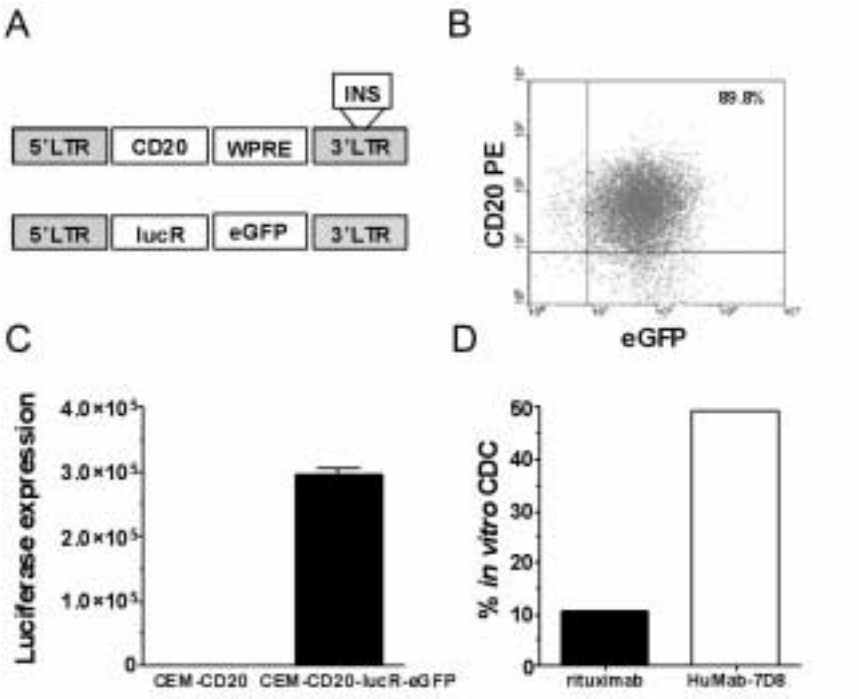

Figure 4. Preparation of luciferase expressing CEM-CD20 cells.

(A) Schematic representation of CD20- and LucR-encoding retroviral vectors. (B) FACS analysis of transduced and purified LucR-tagged CEM-CD20 cells. (C) *In vitro* luciferase activity of LucR-tagged CEM-CD20 cells and control CEM-CD20 cells. (D) *In vitro* CDC assay of LucR-CEM-CD20 cells prior to *in vivo* use.

For *in vivo* imaging of the cells, a retroviral vector was constructed encoding both LucR and eGFP. To obtain CD20-positive and LucR-positive cells, CEM cells were transduced with the CD20-WPRE-INS (28) vector and the LucR-IRES-eGFP vector (figure 3A). The CD20-WPRE-INS retroviral vector was selected because of stable CD20-expression *in vitro* (28). The CD20 and eGFP positive cells were purified by FACS sorting (90% double positive, figure 4B) and luciferase expression was verified (figure 4C). The *in vitro* sensitivity for rituximab- and HuMab-7D8-induced CDC was determined before injection into the mice. For rituximab this was 10% and for HuMab-7D8 50% cell lysis was observed (figure 4D).

At day 1 mice were injected iv with CEM-CD20-LucR cells and ip with 50 µg of antibody / 250 µl of PBS, or with 250 µl PBS alone (control group). Once a week human IgG levels were determined on peripheral blood samples of the mice. Mice lacking human IgG in the serum were excluded from the analyses. Bioluminescent imaging was performed twice a week. The first luciferase signal above background (10^5) was found in the femurs of the mice in the control group at day 7. No bioluminescence signal was detected for both the rituximab- or HuMab-7D8-treated mice at that time point (Figure 5B). Over the next 10 days the signal increased rapidly and became visible in the abdomen and the cervical spine. At day 20, the mice of the control group suffered from hind limb paralysis and were sacrificed (Figure 5A and B). At that same day, the first signal became visible in the treatment groups. The first luciferase signal became visible in the femurs and in time also in the abdomen (soft tissues, e.g. liver and spleen) and cervical spine. At day 40, the mice were sacrificed because of hind limb paralysis. Thus, treatment of the mice with rituximab and HuMab-7D8 prolonged the median survival of the mice from 20 to 40 days (Figure 5D) compared to the control group. However, we found no significant difference in *in vivo* treatment of mice between rituximab and HuMab-7D8. Specific luciferase expression analyses on different parts of the mice revealed that both antibodies were more effective at the site of the abdomen than at the site of the femurs. The relatively low luciferase signal in the femurs (maximally 10 times the background of 10^5) precludes statistical confirmation (data not shown). Mice treated with 5 µg of antibody gave similar results. Antibody treatment at day 3 instead of day 1 also delayed the outgrowth of the cells compared to the non-treated mice. This may suggest that the antibodies do not affect the homing of the cells, but that they eliminate CEM-CD20 cells *in situ*.

After sacrifice, cells were harvested from the bone marrow and cultured for at least two weeks in normal culture medium to eliminate mouse cells. Next, the CD20 expression on

the remaining CEM-CD20 cells was analyzed and no HuMab-7D8 or rituximab was present on the cell surface at that time (data not shown). Cells harvested from the control mice showed similar CD20 expression levels as the original cells that were injected into the mice (figure 6). Cells harvested from mice treated with rituximab still expressed CD20, but at a low level when compared to the original cells. Surprisingly, mice injected with HuMab-7D8 did not exhibit CD20-expression at all. Together, these data suggest that low CD20-expressors escape CDC-mediated lysis by rituximab *in vivo*, while they are efficiently eliminated by HuMab-7D8.

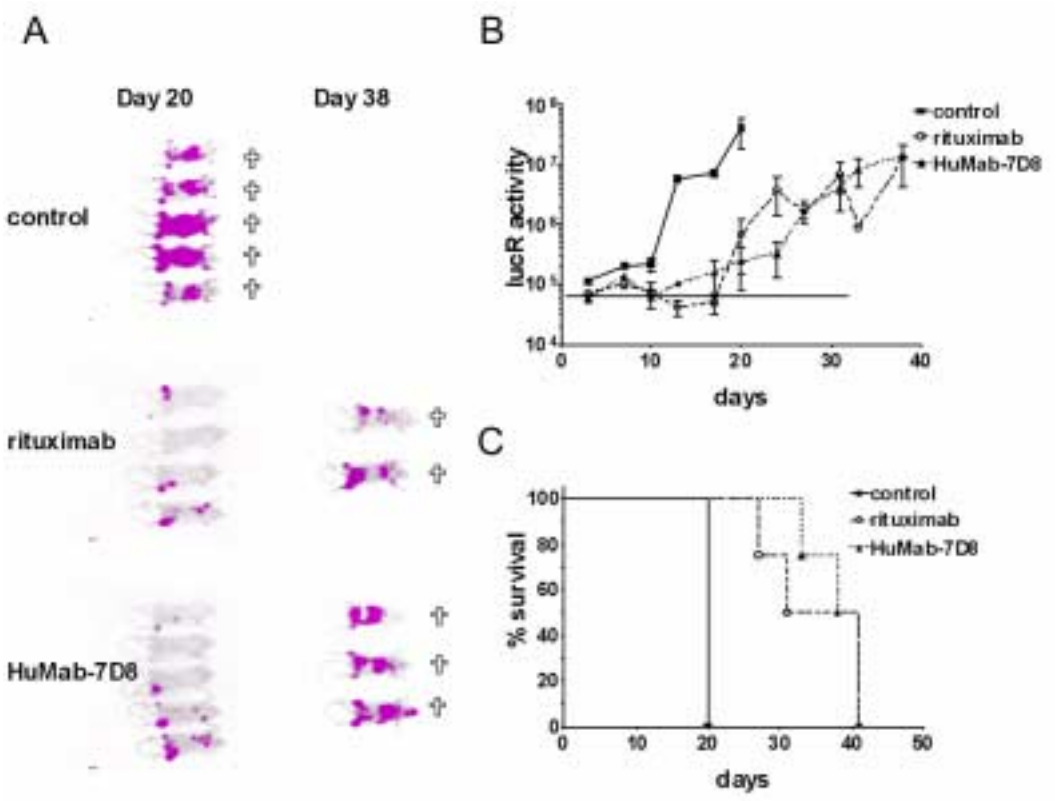

Figure 5. *In vivo* luciferase imaging

(A) *In vivo* imaging at day 20 and day 38 of mice inoculated with LucR-CEM-CD20 cells as described in the materials and methods. Mice were treated either with PBS, rituximab or HuMab-7D8. ✞ Indicates that the mice were sacrified. (B) Real time luciferase activity. (C) Survival curve of mice inoculated with LucR-CEM-CD20 cells treated with PBS (control), rituximab or HuMab-7D8.

Discussion

In this report, we compared the human anti-CD20 antibody HuMab-7D8 with rituximab for its ability to overcome resistance to CD20 antibody therapy in relation to the CD20 expression level of cells. We first demonstrated *in vitro* that compared to rituximab HuMab-7D8 is significantly stronger in killing CD20-transgenic T cells. No significant difference was found in ADCC when the two antibodies were compared, but HuMab-7D8 has improved CDC-activating ability compared to rituximab.

HuMab-7D8 and rituximab both contain human IgG1 constant regions and have the same inherent C1q binding capacity when C1q is deposited on a plastic surface (26). It is, however, not completely understood why the human antibody is more efficient in inducing CDC than rituximab. HuMab-7D8, together with the clinically used ofatumumab, belong to a group of human antibodies that bind to a distinct epitope of the CD20 molecule compared with other mAbs (27). HuMab-7D8 binds to a small 7-mer loop of the CD20 molecule and not to A170xP172-site in the large 44-mer loop that harbours the binding site of rituximab.

Three possible suggestions have been made why HuMab-7D8 acts superior compared to rituximab (27). First, there is superior binding of the human mAb, resulting in a slower off-rate. This suggestion is probably less important because CDC occurs in minutes and the antibody off-rate occurs in hours to days. Another fact that makes the off-rate less important was the creation of the human antibody HuMab-2C6 (IgG1-2C6) by class switching of a human anti-CD20 IgM antibody (27). This human antibody has a faster off-rate than rituximab, but still has a much better capacity to activate complement.

A second mechanism might involve the translocation of CD20 into lipid microdomains. Translocation of the CD20 molecule into lipid rafts is crucial for activation of the complement system (19, 29). However, we demonstrated that efficient raft formation is independent of the number of molecules expressed on the cell surface. Both antibodies are equally efficient in inducing translocation of CD20 into lipid microdomains of cells expressing lower numbers of CD20 molecules. Thus, we conclude that the lower activity of rituximab at low CD20 expression levels is not due to inefficient raft formation.

The third possible explanation is the proximity of the antibody to the membrane. Binding of an antibody to the 7-mer loop probably results in a more proximal localization to the membrane as compared to binding to the large 44-mer loop. This antibody location or

orientation could give rise to a more favorable IgG:IgG interaction or a more proximal membrane positioning of the antibody Fc region. All this could lead to more efficient C1q activation. Complement components are active shortly. Therefore, the close mAb Fc position to the membrane could be important for a rapid and efficient complement-mediated lysis (34-36). This might explain why HuMab-7D8 needs less CD20 molecules than rituximab for induction of cell death and is capable of killing rituximab-resistant cells.

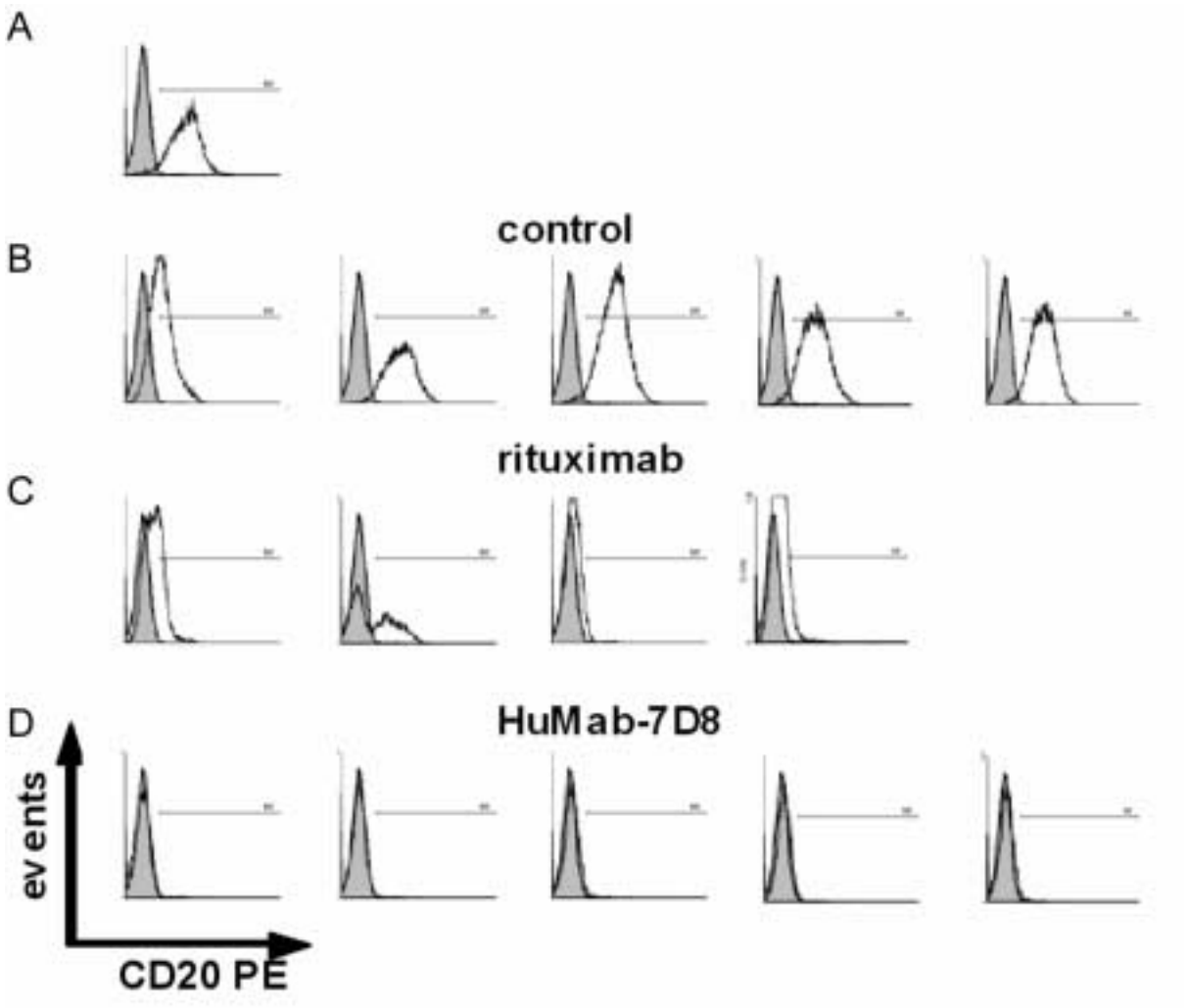

Figure 6. CD20 expression of CEM cells harvested from the bone marrow.
FACS-analyses of CEM cells harvested from BM and cultured for two weeks. Each graph represent cells from an individual mouse. The gray plots represent the non-transduced CEM cells. (A) CD20 expression of a pool of retrovirally CD20 transduced CEM cells that were injected into the mice and kept in culture during the experiment. (B) CD20 expression of CEM cells retrieved from the PBS-treated mice. (C) CD20 expression of CEM cells retrieved from the rituximab-treated group and (D) CD20 expression of CEM cells retrieved from the HuMab-7D8-treated mice.

Next, we found that rituximab-resistant cells remain sensitive to HuMab-7D8-mediated lysis in the presence of complement. Interestingly, rituximab-treated cells were still partly sensitive to re-treatment with rituximab after two weeks of culture. The same phenomenon was seen with HuMab-7D8-treated cells. This is in concordance with the clinical data presented by other groups (13, 14). Rituximab maintenance or re-treatment prolongs survival of patients with (relapsed) B-cell follicular NHL (14, 37). In the patients, however, after the first courses of rituximab, some cells are not ligated by rituximab and the cells that are being killed by maintenance or re-treatment may have experienced their first contact with rituximab. Resistance to rituximab is currently being studied by many investigators and different mechanisms of resistance have been proposed (18, 22, 38-40). From these data and the maintenance therapy we may conclude that there is also a 'transient' resistance. Complement concentration and incubation time were not the limiting factors because increasing both parameters did not improve the cell lysis induced. It is not clear why some cells are 'transiently' insensitive to rituximab or HuMab-7D8. It could be due to transient down regulation of the CD20 molecule or up-regulation of the complement regulatory proteins (CRP) CD46, CD55 or CD59. Notably, re-introducing HuMab-7D8 to rituximab-treated cells leads to improved cell killing.

The *in vivo* studies we conducted, showed similar results. The $RAG2^{-/-}\gamma c^{-/-}$ mice lack B, T and NK cells, but have an active complement system as well as monocytes and macrophages (31). Administration of either rituximab or HuMab-7D8 resulted in a significant increased survival. The mechanism involved in the delayed outgrowth is probably the complement system. Golay et al. nicely demonstrated that complement is the most important effector mechanism *in vivo* for elimination of human CD20-expressing murine lymphoma cells. Depletion of complement by cobra venom factor led to the complete abolishment of the therapeutic effect of rituximab (41). Depletion of NK cells or macrophages did not affect the therapeutic effect of rituximab. It should be noted that the mice still had macrophages. The Kuppfer cells could well be responsible for the delay in outgrowth of CD20-positive cells in the liver compared to the bone marrow. In addition, FcR cross-linking could enhance apoptosis (42). However, we did not find any significant direct effect of rituximab or HuMab-7D8 on the transgenic CD20 cells *in vitro* in the absence of complement, even after hyper-crosslinking (data not shown), which also confirms previous reports (41).
Golay and co-workers were able to kill all human CD20-positive cells with rituximab in their mice leading to 100% survival. We demonstrated an increased time to disease progression of the treated mice, but we were not able to prevent outgrowth of the tumor

cells in the mice. The differences between the two studies were that they used a murine lymphoma cell line which was 100% CD20-positive and used a single dose of 250 μg of rituximab. We used a single dose of 50 μg of rituximab and injected a human CEM cell population, which contained 7.5% CD20-negative cells. In addition, the CD20 expression level was chosen to be rather low as this gave us the opportunity to analyze the CD20 expression level of the cells that had survived treatment in the sacrificed mice. Surviving cells were found in the bone marrow, liver, spleen and lymph nodes. At the end stage of the disease (hind limb paralysis) cells were also found in the peripheral blood. Analysis of harvested cells from the bone marrow and liver showed some interesting differences between rituximab-treated and HuMab-7D8-treated mice. Cells from the rituximab-treated mice still expressed CD20, albeit at a low level, while cells from the HuMab-7D8-treated mice were entirely CD20-negative. From these data we can conclude that HuMab-7D8 is able to kill all CD20-positive cells while rituximab only kills the high density CD20-positive cells. Since the injected cell population still contained 7.5% CD20-negative cells, it is likely that these cells survived HuMab-7D8-treatment. Alternatively, CD20 may have been lost by transient down-regulation of CD20 expression or by shaving of the antibody-CD20 complex by monocytes (43, 44). It is unknown whether there is a difference between the effect on shaving by HuMab-7D8-CD20 or rituximab-CD20 complexes.
Complement is being consumed during rituximab administration to patients and may be the limiting factor for efficient therapy in some B-cell malignancies (45, 46). CDC is one of the main mechanisms of action for the antibodies in these mice. Future experiments will address whether HuMab-7D8- and rituximab-treatment leads to similar levels of depletion of complement.

Summarizing, HuMab-7D8 is able to efficiently kill low CD20-expressing cells, and fully human anti-CD20 antibodies seem good candidates to overcome the CD20 expression level-related resistance to rituximab, either as a first line treatment or as a re-treatment / maintenance treatment strategy after rituximab relapse. Another advantage of HuMab-7D8 is the slower off-rate, which may make it an ideal antibody to carry radioconjugates. Human anti-CD20 mAbs recognizing a unique epitope on the small loop of the CD20 antigen are potent inducers of CDC, and the clinical candidate ofatumumab is already successfully being used in phase I/II trials and has proven to be safe (47-50). In conclusion, HuMab-7D8, or its clinical counterpart ofatumumab, are promising second generation anti-CD20 mAbs in the fight against B-cell malignancies.

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Chapter 7

Discussion

The human CD20 molecule plays a central role in this thesis. In the first part, CD20 is proposed to act as a safety switch for the adoptive transfer of T cells in the allogeneic stem cell transplantation (allo-SCT) setting. Different retroviral vectors have been developed encoding the CD20 molecule. Inclusion of cis-acting elements showed that we obtained long-term, homogeneous and stable CD20 expression. CD20 positive transgenic cells could be purified and subsequently killed by rituximab. Importantly, CD20-expressing T cells were not functionally altered by CD20 expression.

In the second part of this thesis, the mechanisms of action of the anti-CD20 monoclonal antibody rituximab were studied. By using clonally-derived CD20 transgenic T cells we demonstrated that, for complement-dependent cytotoxicity (CDC), the CD20 expression level determined the efficacy of rituximab. The CD20 expression level did not determine the cell kill mediated by antibody-dependent cellular cytotoxicity (ADCC). CDC and ADCC act complementary by increasing the rituximab-mediated cell kill. CDC-resistant cells can be killed by rituximab-induced ADCC and vice versa. Finally, we demonstrated that CD20 expression level-related resistance can be overcome by a new human CD20 monoclonal antibody, HuMab-7D8.

Several questions came forward from the findings in this thesis, and will be summarized and discussed in this chapter.

Can human CD20 be used as a safety switch?

In Chapters 2-4, the CD20 molecule is proposed as a new and improved safety switch to overcome the limitations of the Herpes Simplex Virus thymidine kinase (HSVtk)-ganciclovir system. The major limitations of HSVtk are the immunogenicity in patients, leading to premature elimination of HSVtk transduced cells (1-3) and the fact that ganciclovir is active in dividing cells only (4).
CD20 is of human origin and is therefore non-immunogenic, while rituximab eliminates dividing and non-dividing $CD20^+$ cells *in vivo*. For CD20 purification, clinical grade reagents are readily available. In addition to its suicide properties, it was proposed that CD20 might be used as a selection marker, so no other (marker) genes would be needed within the retroviral construct (5, 6).

One theoretical complication of anti-CD20 therapy could be immune deficiency. In case of Graft versus Host Disease (GvHD), elimination of CD20 positive transgenic T cells by rituximab will also lead to B cell depletion. In general, B cell depletion does not lead to decreased immunoglobulin concentration in the sera of patients for at least six months (7). The immunoglobulin (Ig) producing plasma cells are spared because they do not express the CD20 molecule on the cell membrane. However, this has yet to be found out for the setting of allo-SCT, as it might be problematic in the early engraftment stage, where B cells start to build up the new adaptive host immunity.
Rituximab has also been used in pediatric patients with autoimmune disease. These very young patients, who have also developing immune systems, tolerate a relatively long-lasting reduction of B cell numbers (8). However, there are a few reports concerning opportunistic infections in pediatric patients treated with rituximab (9, 10). This was attributed to neutropenia caused by the rituximab-antigen complexes to neutrophil Fc receptors or by the delayed immune-mediated secondary transient production of auto-antibodies during the acquisition of a new immune system (11). Not only in autoimmune disease neutropenia is a side effect, also in 4.2% of adult patients treated for NHL (12). Long term clinical follow-up studies will point out the effect of long term B cell depletion in rituximab treatment, especially in pediatric patients.

On the other hand, considering preliminary clinical data, elimination of B cells together with the alloreactive T cells may have a positive effect on the course of chronic GvHD (13-15).

Chapter 2 describes the creation of constructs with a stable, homogeneous and long-term expression of CD20 and how these cells can be killed by rituximab and human effectors (peripheral blood mononuclear cells and complement). This demonstrates *in vitro* the feasibility and efficacy of using human CD20 as a suicide gene.

However, as to the use of CD20 as a selection marker, we demonstrated that immunomagnetic selection of transgenic CD20 expressing cells is feasible, but yields low recoveries, as a significant number of cells die within the magnetic column during the selection procedure. The underlying mechanism for this observation is not known. Another disadvantage of using CD20 as a selection marker, is the fact that ligation of CD20 molecules with monoclonal antibodies (eg clone L27) blocks rituximab binding (16, 17). Although the antibodies used for selection do not impair the culture or functionality of the purified T cells (no post-selection induced apoptosis), infused purified T cells can not be eliminated by rituximab during the first days post selection. The antibodies used for immunomagnetic selection will block rituximab binding, but are lost after five days post selection. This may not be a clinical problem, as acute GvHD in general occurs at least 14 days after infusion.
Together, these data indicate that immunomagnetic selection by CD20 is feasible, but the efficacy has yet to be improved before clinical application.

In Chapter 3, we demonstrated that CD20-expressing T cells were able to induce a xenogenic Graft-versus-Host reaction at the same level of the non-transduced T cells and fresh PBMCs. The distribution of the T cells in the organs was comparable, except for the spleen. Remarkably, whereas CD20-negative T cells were scattered throughout to whole organ, CD20-transgenic T cells were found only in the perioarteriolar lymphoid sheath. The mechanism for this difference in distribution is currently under investigation.

How to optimize CD20 expression?

Limiting properties of retroviral vectors are transcriptional gene silencing and variegation of gene expression. Some cells, with integrated virus, do not express the transgene in any way (18). Others are susceptible to position-dependent expression or extinction silencing, which is progressive silencing during long-term culture (19). Variegation of gene expression by retroviruses occurs if genetically identical sister cells inherit the same provirus, but show different levels of expression (20, 21).

What can be done to optimize transgene expression and make it stable over time?
Purification of the transduced population will eliminate most of the 'silent' provirus integration. Secondly, transducing cells at a high multiplicity of infection (MOI) decreases the chance of silencing, but this will enhance the chance of insertional mutagenesis (22). Therefore, at the same time some adjustments have to be made at the molecular level.

Silencing occurs at the transcriptional level and DNA methylation is involved in the process. Silencing elements are found in the Long Terminal Repeat (LTR) region of the retrovirus. Mutations of the responsible elements did lead to more stable transgene expression in time (23, 24). Also Self-Inactivating (SIN) retroviral vectors, initially constructed for clinical safety, are less susceptible to silencing. SIN vectors delete the enhancer/promoter from the 3'LTR upon integration (25, 26).

To make expression more stable and to reduce position dependent effects different cis-acting elements can also be included into the retroviral backbones.

The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) has been widely proven to enhance transgene expression under control of several different promoters and in different cell lineages (27, 28). It has been suggested that the WPRE element acts on both the transcriptional and translational level. It promotes mRNA transport from the

nucleus to the cytoplasm, and may facilitate protein expression from mRNAs that normally would be degraded within the nucleus (29-31).

Inclusion of an insulator element into the 3'LTR of the vector also leads to a more stable expression. Insulators are currently used in retroviral vectors for the treatment of γ-globin deficiencies and have been shown to reduce position effects and protect transgene expression from silencing (32-38). Insulators also have shielding capacity and block transgene expression by interfering with promoter-enhancer activity. As a consequence, insulators reduce the influence of neighbouring promoters/enhancers on transgene expression and provide a more homogeneous transgene expression (32, 33, 35, 39-42). Interferon-β scaffold attachment region (IFN-SAR) have also the capacity to increase transgene expression (43).

We inserted the WPRE element for the first time in T cells downstream of the CD20 gene and the insulator element into both LTRs of the retroviral vector, which resulted in stable and significantly more homogeneous expression during long-term culture.

However, despite these successful optimizations, retroviruses still have one major disadvantage: for integration into the genomes of the cells, the cells need to divide.
For the transduction of T cells this means that the T cells have to be stimulated. Anti-CD3/anti-CD28 costimulation *in vitro* seems to preserve T cell function the most. Anti-CD3/CD28 costimulation enhances gene transfer and transgene expression (44-46), preserves the CD4:CD8 ratio (47), reduces apoptotic-induced cell death (48) and prevents TCR Vβ skewing (49, 50) compared to stimulation with anti-CD3 alone. Extended culture time also has a profound effect on T cell functionality *in vivo* (51).

Despite the achievements to optimize T cell stimulation and culture, in general, activation of T cells results in loss of the naïve T cell population and skewing to a memory phenotype (52), while triggering T cells for expansion will initiate activation-induced cell death (52, 53).

Lentiviral vectors do not need target cell division for infection. Lentiviral vectors are human immunodeficiency virus type 1 (HIV-1) based vectors, which lead to a more efficient expression of the transferred gene compared to retroviruses (54, 55). The first lentiviral vector was generated only recently in 1996 by Naldini et al (56), so there is not much experience with this backbone. Currently, the origin of the lentiviral backbone (HIV) is still holding back its clinical use, although this may largely be based on emotional grounds. Future (pre)clinical studies will have to demonstrate the safe application of gene therapy with lentiviruses that may eventually lead to a more stable and efficient way of gene transfer in hematopoetic stem cells and T cells (57).

How safe is gene therapy?

In 1963 Joshua Lederberg stated that the ultimate application of molecular biology would be the control of gene expression in human chromosomes (58). After only a short history of three decades from bench to bedside, the first gene therapy trial was conducted in 1989 (59). Between 1989 and 2007, more than 1340 clinical trials have been approved (60). The majority of these trials deal with the treatment of cancer.

The success and the drama of gene therapy lie within the treatment of a single congenital genetic disorder. The first therapeutic clinical trial was performed in two girls suffering from severe combined immunodeficiency (SCID) characterized by adenosine deaminase deficiency (ADA) (61). Results of this trial showed a safe and effective correction of the genetic disorder. A major success came from the treatment of X-linked SCID in 2000. This rare disease is caused by mutations of the gene encoding the γ-chain cytokine receptor subunit of interleukin-2, -4, -7, -9, and -15 receptors leading to a block in T and NK cell development (62). Autologous stem cells were transduced with a retrovirus containing the γ-chain and were re-infused into the patients. Ten out of 17 γ-SCID patients were cured completely, preventing them from treatment with allo-SCT.

However, in 2003 the authors reported that 2 out of the 10 patients had developed T cell leukemia (63). The malignant transformation could be attributed to integration of the retrovirus in close proximity of the LMO-2 proto-oncogene promoter (64). Later, leukemia developed in a third child, who was shown to have three retroviral insertions and two additional insertions of the transgene next to oncogenes (65). In the meantime, leukemia development was reported in an experimental mouse study, demonstrating that retroviral insertion was responsible for the activation of the Evi1 gene (66). The oncogenic mutagenesis was caused by the integration of retroviruses near transcriptional active places (67) leading to clonal dominance of the transduced cells (68, 69).

These events led to the temporary hold of stem cell gene therapy trials in the US, France, Italy, Germany and the Netherlands (70). Nowadays, following a critical examination of the risk/benefit ratio of gene therapy, clinical trials are carefully being re-started with pre-clinical and clinical studies focusing on safety of gene transfer.

So far, adverse events in retroviral gene therapy only occurred with *ex vivo* manipulated pluripotent stem cells. However, genetic manipulation of T cells is also not free of risks, especially since T cells have a high proliferative potential. Murine Leukemia Virus (MLV)-based retroviral insertion sites in T cells have been examined, and it was demonstrated that also in T cells the integration sites lie near transcription sites and in gene-dense chromosomes (71).

Although retroviruses insert the transgenes into unwanted integration sites, they were the first and are still the best vehicles to carry transgenes to the target cells.
In future, other gene delivery systems could be used to manipulate cells in order to express therapeutic genes.
Examples are adenoviruses, adeno-associated viruses or non-viral vectors. Adenoviruses are the most widely used vectors (60). Adenoviral vectors can carry larger transgenes than retroviral vectors. Adenoviral vectors have a high efficiency of transduction and a high level of gene expression. They do not integrate into the chromosomes, and therefore do not induce insertional mutagenesis. However, the transgene expression is transient. Also, in a

previous clinical gene therapy trial, in which adenoviruses were infused directly into the patients in stead of gene-modified cells, the patient died because of an inflammatory reaction towards the adenoviruses (72).

Adeno-associated viral (AAV) vectors can be used to transduce a wide variety of tissue, although they were not yet widely used in blood (precursor) cells. Their capacities differ significantly from the other mentioned vectors. Transgene expression is stable for a long time and the expressed viral molecules show a lower immunogenicity than molecules expressed by other viral vectors (73). *In vitro*, without helper virus, AAV establishes latency by integrating into human chromosome 19, but after co-transfection with HSV, AAV processes to a lytic productive phase (74, 75). *In vivo*, a site-specific integration appears to be less frequently observed, as AAV persists mostly in episomal form (76, 77). A disadvantage of AAV is that not always the desired therapeutic transgene expression is obtained and the transgene size may not exceed 5 kb. Future optimization experiments will make the AAV vector a very useful tool for transgene delivery.

Non-viral vectors, eg naked DNA, have also been used to overcome the risks of retroviruses, but to date expression of transgenes is low and transient.

How to make retrovirus-mediated gene therapy safer?

Activation of specific (onco) genes following retroviral insertion is likely to be influenced by a number of factors.
In the first place, the vector system and transduction/culture protocol seem to be important. Two gene therapy trials for treating X-linked SCID have been compared for their transduction protocols and their vectors. In the “French” trial four out of ten patients developed an acute leukemia (78). On the other hand, none of the six patients in the “British” trial developed a hematological malignancy (79). Examination of both transduction protocols and the vectors that were used revealed that in the French trial more interleukin-3 and protamine sulphate were used in the transduction medium. The difference

in the retroviral vector was the pseudotyping of the envelope protein. The French used the amphotropic envelope protein whereas the British used the gibbon ape leukemia virus envelope protein. In addition, the French vector had the B2 mutation in the primer binding site of the LTR. This B2 mutation has been associated with the prevention of transgene silencing (80).

Secondly, regulatory elements in the vectors may play an important role. Within the two intact LTRs of the conventional retroviral vectors strong promoter and enhancer elements are located. The strong promoter is able to read-through transcription of downstream sequences (genes), and the enhancer can interact with nearby endogenous promoters. SIN vectors, which have deletions in these elements of the LTR, may be a solution for the prevention of (trans)activation of proto-oncogenes (81). On the other hand, the polyadenylation elements in the LTR are relatively weak and will therefore also allow read-through transcription and the possible activation of proto-oncogenes (82). Addition of a strong polyadenylation signal could prevent the read-through transcription. Also, potential oncogenes and oncogene activators must be excluded from a retroviral vector backbone (66, 83-85).

Inclusion of insulator elements may have enhancer blocking activities. Insulator elements shield promoters from the influence of neighbouring regulatory elements and protect retroviral transgenes from silencing. Insulators may prevent oncogene activation after chromosomal insertion of a retrovirus (32, 33, 35).

Thirdly, the number of retroviral vector integrations must not exceed one. The higher the retroviral vector copies, the higher the chance of proto-oncogenic insertions (22). In addition, the more transgenic cells are transplanted, the higher the chance of insertional mutagenesis. However, a certain therapeutic number of cells is needed, so this is difficult to regulate.

Another way of thinking may be to define safe integration sites and create vectors that insert themselves into these safe sites.

Finally, in all vectors a safety switch must be included. The co-expression of a suicide gene enables the specific elimination in case of clonal transformation. The non-immunogenic human CD20 would be a good candidate.

These results indicate the importance of retroviral vector design and transduction/culturing protocols. *In vitro* culturing protocols may also predict non-controlled cell growth. For instance, retroviral transduction may immortalize bone marrow cells and convert growth factor dependent cells into growth factor independent cells (81, 86-88). In addition, the development of animal models will be helpful to test new vectors for their potential to induce insertional mutagenesis.

Although there are risks to gene therapy, it is in some cases the only curative option. We may have to accept that risk, just as we have accepted the potential risks of allogeneic stem cell transplantation. As in pharmacology, no true effect is possible without inducing side effects. However, gene therapy remains experimental medicine, and future preclinical and clinical studies will be necessary to prove the efficacy and safety of gene therapy in the treatment of human diseases.

What is the mechanism of anti-CD20 immunotherapy?

In the second part of this thesis we investigated the different mechanisms of action of the anti-CD20 antibody rituximab in an experimental model using CD20-transgenic T cells. The system we used was not interfered by the expression of complement regulatory proteins (CRPs) CD35, CD46, CD55 and CD59.

The three mechanisms of action of rituximab are generally considered to be CDC, ADCC and the induction of apoptosis. In concordance with results of studies involved in B-cell Non-Hodgkin's Lymphoma (NHL) and Chronic Lymphocytic Leukemia (CLL), our *in vitro* studies indicate that the main mechanisms of action are CDC and ADCC (89-94).

CDC

In Chapter 5 and 6 we demonstrate by using CD20-transgenic clones that the CD20 expression level is related to the CDC efficacy of rituximab.

In some reports, the *in vitro* CDC-activity of rituximab has been correlated with the CD20 expression level on B cell lymphomas (95, 96). However, others could not confirm these results (93). In addition, the *in vitro* CDC-activity of rituximab against patient lymphoma samples did not correlate with the clinical response (97) and CD20 expression level of lymphoma samples did not clearly relate to the *in vivo* rituximab response (98). Apparently, additional mechanisms of resistance contribute to the variable responses to rituximab.

In chapter 5, we demonstrated in a system with only one variable parameter, namely the CD20 molecule, the direct correlation of CD20 expression with rituximab-induced CDC. This means that at least *in vitro*, CD20 expression level should be considered as one of the critical factors that determine rituximab sensitivity.

ADCC

We demonstrated that CD20 expression level did not correlate with the cell kill mediated by effector cells (ADCC). This is in concordance with previous findings (93). The ADCC activity depends on the phenotype of the effector cells (99-101). In our experiments we used several different donor effector cells (peripheral blood mononuclear cells) but we did not check for FcγRIIIA genetic polymorphism (99-101). No significant difference was found for rituximab-mediated cell kill on the different clones with different donor effectors cells.

CDC and ADCC

CDC and ADCC together had complementary effects on rituximab-mediated cell kill. One explanation is that complement activation has other effects beside lysis of target cells. Depositions of C3 and C3b on the cell membrane can act as targets for the C3 receptor expressed on effector cells. This could lead to enhanced ADCC activity (102).

In addition, as demonstrated in chapter 5, we showed for the first time that CDC-resistant cells could be killed by ADCC and vice versa. This means that tumor-related resistance (CDC) may be overcome by ADCC and that host-related resistance (ADCC) may be overcome by CDC. This means that multiple ways of resistance must be incorporated in one rituximab-resistant cell. This also suggests that the triggering of other killing mechanisms may overcome apparent rituximab resistance.

How can we overcome rituximab resistance?

There are different mechanisms of resistance towards rituximab. Here, several of these mechanisms and possible solutions to the problem are discussed.

Cellular microenvironment

The cellular microenvironment contributes to the dominant effector and resistance mechanism of rituximab (103). Circulating B cells are depleted mainly through the reticuloendothelial system, while B cells within the marginal zone compartment depend on CDC rather than FcγR-mediated depletion. B cells residing in the lymphoid tissues depend on the vasculature for the accessibility of effector cells. The significance of the microenvironment in rituximab-induced cell death is also indirectly observed by differential responses to rituximab therapy in distinct histology of CD20 positive lymphomas (which have unique microenvironments), and the fact that molecular remissions in the blood and bone marrow induced by rituximab can occur in the setting of progressive nodal disease. Investigation and/or manipulation of the microenvironment may enhance or decrease rituximab resistance.

Rituximab and chemotherapy

Although rituximab has a chemosensitization effect to drug-induced apoptosis by inhibiting anti-apoptotic pathways (104, 105), chemotherapy can also deplete effector cell populations (106). Less resistance was observed with initial single-agent rituximab therapy and no previous chemotherapy in patients with indolent NHL (107) than in indolent NHL patients

treated with rituximab in the relapsed phase (7). In addition, *in vitro* results demonstrated that dexamethasone impaired the ADCC effectivity (108). Changing the timing or combining of antibodies to chemotherapy schedules may enhance the rituximab effect by an improved activation of the different mechanisms.

Overcoming apoptosis resistance

Recent data by Vega et al. suggested that rituximab is involved indirectly in apoptotic death by up-regulation of FAS expression (109). They demonstrated in FAS-resistant NHL cell lines that rituximab sensitizes the cells for FAS-induced apoptosis by inhibiting NF-κB activity (109). *In vivo*, this would mean that FAS/Ligand-expressing cells (T, NK and macrophages) could indirectly induce apoptosis. Mutations in FAS signal transduction pathways or in the receptors could therefore be responsible to rituximab resistance (110).

Not only is the FAS ligand/FAS death pathway sensitized by rituximab, rituximab also works as a chemosensitizing agent (111). For example, bcl-2 is an anti-apoptotic protein that confers chemotherapy resistance. Bcl-2 over-expression in CLL patients has been associated with apoptosis resistance. Dysregulation of bcl-2 (t14;18 in follicular NHL) led to increased sensitivity to rituximab (112, 113). In patients treated with rituximab, down-regulation of interleukin-10 was observed and associated with a decreased DNA binding activity of STAT3 and decreased bcl-2 expression (114).
In case of over-expression of bcl-2, down-regulation of bcl-2 by antisense oligonucleotides may enhance anti-CD20 efficacy (115).

Several other signaling pathways have been attributed to rituximab-induced apoptosis, including down-modulation of the ERK1/2 and NF-κB transcription factors, leading to downregulation of bcl-xl (111). In future studies, apoptotis-induced cell death may be targeted at each level of the different signaling pathways.

Overcoming ADCC resistance

In vivo, polymorphisms in the FcγRIIIA gene have been correlated with clinical responses (99-101), which indicate that the nature of the interaction of the antibody with FcγR could be altered by either the antibody or the FcγR. The FcγRIIIA polymorphism may be used to predict clinical outcome and antibodies may be generated to overcome the polymorphism related resistance.

In case of impaired effector cell population by systemic therapy, the effector cell population can be restored with cytokines such as IL-2, 12 or 15 and myeloid growth factors (116). In addition, CpG sequences are short DNA sequences that have immunostimulatory effects and could have a synergistic effect with antibodies (117, 118).

On the other hand, effector cells can kill by direct lytic action of enzymes such as granzyme and perforin. Tumor cells can be resistant to such enzymes by expression of protease inhibitor 9 (119). Blocking this process could also lead to enhanced ADCC.

Recently, it has been demonstrated that phagocytes can remove the rituximab/CD20 complex from mantle cell lymphoma and CLL cells (120). This process, removing the extracellular part of the CD20 protein, was called shaving. The authors suggest that decreasing the rituximab dose leads to a decreased number of 'shaved' CD20 cells (121). To enhance rituximab response, (pre)-clinical studies could investigate the effects of different rituximab dose levels.

Overcoming CDC resistance

As demonstrated in this thesis, there is a clear correlation between CD20 expression level and rituximab-mediated CDC. Complement Regulatory Proteins (CRPs) regulate complement fixation and blocking of CRPs by antibodies lead to enhanced CDC (92, 95, 96). Fludarabine, a nucleoside analog clinically active against CLL, down-regulates CD55 expression (122). *In vivo*, the combination with rituximab may have a beneficial effect.

Enhancing CD20 expression can be effectuated by cytokines. For example, CD20 up-regulation was observed with IL-4, TNF-α, and GM-CSF treatment of patients with CLL (123) and INF- α also increased CD20 expression in CLL patients (124). In addition, immune modulator agents such as bryostatin-1 up-regulate CD20 expression through ERK-dependent mechanisms (125) and low doses of external beam radiotherapy also increased CD20 expression *in vitro* (126).

Enhancing complement fixation by an anti-C3b(i) monoclonal antibody may also enhance the CDC activity (127).

In addition, tailoring antibodies with improved complement fixation (128) or new antibodies can also be employed to improve *in vivo* CDC activity (129, 130).

New antibodies

We analysed a new human anti-CD20 antibody, which binds to a distinct CD20 epitope compared to rituximab, and demonstrated improved CDC (129, 130). *In vivo* HuMab-7D8 was able to eliminate all CD20 expressing cells whereas rituximab did not eliminate the low-expressing cells. First results with a similar anti-CD20 antibody HuMax-2F2 (Ofatumumab) recently demonstrated safe application in patients (131). Also recently, a novel antibody has been proposed, the humanized anti-CD20 antibody GA101. This is a type II anti-CD20 monoclonal antibody. Type II anti-CD20 antibodies do not translocate the CD20 molecules into lipid microdomains and have no CDC activity. However, these antibodies have a very strong induction of direct cell death. In cynomolgus monkeys, GA101 was shown to have a superior efficacy for tissue B-cell depletion compared to rituximab (132).

To conclude, rituximab has multiple complicated mechanisms of action. Although rituximab treatment is very successful, resistance can occur at different levels. Various strategies to overcome each single resistance mechanism have been mentioned above. In addition, it will be critical to understand the interactions of these mechanisms of action.

Investigating the resistance mechanisms will also be the key issue for development of new and improved antibodies or the use of combinations of different antibodies.

Perhaps if we understand resistance, we can treat patients in an individual way.

General conclusion

This thesis describes the investigation of human CD20 as a suicide gene and adds to clarify the mechanism of the anti-CD20 antibody rituximab, using CD20 gene transfer.

In all future gene therapy trials, a suicide gene should be co-expressed in transgenic cells to maximize safety of clinical applications. Based on our results, we suggest human CD20 as a suicide gene for T cells.

Rituximab triggers different effector mechanisms. The observation that CDC-resistance cells are sensitive to ADCC and vice versa suggests that the triggering of other killing mechanisms may overcome apparent rituximab resistance. The clear CD20 expression level related resistance of rituximab may be overcome by new human anti-CD20 antibodies, like HuMab-7D8

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Summary

Leukemia and lymphoma are hematological malignancies, which are characterized by an uncontrolled proliferation of white blood cells. The current therapy of these diseases consists of high-dose chemotherapy and/or radiotherapy. In certain patients this treatment is consolidated with a transplantation of stem cells and immune cells from a healthy and matched donor (allogeneic stem cell transplantation). The goal of this treatment is to replace the patient's bone marrow. The immune cells from the donor can track down and eliminate remaining malignant cells, the so-called graft-versus-leukemia or lymphoma effect.

Treatment with chemo- and/or radiotherapy is limited by the damage that is afflicted to the healthy tissues and organs of the patients. Also, in a significant number of patients, despite these intensive treatments, the disease returns. A more effective therapy of hematological malignancies should therefore be specific for malignant cells and spare the healthy cells.

Immune therapy and gene therapy are the two most innovative treatment strategies developed over the last years, aiming at a more effective and specific treatment of hematological malignancies.

Adoptive immune therapy is the application of donor immune cells (T lymphocytes or T cells) to a patient. The most applied type of adoptive immune therapy is allogeneic stem cell transplantation, as mentioned above. The donor T cells can track down malignant cells in the host and eliminate them. Unfortunately, also the healthy tissues of the host can be attacked. This serious and potentially life-threatening complication is called graft-versus-host disease. Gene therapy of the donor T cells can offer a solution to this problem. Through genetic manipulation of T cells in the laboratory before application to the patients, the T cells can be provided with a gene that makes it possible to selectively recognize and eliminate the T cells in case of graft-versus-host disease. This specific form of adoptive immuno-gene therapy is called suicide gene therapy.

Another specific therapy is immune therapy with antibodies. Antibodies are produced by another type of immune cells, the B cell. Antibodies have a structure that enables them to recognize and attach to specific molecules (proteins) on the cell membrane of malignant cells. These antibodies can then have a direct toxic effect on the malignant cell or can activate other immune mechanisms of the body to eliminate the cells. Antibody therapy is world-wide one of the most successful forms of immune therapy. The most famous type is

anti-CD20 antibody therapy directed against the CD20 molecule on the membrane of lymphoma cells.

The goal of this thesis is to improve antibody-mediated immune therapy and gene therapy of hematological malignancies by investigation of the human CD20 molecule. In the first part of this thesis the human CD20 molecule is used as a suicide gene in donor T cells to prevent graft-versus-host disease. In the second part of the thesis, the mechanism of and resistance to anti-CD20 antibody therapy is studied. In addition, the effect of new anti-CD20 antibodies is investigated.

Summary of the chapters

In **Chapter 1** (Introduction), the function of the CD20 molecule is explained as well as the background of the research in this thesis.

CD20 is normally expressed on B cells. B cells are immune cells involved in the adapted immunity. B cells originate in the bone marrow and during maturation they travel to the peripheral blood and lymph nodes. At these sites they recognize non-self material from for instance bacteria or viruses (antigen) and can differentiate to ‘memory’ cells or to plasma cells. Plasma cells produce antibodies. These antibodies bind to the specific antigen, leading to activation of the immune system and the attack and elimination of the micro-organisms connected to the antigen.

Young and mature B cells express the CD20 molecule. B cells that differentiate to plasma cells lose the CD20 expression. The precise function of CD20 is still unclear. At least, CD20 functions as a calcium channel. Calcium conduction can activate cells, which for instance could promote the differentiation of B cells. The CD20 molecule is largely localized in the cell membrane, but a small part is extracellular. This part can be recognized by specific antibodies.

If the CD20 molecule is expressed by malignant B cells, the cells can be targeted by anti-CD20 antibodies and in this way be eliminated. Antibodies can kill the malignant B cell directly, but also by activation of the classic complement system or of effector cells of the immune system, like macrophages and monocytes.

In the last part of this chapter, it is explained how the CD20 molecule can be used as a suicide gene in adoptive T cell immune therapy. Like B cells, T cells are involved in adapted immunity. T cells are capable of the direct killing of virus-infected or malignant cells.

In the laboratory, a gene can be inserted into the T cell genome (a suicide gene). Expression of this gene makes the T cell express a certain molecule on the cell membrane. In case of complications after adoptive T cell immune therapy, like graft-versus-host disease, a drug can be administered to the patient that recognizes only the expressed molecule on the cell membrane and therefore selectively kills the T cell. The feasibility of this strategy was demonstrated for the Herpes Simplex Virus thymidine kinase gene (HSVtk) as a suicide gene in donor T cells. However, an important disadvantage in patients appeared to be a preliminary elimination of the infused donor T cells, which was caused by an immune reaction of the patient to this (viral) molecule as soon as immune competence of these patients was regained.

T cells normally do not express CD20. In this thesis, it is demonstrated how the CD20 gene is built into T cells as a suicide gene. It can be expected that CD20 as a self-protein does not have the disadvantage of preliminary elimination by the own immune system of the patient.

A suicide gene is built into a cell by a retroviral vector. This is a retrovirus of which the harmful elements are replaced in the laboratory by the specific gene. In this way, infection (transduction) of T cells with retroviruses containing the CD20 gene leads to expression of the CD20 molecule.

In **Chapter 2**, the construction is described of several retroviral vectors that can build the CD20 gene into T cells. The results show that the CD20 molecule can efficiently be expressed on the T cell membrane. Building-in a post-transcriptional regulation element (WPRE) and an insulator element next to the CD20 gene into the retroviral vector leads to a higher, more stable and homogeneous CD20 expression.

Also in this chapter it is described that CD20-positive T cells can be efficiently killed by binding to the anti-CD20 monoclonal antibody rituximab (Mabthera®, Rituxan®). Rituximab eliminates CD20-positive T cells by activation of human complement and human effector cells.

In **Chapter 3**, the influence of CD20 expression on T cell function is described. As mentioned above, CD20 functions as a calcium channel. Calcium in cells triggers certain cellular processes and an increased level of intracellular calcium can induce cell death.
CD20 expression on T cells did not induce early cell death, not even after activation of CD20 by the antibody rituximab. Also, it was demonstrated that the antigen response of CD20-positive T cells was similar to the response of CD20-negative T cells.

In a mouse model, CD20-positive T cells were able to induce graft-versus-host disease to a similar level as CD-20 negative T cells. A remarkable finding after histopathological analysis in this mouse model was the fact that the CD20-positive T cells, compared to the CD20-negative T cells, were localized preferentially at a different site in the spleen.

For the down tracking of remaining malignant cells, a sufficient number of CD20-positive T cells should be administered to the patient. Also, all infused T cells should have CD20 expression, because all cells are potentially able to cause graft-versus-host disease.

In **Chapter 4** it is described that for practical and safety reasons only 30% of the donor T cells can be transduced with CD20. This means that after transduction, the CD20-positive T cells have to be purified to 100% before they can be administered to the patient. CD20-positive cells can be purified by binding an antibody to the CD20 molecule and attaching a small metal bead to the antibody. When this complex is pulled alongside a magnetic column, all CD20-positive cells will stick to the column. By switching off the magnetic field, the CD20 positive cell population is selected.

The results described in this chapter show that the cells can be purified well in this way, but that a large part of the CD20-positive T cells dies during the procedure. To obtain enough CD20-positive T cells for clinical application, it was tried to provide the T cells with a selection gene next to the CD20 gene. Selection with the help of such a gene led to a higher number of purified T cells, but CD20 expression was lower on these cells, resulting in an insufficient elimination of the T cells with low CD20 expression by rituximab. Additional experiments will have to be performed to optimize this system.

In **Chapters 5 and 6** the experiments for investigation of the mechanism of anti-CD20 antibody therapy are described. In these experiments a unique system was used, in which

the CD20 molecule was transduced to a T cell line resulting in different CD20 expression levels.

In Chapter 5, the results are described of the experiments that showed for the first time the direct relationship between rituximab-mediated cell death due to complement activation and the number of CD20 molecules expressed on the cell membrane. This is not the case for rituximab-mediated cell death induced by effector cells. The combination of complement activation and effector cell activation by rituximab leads to a higher cell kill than complement- or effector cell activation alone. Moreover, complement-resistant cells are still sensitive to cell death by effector cells and vice versa.

In Chapter 6 it is described how the cells that are resistant for rituximab-mediated complement activation can be killed by a new and completely humanized anti-CD20 antibody (HuMab-7D8). *In vitro* experiments showed that, by Humab-7D8, cells with a low CD20 expression were killed more efficiently in the presence of complement than by rituximab.
In mice, this observation was confirmed by the demonstration that HuMab-7D8 could eliminate all CD20-positive T cells, while with rituximab the cells with a low CD20-expression remained alive.

In the last chapter (**Chapter 7**) the questions that came up during the development of this thesis are discussed.

An important point concerning the field of gene therapy in general, is safety. It has become clear in the past that the insertion of a vector into the genome of a cell can induce malignant transformation. It will be necessary in the future to protect against this insertional mutagenesis by for instance providing the retroviral vectors with an insulator element or a suicide gene.

For anti-CD20 antibody therapy, it can be concluded that the maximum effect of treatment does not seem to be reached. The use of improved second generation antibodies or ways to increase the CD20 expression can lead to a more efficient therapy and may overcome disease resistance.

In conclusion, this thesis shows the development of the CD20/anti-CD20 suicide system for adoptive T cell immunotherapy and the investigation of the mechanism of CD20/anti-CD20 antibody therapy. The results may lead to a more effective treatment of hematological malignancies.

Samenvatting

Leukemie en lymfklierkanker zijn hematologische maligniteiten, die gekarakteriseerd worden door een ongecontroleerde proliferatie van witte bloedcellen. De huidige behandeling van deze ziekten bestaat uit hoge dosis chemotherapie en/of radiotherapie. In bepaalde gevallen wordt aansluitend een transplantatie verricht met stamcellen en afweercellen van een gezonde en gematchte donor (allogene stamceltransplantatie). Deze behandeling heeft als doel het beenmerg van de patiënt te vervangen. De afweercellen van de donor kunnen eventueel resterende maligne cellen opsporen en vernietigen, het zogenoemde graft-versus-leukemie effect.

Behandeling met chemo- en/of radiotherapie wordt beperkt door schade aan gezonde weefsels en organen van de patiënt. Daarnaast komt, ondanks deze zware behandelingen, in een significant deel van de patiënten de ziekte terug. Een effectievere behandeling van hematologische maligniteiten moet dus specifiek zijn voor maligne cellen en de gezonde cellen te sparen.

Immuuntherapie en gentherapie zijn de twee meest innovatieve behandelingsstrategieën die zijn ontwikkeld in de laatste jaren met als doel een meer effectieve en specifieke behandeling van hematologische maligniteiten.

Adoptieve immuuntherapie is het toedienen van donor afweercellen (T lymfocyten/T cellen) aan een patiënt. De bekendste vorm van adoptieve immuuntherapie is die bij allogene stamceltransplantatie, zoals hierboven genoemd. De donor T cellen kunnen de maligne cellen opsporen en vernietigen. Helaas kunnen de T cellen ook de gezonde weefsels van de patiënt aanvallen. Deze ernstige en mogelijk zelfs letale complicatie wordt graft-versus-host ziekte genoemd. Gentherapie van de T cellen kan hiervoor een oplossing bieden. Met behulp van genetische manipulatie van de donor T cellen in het laboratorium vooraf aan toediening aan de patiënt kunnen de T cellen worden uitgerust met een gen dat het mogelijk maakt om de cellen selectief te herkennen en uit te uitschakelen wanneer deze cellen graft-versus-host ziekte veroorzaken. Deze specifieke vorm van adoptieve immuno-gentherapie wordt suïcide gentherapie genoemd.

Een andere specifieke behandeling is immuuntherapie met behulp van antilichamen. Antilichamen worden gemaakt door een ander type afweercel, de B cel. Antilichamen zijn zo gemaakt dat ze een specifiek molecuul (eiwit) op het celmembraan van de maligne cellen kunnen herkennen en aan dit molecuul kunnen binden. Deze antilichamen kunnen dan een direct toxisch effect hebben op de maligne cel of kunnen de afweermechanismen

van het lichaam zelf hiervoor activeren. Antilichaamtherapie is wereldwijd een van de meest succesvolle vormen van immuuntherapie. De bekendste vorm is anti-CD20 antilichaamtherapie gericht tegen het CD20 molecuul op het membraan van lymfklierkankercellen.

Dit proefschrift heeft als doel om antilichaam-gemedieerde immuuntherapie en gentherapie van hematologische maligniteiten te verbeteren door onderzoek naar het humane CD20 molecuul. In het eerste deel van het proefschrift wordt het CD20 molecuul gebruikt als suïcide gen in donor T cellen om graft-versus-host ziekte te voorkomen. In het tweede deel van het proefschrift worden het mechanisme en de resistentie van anti-CD20 antilichaam therapie bestudeerd en is onderzocht of deze therapie verbeterd kan worden met het gebruik van nieuwe anti-CD20 antilichamen.

Samenvatting van de hoofdstukken

In **Hoofdstuk 1** (Inleiding) wordt beschreven wat de functie van het CD20 molecuul is en wordt het doel van het onderzoek in dit proefschrift uiteengezet.

CD20 komt normaal tot expressie op B cellen. B cellen zijn witte bloedcellen die betrokken zijn bij de verworven immuniteit. B cellen ontstaan in het beenmerg en migreren bij rijping naar het bloed en de lymfklieren. Op deze plaatsen herkennen zij lichaamsvreemd materiaal (antigeen), zoals bacteriën en virussen, en kunnen dan differentiëren tot ‘memory’ cellen of tot plasmacellen. Plasmacellen produceren antilichamen. Deze antilichamen binden aan het specifieke antigeen wat leidt tot activatie van het immuunsysteem en het aanvallen en verwijderen van het bijbehorende micro-organisme.

Jonge en rijpe B cellen brengen het CD20 molecuul tot expressie. Als B cellen differentiëren tot plasmacellen verliezen zij de CD20 expressie. De functie van CD20 is nog niet geheel duidelijk. In ieder geval fungeert CD20 als een calcium kanaal. Door calcium geleiding kunnen cellen worden geactiveerd, wat bijvoorbeeld kan leiden tot differentiatie van de B cellen. Het CD20 molecuul zit grotendeels gelokaliseerd in het celmembraan, maar een klein deel is extracellulair te vinden. Dit deel kan herkend worden door specifieke antilichamen.

Als het CD20 molecuul tot expressie wordt gebracht door maligne B cellen, kunnen de cellen worden getarget door anti-CD20 antilichamen en zo worden geëlimineerd. Antilichamen kunnen de maligne B cel direct doden, maar ook het klassieke complementsysteem en effector cellen van het immuunsysteem, zoals monocyten en macrofagen, kunnen hiertoe geactiveerd worden.

In het laatste deel van Hoofdstuk 1 wordt uiteengezet hoe het CD20 molecuul gebruikt kan worden als suïcide gen bij adoptieve immuuntherapie met T cellen.
Net als B cellen spelen T cellen een rol in de verworven afweer. T cellen zijn verantwoordelijk voor het rechtstreeks uitschakelen van virus-geïnfecteerde cellen en maligne cellen.

In het laboratorium kan een gen worden ingebouwd in T cellen (een suïcide gen). Expressie van dit gen zorgt ervoor dat een T cel een bepaald molecuul tot expressie brengt op het celmembraan. Op dit molecuul kan worden aangegrepen door een medicament dat de desbetreffende T cel selectief doodt, indien dit nodig is in het geval van bijwerkingen van adoptieve T cel immuuntherapie (graft-versus-host ziekte). In de praktijk werd gebruik gemaakt van het Herpes Simplex Virus thymidine kinase gen (HSVtk) als suïcide gen in donor T cellen. Echter dit (virale) gen heeft onder andere als belangrijk nadeel in patiënten dat de cellen met het HSVtk molecuul op het oppervlak vroegtijdig kunnen worden uitgeschakeld door de eigen afweercellen van de patiënt.

T cellen brengen CD20 niet van nature tot expressie. In dit proefschrift wordt beschreven hoe het CD20 gen is ingebouwd als suïcide gen in T cellen. Het is de verwachting dat het lichaamseigen CD20 molecuul het nadeel van herkenning door het eigen immuunsysteem van de patiënt niet heeft.

Een suïcide gen wordt in een cel ingebouwd door gebruik te maken van retrovirale vectoren. Dit zijn in het laboratorium gemodificeerde retrovirussen waarvan alle schadelijke componenten zijn vervangen door het desbetreffende gen. Zo leidt infectie (transductie) van T cellen met retrovirussen die het CD20 gen bevatten tot expressie van het CD20 molecuul.

In **Hoofdstuk 2** wordt de constructie van een aantal retrovirale vectoren beschreven die het CD20 gen kunnen inbouwen in T cellen. De resultaten laten zien dat het CD20 molecuul hiermee efficiënt op T cellen tot expressie kan worden gebracht. Inbouwen van een post-

transcriptioneel regulatie element (WPRE) en een insulator element naast het CD20 gen in de retrovirale vector leidt tot een hogere, meer stabiele en homogene CD20 expressie.

Verder wordt in dit hoofdstuk beschreven dat CD20-positieve T cellen efficiënt kunnen worden gedood door binding aan het anti-CD20 monoklonale antilichaam rituximab (Mabthera®, Rituxan®). Rituximab elimineert CD20-positieve T cellen door activatie van humaan complement en humane effector cellen.

In **Hoofdstuk 3** wordt beschreven dat CD20 expressie op T cellen de functie van T cellen niet verandert. CD20 komt normaal tot expressie op B cellen en fungeert als calcium kanaal. Calcium is in cellen nodig voor het aansturen van bepaalde cellulaire processen en een verhoogd intracellulair calcium kan leiden tot celdood.

CD20 expressie op T cellen leidt niet tot een vervroegde celdood, ook niet als CD20 geactiveerd wordt door het antilichaam rituximab. Ook vertonen CD20-positieve T cellen dezelfde afweerrespons tegen een antigeen als CD20-negatieve T cellen.
In een muizenmodel zijn CD20-positieve T cellen in dezelfde mate als de CD20-negatieve T cellen in staat om graft-versus-host ziekte te veroorzaken. Opmerkelijk is wel dat na histologische analyse bleek dat de CD20-positieve T cellen zich in de milt preferentieel op een andere locatie bevinden dan de CD20-negatieve T cellen.

Voor het goed opsporen van maligne cellen moeten voldoende CD20-positieve T cellen in de patiënt worden ingebracht. Ook moeten alle T cellen CD20 tot expressie brengen, omdat iedere T cel graft-versus-host ziekte kan veroorzaken.

Hoofdstuk 4 beschrijft dat om praktische en veiligheidsredenen maar 30% van de donor T cellen met CD20 getransduceerd kan worden. Dit betekent dat na transductie de CD20-positieve T cellen moeten worden opgezuiverd tot 100% voordat de cellen aan de patiënt kunnen worden toegediend. CD20-positieve cellen kunnen worden opgezuiverd door aan het CD20 molecuul een antilichaam te binden en dit antilichaam te voorzien van een metalen bolletje. Wanneer dit complex over een magnetische kolom wordt gehaald blijven alle C20-positieve cellen achter op de kolom. Door het magnetische veld uit te schakelen wordt de CD20-positieve populatie opgezuiverd.

De resultaten beschreven in dit hoofdstuk laten zien dat de populatie goed kan worden opgezuiverd, maar dat een groot deel van de CD20-positieve T cellen dood gaat tijdens deze procedure.
Om toch een voldoende aantal CD20-positieve T cellen voor klinische toepassing te verkrijgen, is getracht om naast het CD20 gen selectiegenen in de cel te brengen. Selectie met behulp van deze additionele genen geeft een hoger aantal opgezuiverde T cellen, maar CD20 expressie is weer lager op deze cellen, waarbij er een onvoldoende eliminatie is van de T cellen met zwakke CD20 expressie door rituximab. Additionele experimenten zullen nog gedaan moeten worden om dit systeem te optimaliseren.

In **Hoofdstuk 5 en 6** zijn de experimenten beschreven die betrekking hebben op het mechanisme van anti-CD20 antilichaamtherapie. In de experimenten is gebruik gemaakt van een uniek systeem waarbij het CD20 molecuul is getransduceerd op een T-cellijn tot verschillende niveau's van CD20 expressie.

In Hoofdstuk 5 wordt voor het eerst aangetoond dat er een directe relatie is tussen rituximab-gemedieerde celdood als gevolg van complement-activatie en het aantal CD20 moleculen dat op het celmembraan tot expressie wordt gebracht. Dit geldt niet voor rituximab-gemedieerde celdood door effector cellen. De combinatie van complement-activatie en effector cel activatie door rituximab leidt tot een hogere celdood dan complement- of effector cel activatie alleen.
Bovendien blijken complement-resistente cellen nog wel gevoelig voor celdood door effector cellen en vice versa.

In Hoofdstuk 6 wordt beschreven hoe cellen die resistent zijn voor rituximab-gemedieerde complement-activatie wel kunnen worden uitgeschakeld door een nieuw en volledig humaan anti-CD20 antilichaam (HuMab-7D8). *In vitro* experimenten laten zien dat cellen met een lage CD20 expressie in aanwezigheid van complement efficiënter worden gedood met HuMab-7D8 dan met rituximab.
In muizen is deze bevinding bevestigd door aan te tonen dat HuMab-7D8 in staat is om alle CD20-positieve T cellen te elimineren terwijl bij rituximab de cellen met zwakke CD20-expressie blijven leven.

In het laatste hoofdstuk (**Hoofdstuk 7**) worden de vragen die naar boven kwamen tijdens de ontwikkeling van dit proefschrift bediscussieerd.

Een belangrijk punt met betrekking tot gentherapie in het algemeen is veiligheid. Omdat is gebleken dat het genetisch veranderen van een cel maligne transformatie tot gevolg kan hebben is het noodzakelijk om in de toekomst de vectoren te beveiligen met bijvoorbeeld een insulator element of een suïcide gen.

Voor anti-CD20 antilichaamtherapie geldt dat het maximale effect van de behandeling nog niet lijkt te zijn bereikt. Het gebruik van verbeterde tweede generatie antilichamen of het omhoog brengen van de CD20 expressie kan leiden tot een efficiëntere therapie en het overwinnen van ziekteresistentie.

Concluderend laat dit proefschrift de ontwikkeling zien van het CD20/anti-CD20 suïcide systeem voor adoptieve T cel immuuntherapie en van het onderzoek naar het mechanisme van CD20/anti-CD20 antilichaamtherapie. De resultaten kunnen leiden tot een effectievere behandeling van hematologische maligniteiten.

Dankwoord, Curriculum Vitae, List of Publications

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Tom

Curriculum Vitae

Tom van Meerten werd geboren op 3 februari 1980 te Veenendaal. In 1998 werd het HAVO diploma behaald aan het Christelijk Lyceum Veenendaal. Vanaf 1998 volgde hij de opleiding Moleculaire Biologie aan de Hogeschool van Utrecht, waarvan hij in 2002 het Bachelor diploma behaalde. Tijdens deze studie werd de wetenschappelijke stage gedaan op het Jordan Laboratorium van de afdeling Hematologie (Universitair Medisch Centrum Utrecht, dr. ACM Martens) met als onderwerp locale secretie van GM-CSF-DT fusietoxine door retroviraal genetisch gemodificeerde stamcellen voor de behandeling van MRD in AML. Aansluitend is hij in 2002 begonnen als assistent in opleiding bij de afdelingen Hematologie en Immunologie (Universitair Medisch Centrum Utrecht, dr. SB Ebeling en prof. dr. A. Hagenbeek). Het onderzoek verricht in deze periode (2002-2006) is beschreven in dit proefschrift. In 2006 is hij begonnen aan de studie Geneeskunde, volgens de Master opleiding tot Arts/Onderzoeker (SUMMA, Selective Medical Master Utrecht).

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Submitted.