Aspects of the mode of action of bispecific T cell engager (BiTE) antibodies

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"Der erste Trunk aus dem Becher der Naturwissenschaft macht atheistisch,
aber auf dem Grund des Bechers wartet Gott."

Werner Heisenberg

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1 Introduction

1.1 Monoclonal antibodies in cancer therapy

In past decades, many new strategies have been developed to relieve cancer patients of their tumor burden load. It was found that special care has to be directed towards tumor stem cells and residual tumor cells, which remain at the tumor site after surgery, in order to prevent recurrence of tumors. Alongside the classical chemo- and radiation therapy, monoclonal antibodies were developed as a promising means to fight tumors. Therapeutic antibodies are directed against tumor-associated antigens, which arise from over-expression of normal proteins in malignant cells or are derived from differentiation antigens, which are characteristic of a certain stage of normal tissue differentiation.

Monoclonal antibodies exert their cytotoxic effect via different pathways. Bevacizumab (Avastin) sequesters VEGF, which is necessary for angiogenesis in and around growing tumors (Ferrara et al. 2005). Gemtuzumab (Mylotarg) is conjugated to a chemotherapeutic drug (Duong and Sekeres 2009), ibritumomab (Zevalin) and tositumomab (Bexxar) to radioactive isotopes (Smith and Sweetenham 2007; Otte et al. 2009); each delivering death to their binding partners, the tumor cells.

In another approach, the immunological activity of antibodies is used to engage the patient's own immune system to eliminate tumor cells by using the natural mechanism of antibody-dependent cellular cytotoxicity (ADCC). Monoclonal antibodies bind to the tumor tissue flagging it for destruction by immune effector cells. Natural killer (NK) cells, macrophages and neutrophils are activated by the binding of the antibodies' Fc portions with their Fc receptors resulting, subsequently, in the secretion of cytotoxic granules (Bonnema et al. 1994; Tapper 1996). Macrophage- and neutrophil-derived granules contain hydrolytic enzymes, reactive oxygen intermediates and nitric oxide, whereas NK cells lyse malignant cells via perforin and granzymes.

The four most prominent members of tumor tissue-targeting monoclonal antibodies are: the anti-CD20 antibody rituximab (Rituxan/MabThera) (Vidal et al. 2009); trastuzumab (Herceptin), directed against the Her2/neu tyrosine kinase receptor (Tolaney and Krop 2009); cetuximab (Erbitux) targeting EGFR (Mehra et al. 2008); the anti-CD52 antibody alemtuzumab (Campath-1H) (Alinari et al. 2007).

However, approved antibodies have had limited success in eradicating solid tumors when administered as a monotherapy and they cover only a small percentage of cancer patients according to their binding specificities.

Conventional antibody therapy recruits effectors of the innate immune system but cannot engage the most potent effectors, the T cells, as these lack an Fcγ receptor. A novel class of antibodies, bispecifics, is being developed to recruit T cells for elimination of tumor cells. They are equipped with two non-identical binding arms. Bispecific antibodies providing a binding entity for CD3, which is a potent signaling protein on the surface of T cells, will mobilize any antigen-experienced pre-existing T cell as effector cell. The second arm, binding to a tumor-associated antigen, will ensure that T cell activity is restricted to the tumor tissue. Bispecific antibodies bind to the tumor forming at the same time a matrix of binding sites for T cells. A passing T cell will establish a connection with tumor-associated antibodies and subsequently release the contents of its lytic granules leading to apoptosis of the bound partner. The first, and so far only, approved member of the group of bispecific antibodies is catumaxomab (Removab), which targets CD3 and EpCAM and is indicated for the intraperitoneal treatment of malignant ascites in patients with EpCAM-positive carcinomas (Sebastian et al. 2009). Removab is also termed trispecific as its intact Fc region provides a third functional binding site for cells with an Fc receptor.

1.2 Bispecific T cell engager in cancer therapy

The bispecific T cell engager (BiTE) displays a very specific design within the group of bispecific antibodies. It is composed of the two binding domains (variable heavy- and light chain domains) of two different human IgG antibodies flexibly linked by a short nonimmunogenic peptide. Figure 1 depicts the composition of a BiTE molecule.

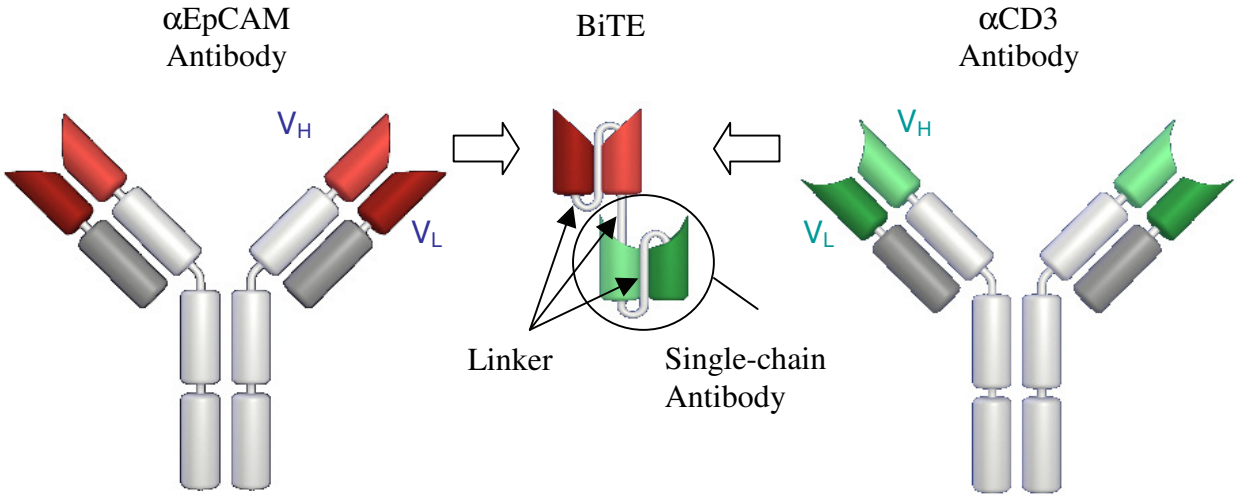


Figure 1 Construction of a BiTE molecule from two monoclonal antibodies
Via recombinant DNA technology, variable V_H and V_L domains of each antibody are fused into a single-chain antibody that are interconnected by a short peptide linker to give the BiTE construct (Baeuerle et al. 2008).

BiTE molecules consist of a single nonglycosylated polypeptide chain of 55 to 60 kDa molecular weight. Their small size allows them to bring T cells and tumor cells into close proximity when both cell populations are bound to the respective binding arm.

With their binding specificity for CD3, which is invariably present on all mature T cells, BiTE molecules recruit T cells regardless of their T cell receptor (TCR) specificity. This feature renders BiTE-mediated tumor cell lysis independent of intracellular processing and surface presentation of tumor peptide antigens, which are major weak points in tumor cells.

T cell activation induced by a BiTE molecule does not require a co-stimulatory signal by CD28 or CD4/CD8. However, it does mostly involve effector memory T (T_{EM}) cells that have already encountered an antigen but not naïve T cells. As the whole population of T_{EM} cells could possibly function as effector cells, the resulting immunologic reaction is referred to as a polyclonal T cell response.

Upon establishment of the T cell-BiTE-tumor cell tripartite unit, an immunological synapse forms between effector cell and target cell that shows all the hallmarks of synapses induced by MHC-restricted lysis (Offner et al. 2006).

1.3 Lysis of pathogenic cells by T cells

The immune system naturally provides several populations of effector cells to eliminate diseased cells, the most effective being T cells and NK cells.

NK cells are part of the innate immune system. They do not proliferate significantly upon a stimulation signal but they can mediate cytotoxicity within minutes because they contain pre-formed granules filled with cytolytic proteins, such as granzymes (granule enzymes) and perforin (Moretta et al. 2008).

T cells belong to the adaptive immune system and are further subdivided into $CD4^+$ and $CD8^+$ T cells, referring to co-receptors on their surfaces. Naïve $CD8^+$ cytotoxic T lymphocyte (CTL) precursors do not show lytic activity. $CD8^+$ T cells reach maximal activity after 1 - 3 days of persistent stimulation of the T cell receptor with MHC + peptide antigen and a CD28 co-stimulus. TCR stimulation results in formation of granzyme- and perforin-containing granules that equip the cells for elimination of multiple cells. At the next encounter with a target cell presenting the T cell's cognate antigen in MHC class I context, the T cells kill target cells within minutes by re-orientation and release of granules. $CD4^+$ T cells recognize MHC class II on target cells and antigen-presenting cells (APC). Besides cytotoxic activity, they also mediate activation of macrophages and B cells.

$CD4^+$ and $CD8^+$ T cells both use two different effector pathways to kill infected or malignant cells: death receptor-mediated and granule-mediated killing. The latter being the main pathway of destruction (Yasukawa et al. 2000; de Vries et al. 2007). In the death receptor-mediated pathway, expression of genes of the tumor necrosis factor (TNF) family of ligands is induced in T cells in response to TCR signaling in a process that needs 1 - 2 h (Henkart 1994). Ligands are transported to the surface of effector cells where they interact with members of the TNF receptor gene superfamily on target cells (Locksley et al. 2001). These receptors share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" (Ashkenazi and Dixit 1998). Pairs of ligands and receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Chicheportiche et al. 1997; Ashkenazi and Dixit 1998; Peter and Krammer 1998; Suliman et al. 2001; Rubio-Moscardo et al. 2005). Upon establishment of binding between a ligand and its receptor, a caspase cascade is initiated inside the target cell (Launay et al. 2005). Receptor-mediated induction of the caspase cascade is referred to as the extrinsic pathway of caspase activation (Figure 3).

Granule exocytosis has been reported for both $CD4^+$ and $CD8^+$ T cells as a means of eliminating target cells (Hahn et al. 1995; Appay et al. 2002). Upon TCR stimulation, the expression of granule components is induced in T cells. Perforin and various granzymes are produced as effector molecules for the lysis of target cells. Granzymes are a subclass of serine proteases displaying the chymotrypsin fold (Smyth et al. 1996). Among these proteases, granzyme B is the best studied. Granzyme A can also induce target cell death when applied together with perforin (Froelich et al. 2009). Three more granzymes have been found in humans: granzyme H (a chymase) (Edwards et al. 1999), granzyme K (a tryptase) and granzyme M (a metase) (Kam et al. 2000). All of them can contribute to the lysis of malignant cells, albeit via different pathways (Chowdhury and Lieberman 2008). Granzymes need to be translationally modified. First a signal peptidase removes the signal peptide, then a second enzyme removes a short pro-sequence. For granzymes A and B, dipeptidyl peptidase I (DPPI) cleaves off the pro-sequence (McGuire et al. 1993; Smyth et al. 1995). After cleavage, granzymes are glycosylated and sorted into specialized granules in the Golgi apparatus (Griffiths and Isaaz 1993; Griffiths 1997). Highly positively-charged granzymes are packed in the granules using serglycin as a scaffold (Galvin et al. 1999).

Similarly, perforin needs to be processed into an active form; the 70 kDa precursor is cleaved at the carboxylterminus to yield the 60 kDa active form (Uellner et al. 1997).

When a T cell receptor recognizes its cognate antigen on an MHC molecule of a target cell and CD4/CD8 co-receptors bind to the MHC molecule, a structure named an immunological synapse forms between the two cells. The junction is initiated by the T cell receptor binding MHC on the target cell but maintained by adhesion molecules like ICAM-1 on target cells and LFA-1 on T cells. The granules inside the effector cell migrate towards the site of contact and fuse with the plasma cell membrane, their contents then being secreted into the tight intercellular junction.

Free calcium near the region of contact causes perforin to polymerize in the target cell membrane forming ring-like structures with a central pore of 5 - 20 nm diameter (Millard et al. 1984; Tschopp et al. 1986; Sauer et al. 1991). This structure is considered too small for the passage of granzymes, which range in size from 30 to 65 kDa and are even larger in complex with serglycin. It is believed that other critical death-inducing enzymes might pass through it, but, importantly, extracellular Ca^{2+} can enter the cell via the pore. The resulting rise of the Ca^{2+} level in its cytosol is sensed by the target cell as a sign of a damaged membrane (Keefe et al. 2005). Endosomes, lysosomes and other intracellular vesicles are directed to the site of damage with their membranes used to seal the holes (Miyake and McNeil 1995; Reddy et al. 2001). At some point in this process, perforin and granzymes are co-endocytosed and stored in large endosomes (Pipkin and Lieberman 2007). Endocytosis of granzyme B might happen via binding to the cation-independent mannose-6-P receptor (CI-MPR) in the immunological synapse (Motyka et al. 2000), or via electrostatic interactions between positively charged granzyme B and anionic components of the cell surface (Shi et al. 2005). Granzyme B is internalized and released to the endolysosomal compartment. Perforin then perturbs the endosomal membrane to release granzymes into the cytoplasm of the target cell (Froelich et al. 1996).

1.4 Role of granzyme B in T cell-mediated cell death

Granzyme B contributes to apoptosis via multiple pathways. The proteinase cleaves inactive pro-caspase 3 resulting in functional caspase 3 (Adrain et al. 2005), which is the key executioner cleaving apoptosis regulators, house-keeping proteins and the endonuclease CAD. When CAD is released from its inhibitor ICAD, it leads to fragmentation of DNA (Gorelik and Flavell 2002). Granzyme B can also directly cleave the CAD/ICAD complex without the help of caspase 3 (Thomas et al. 2000; Sharif-Askari et al. 2001).

Granzyme B-mediated cleavage of Bid links the proteinase to the intrinsic pathway of caspase activation (Barry et al. 2000; Sutton et al. 2000). Cleavage product truncated Bid (tBid)

inserts into the mitochondrial outer membrane, interacts with its receptors Bak and Bax and acts as a death ligand capable of inducing oligomerization of Bak (Marsden and Strasser 2003). Oligomerized Bak forms a channel that permits release of cytochrome *c* (Wang et al. 2001), which when present in the cytosol induces the formation of the apoptosome with Apaf-1 and pro-caspase 9 thereby triggering autoactivation of pro-caspase 9 (Li et al. 1997). Active caspase 9 activates caspases 3, 6 and 7 (Figure 2).

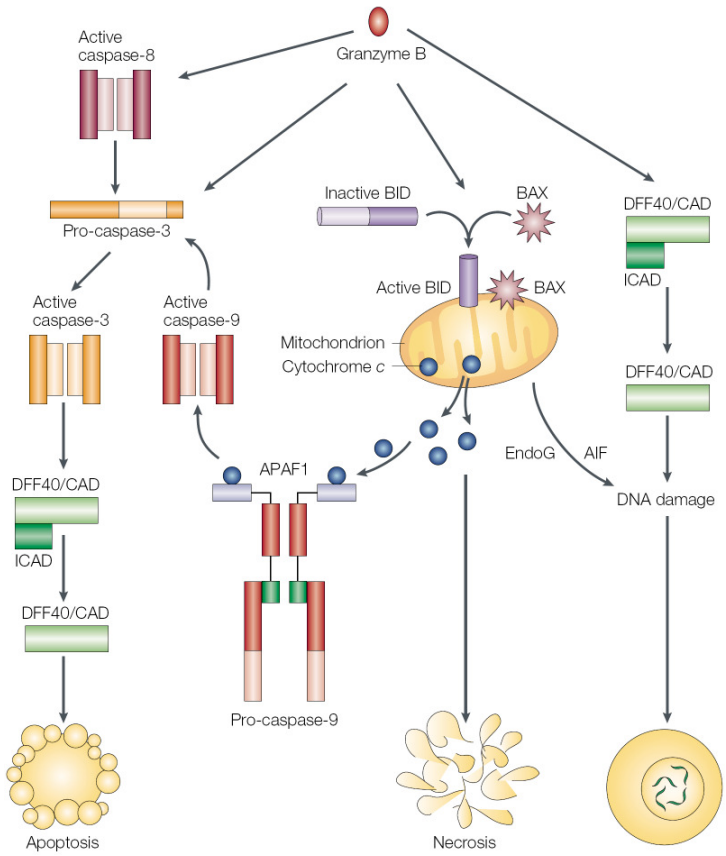


Figure 2 Granzyme B-mediated apoptotic signals
See text for detailed explanations (Barry and Bleackley 2002).

1.5 Role of caspases in T cell-mediated cell death

Caspases are cysteine proteases that can cause cell death via an extrinsic or an intrinsic pathway, both of which are interconnected (Launay et al. 2005).

The intrinsic caspase pathway is usually induced by developmental signals, chemotherapeutic agents or withdrawal of survival factors causing release of cytochrome *c* from mitochondria. Cytosolic cytochrome *c* binds to Apaf-1 (Zou et al. 1997), which then associates with pro-caspase 9 to form the apoptosome, a platform for autoactivation of pro-caspase 9 (Li et al. 1997). Active caspase 9 activates effector caspases (caspases 3, 6 and 7).

In the extrinsic pathway, initiation of the caspase cascade is triggered by a death receptor binding its cognate ligand. Upon binding, a cytoplasmic adapter protein, e.g., FADD, and pro-caspase 8 (or 10) are recruited to the death domain of the receptor forming the death-inducing signaling complex (DISC) (Medema et al. 1997). FADD brings into close proximity two or more pro-caspase 8 (or 10) molecules leading to auto-catalytic activation of caspase 8 (or 10) (Kischkel et al. 1995; Nunez et al. 1998). These enzymes (initiator caspases) activate caspases 3, 6 and 7 (effector caspases).

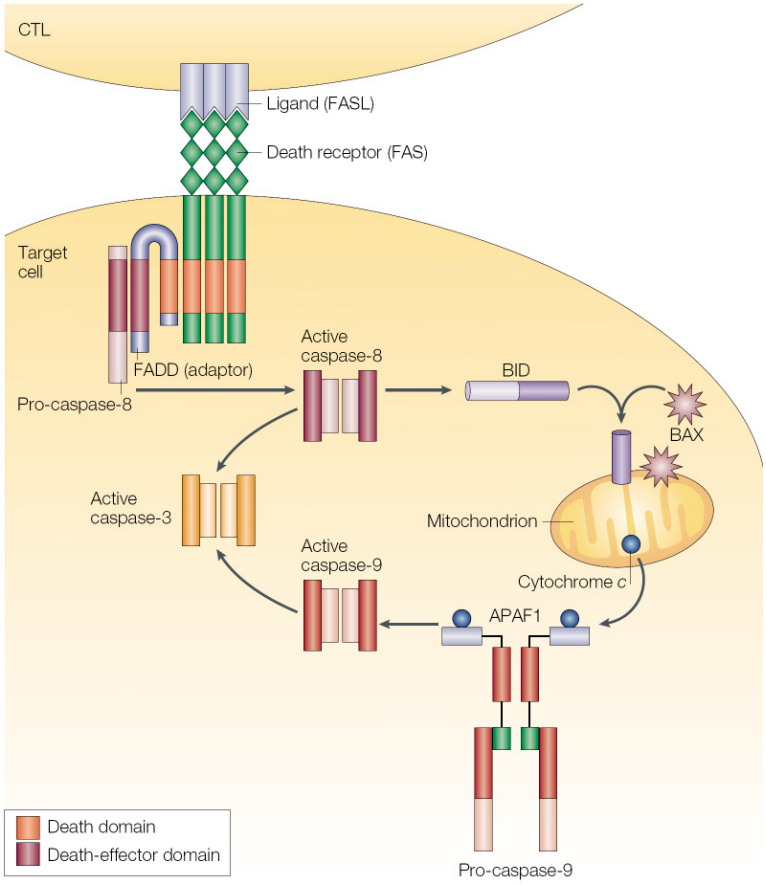


Figure 3 Fas-mediated apoptotic signal and caspase cascade
See text for detailed explanations (Barry and Bleackley 2002).

Functional caspases 8 and 10 link the two pathways by cleaving Bid, a pro-apoptotic member of the Bcl-2 family (Nunez et al. 1998). Cleaved Bid binds to mitochondria and induces the release of cytochrome *c* triggering the formation of the apoptosome by Apaf-1 and pro-caspase 9 (Li et al. 1998; Luo et al. 1998).

Both pathways result in activation of effector caspases. Activated caspase 3 cleaves various substrates including cytokeratins, poly (ADP-ribose) polymerase (PARP), and cytoskeletal and nuclear proteins (Slee et al. 2001). Most importantly, it activates the cytoplasmic

endonuclease caspase-activated DNAse (CAD) (Sakahira et al. 1998). CAD exists as a heterodimer with Inhibitor of CAD (ICAD). Cleavage from ICAD allows CAD to assemble into its active form (Liu et al. 1997; Wolf et al. 1999), which then degrades chromosomal DNA and causes chromatin condensation.

Chromosomal DNA is fragmented into stretches of ~ 300 kb and subsequently into smaller DNA pieces. The target cell starts to disassemble and vesicles containing cytoplasm and plasma membrane are formed internally. The vesicles and the remaining core cell are taken up by phagocytes so their constituents can be reused.

1.6 Tumor escape mechanisms

Tumor cells have evolved a multitude of ways to escape the recognition, interaction and most importantly destruction by T cells. These escape mechanisms can be divided into several categories.

(1) Altered machinery for generation and presentation of peptide antigens to T cells.

Tumor cells stop or greatly reduce expression of the MHC class I receptor (Zheng et al. 1999). They express a nonfunctional variant of the transporter associated with antigen processing (TAP) or stop expression completely; TAP is a protein that is responsible for transporting peptides of cytosolic proteins into the lumen of the endoplasmic reticulum (Johnsen et al. 1999). They fail to produce β_2-microglobulin, the light chain of MHC I (Momburg and Koch 1989).

(2) Tumor cells express proteins that impede survival, differentiation or activation of T cells.

Most human epithelial tumors express B7-H1, a receptor for the membrane protein PD-1 that is found on effector T cells (Dong et al. 2002; Curiel et al. 2003). B7-H1 is a member of the B7 family but acts in a co-inhibitory rather than co-stimulatory manner. When bound to PD-1, it causes apoptosis of effector T cells.

Expression of indoleamine 2,3-dioxygenase (IDO) reduces free tryptophan in the tumor environment. IDO catalyzes the oxidative catabolism of tryptophan. As this amino acid is essential for T cell proliferation and differentiation, clonal expansion of T cells is prevented and T cell death promoted (Munn et al. 2002).

Nitric-oxide synthase 2 (NOS2) oxidizes L-arginine (L-Arg) to citrulline and nitric oxide. Nitric oxide is important for neo-angiogenesis, cell growth and differentiation, and favours tumor metastasis. Furthermore, NOS2 impedes phosphorylation of STAT5, Akt and Erk preventing signalling through the IL-2 receptor and activation of several signaling molecules resulting in T cell apoptosis (Bronte et al. 2005).

Arginase-1 (ARG1) depletes the milieu of L-Arg by hydrolizing it to urea and ornithine (Bronte et al. 2005). Tumor-induced expression of ARG1 by myeloid suppressor cells impairs antigen-specific T cell responses and the development of a T cell memory phenotype (Bronte et al. 2003).

Transforming growth factor-beta (TGF-β) inhibits the activation, proliferation and activity of lymphocytes *in vivo* (Fontana et al. 1989).

(3) Tumor cells directly influence effector mechanisms of T cells.

By expression of anti-apoptotic proteins like Bcl-x_L and Bcl-2 (Vaux et al. 1988), or by down-regulation or mutation of pro-apoptotic proteins like Bax and Bak, tumor cells aquire resistance to apoptosis (O'Connell et al. 2000).

Over-expression of proteinase inhibitor 9 (PI-9) is another method by which cancer cells inhibit the induction of apoptosis. The serpin PI-9 is a potent inhibitor of granzyme B, forming a tight complex with it. PI-9 is found in the cytoplasm and nuclei of CTLs and is believed to protect CTLs from granzyme B that is misdirected to the effector cell after granule secretion (Sun et al. 1997; Bird et al. 1998).

Tumor cells can down-regulate Fas receptor (Landowski et al. 1997), express nonfunctioning Fas receptor (Landowski et al. 1997), or secret high levels of a soluble form of the Fas receptor (Cheng et al. 1994). Some tumor cells even express Fas ligand on their surface which renders T cells apoptotic (Koyama et al. 2001).

TGF-β suppresses CTL function through an anticytotoxic program of transcriptional repression that inhibits expression of perforin, granzyme A, granzyme B, Fas ligand and IFN-γ (Thomas and Massague 2005).

(4) Cancer cells produce agents that have an influence on differentiation and function of dendritic cells (DC) that are the most potent among antigen presenting cells (APC). They take up, process and present antigen to other immune cells (Zou 2005). Tumor cells can be major producers of vascular endothelial growth factor (VEGF) (Carmeliet and Jain 2000; Kryczek et al. 2005), which suppresses differentiation and maturation of DCs (Gabrilovich et al. 1996). A range of human tumors express cyclooxygenase-2 (COX2) (Wolff et al. 1998; Joki et al. 2000; Shono et al. 2001), a promoter of prostaglandin E2 (PGE2) production. PGE2 suppresses differentiation and function of DCs (Kalinski et al. 1998; Sombroek et al. 2002; Jozefowski et al. 2003; Akasaki et al. 2004).

Tumor factors present in the local environment can induce DCs to express B7-H1 as a membrane-bound receptor. Tumor-associated T_{reg} cells bind B7-H1 with their ligand PD-1 and subsequently suppress IL-12 production by DCs (IL-12 being a promoter of NK and T

cell activity) (Curiel et al. 2003). It is even hypothesized that tumor antigen presentation by cancer cells leads to T cell tolerance when accompanied by cross-presentation of tumor antigen by bone marrow-derived APCs (Horna and Sotomayor 2007). Interleukin 10 (IL-10) and TGF-β expressed by tumor cells suppress DC maturation and function (De Smedt et al. 1997; Zou 2005).

TGF-β is a very potent immunosuppressive agent that acts on several levels and its production and activation is considered one of the most potent mechanisms employed by tumor cells to avoid clearance (Wojtowicz-Praga 2003). Besides decreasing the proliferation of T cells, suppressing CTL function and reducing the amount of antigen presentation by DCs, TGF-β inhibits the activity of IFN-γ, suppresses cytotoxic activity of NK cells and stimulates the proliferation of T_{reg} cells (Zitvogel et al. 2006).

BiTE-activated T cells do not rely on the tumor peptide-processing and -presenting machinery nor are they dependent on DCs. BiTE-mediated tumor cell lysis can therefore be triggered even in the presence of the related tumor escape mechanisms. Nonetheless, other types of tumor escape mechanisms might possibly be influential.

1.7 The target antigen EpCAM

This cell surface molecule gained interest when it became evident that it is differentially expressed on healthy and malignant tissue. Accordingly, it was chosen as the target antigen for the BiTE molecule MT110.

1.7.1 Structure

Epithelial Cell Adhesion Molecule (EpCAM) is a glycoprotein of about 40 kDa that is present on almost all normal epithelia and forms a noncovalent *cis*-dimer in the membrane. It does not belong to one of the four CAM families (cadherins, selectins, integrins or the immunoglobulin CAM superfamily) as it does not share any of their structural patterns. EpCAM consists of an extracellular portion, a single-spanning transmembrane domain and a cytoplasmic sequence. The intracellular domain contains several alpha-actinin binding sites (Balzar et al. 1999).

The extracellular portion consists of a leading signal sequence, an epidermal growth factor (EGF)-like repeat, a human thyroglobulin (TY) repeat and a cysteine-poor domain (Balzar et al. 1999). As N- and C-termini of TY domains were found to be in close proximity in many proteins, domains within the EpCAM molecule are suggested to be arranged as depicted in Figure 4 (Baeuerle and Gires 2007).

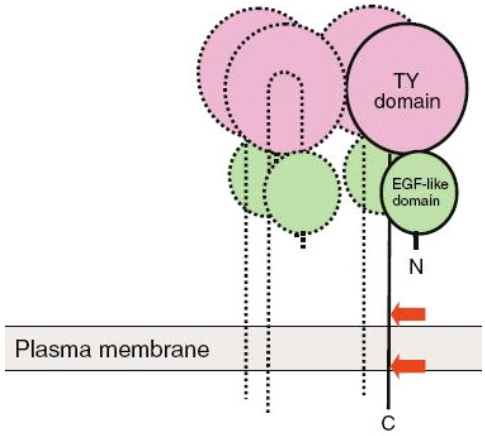


Figure 4 Structural model of EpCAM
An EpCAM tetramer is shown with one continuous-line subunit and three additional subunits indicated by dotted lines. Red arrows mark cleavage sites for proteases (Baeuerle and Gires 2007).

The molecule's proteolytic cleavage sites regulate the cells' adhesiveness. When an adenocarcinoma cell line from the rat was treated with protease, cell-cell adhesion was strengthened (Wurfel et al. 1999). The EGF-like repeat might be a target for differential glycosylation. Carcinoma cells not only show a different glycosylation pattern from normal epithelial cells but glycosylation can also vary between individual carcinomas (Pauli et al. 2003). Variable glycosylation of EpCAM may decide its specific binding partner and consequently regulate adhesiveness of $EpCAM^+$ cells.

1.7.2 Tissue distribution

Normal epithelia express EpCAM at variable levels, mostly on the basal or basolateral cell membrane. In dysplasia and malignancy, EpCAM is also found on the apical membrane (Winter et al. 2003). Its accessibility for an antibody is therefore high on tumor tissue and almost absent on healthy tissue. Virtually all tumors of epithelial origin express EpCAM at a high level. Sarcomas, melanomas, lymphomas and other tumors of mesodermal and ectodermal origin do not express EpCAM (Momburg et al. 1987). Distribution of EpCAM varies in carcinoma depending on the differentiation status and type of carcinoma. For example, adenocarcinoma cells in colon carcinoma show a homogeneous basolateral distribution of EpCAM when they are well-differentiated but a membranous, cytoplasmic and luminal expression when only moderately differentiated (Ogura et al. 1998). In general, well-differentiated tumors are more expressive than those less differentiated (Chaudry et al. 2007).

1.7.3 Binding partners and function

In homotypic cell-cell adhesion, the extracellular domain of EpCAM can interact with a second EpCAM molecule on another cell. Indeed dimers on opposing membranes can interact and form a tetramer. This might play a role in the immune response against mucosal infections. Through its alpha-actinin binding sites in its cytoplasmic domain, EpCAM is linked to the actin cytoskeleton (Guillemot et al. 2001). Its direct interaction with CD44v4-v7, tumor metastasis-promoting variants of CD44, and with claudin-7, a tight junction protein, potentially influences cell-cell and cell-matrix adhesion and induces apoptosis resistance and metastasis (Schmidt et al. 2004; Le Naour and Zoller 2008).

EpCAM indirectly associates with the cell adhesion molecule E-Cadherin via adaptor proteins. Cadherins are crucial for the establishment and maintenance of epithelial cell polarity and regulation of cell proliferation and apoptosis. They are linked with the actin cytoskeleton by alpha-, beta- and gamma-catenins. Beta-catenin is a member of the Wnt pathway. Upon binding of Wnt to a receptor of the Frizzled family, beta-catenin is stabilized, accumulates in the cytoplasm and enters the nucleus where it associates with a lymphoid enhancer factor/T cell factor transcription factor. The complex activates expression of target genes like c-Myc, VEGF and cyclooxygenase-2. As EpCAM up-regulates the proto-oncogene c-myc it is hypothesized that EpCAM inhibits E-Cadherin causing the level of free beta-catenin in the cell to increase and finally the expression rate of its target genes to augment (Osta et al. 2004).

EpCAM acts as a functional antagonist for E-Cadherin (Litvinov et al. 1997). In disrupting the link between alpha-catenin and F-actin, EpCAM loosens the tight intercellular adhesions and modulates proliferation, differentiation and tissue maintenance (Winter et al. 2003; Osta et al. 2004). Over-expression of EpCAM in carcinoma cells might therefore be a mechanism to disrupt cell-cell contact to enable cell migration required for metastasis.

EpCAM furthermore up-regulates the expression of nuclear factor (NF)-κB, cyclin A and E (Munz et al. 2004) and epidermal fatty acid-binding protein (E-FABP), a target gene of c-myc (Munz et al. 2005). In summary it is therefore likely that EpCAM is involved in cell migration, metastasis and cell signaling.

EpCAM is expressed at higher levels on malignant than on healthy tissue. Therefore tumor cells are the primary target of MT110 before other systemic cells. Additionally, EpCAM in non-malignant tissue is hardly accessible for antibodies as it is predominantly expressed at the basolateral membrane. Only cancerous tissue shows EpCAM expression on the apical membrane.

1.8 The target antigen CD19

The cell surface molecule CD19 is expressed only by B lymphocytes and follicular dendritic cells of the hematopoietic system. It is composed of a 240 amino acid cytoplasmic tail and a 280 amino acid extracellular domain that contains two C2-type Ig-like domains separated by a smaller potentially disulfide-linked domain. CD19 assembles with the antigen receptor of B lymphocytes in order to decrease the threshold for antigen receptor-dependent stimulation (Carter and Fearon 1992). Unlike EpCAM, the cell surface molecule CD19 is not differentially expressed on malignant and healthy tissue.

T cells activated with the BiTE molecule MT103 eliminate all B cells regardless of their malignancy status. However, complete eradication of B cells does not seem to pose a problem for patients as production of B cells in the bone marrow does not cease.

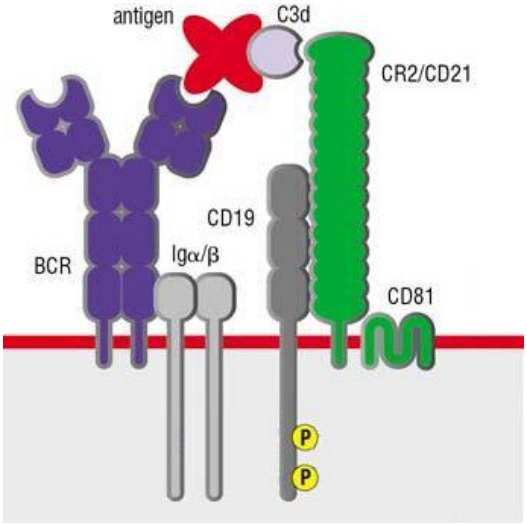


Figure 5 The B cell receptor and its co-receptor
The B cell receptor complex consists of cell-surface immunoglobulin associated with the invariant signaling proteins Igα and Igβ. B cell antigen receptor signaling is modulated by a co-receptor complex of at least three cell-surface molecules, CD19, CD21, and CD81. CD21 (complement receptor 2 (CR2)) is a receptor for the C3d fragment of complement. Antigens bound to C3d can cross-link the B cell receptor with its co-receptor. This induces phosphorylation of tyrosine residues in the cytoplasmic domain of CD19 by B cell receptor-associated kinases resulting in augmentation of signaling through the B cell receptor (DeFranco et al. 2007).

1.9 Finding the initial dose for a first-in-man clinical trial

In order to proceed with clinical testing of the BiTE molecule MT110, the first clinical trial had to be approved. To receive approval, a detailed study plan had to be designed with precise indications of duration of treatment, concentration of MT110 and possible adverse effects. According to usual procedure, initial treatments were planned with a low concentration (starting dose) of MT110 that would under normal circumstances be subsequently escalated. Finding the starting dose is subject to strict rules.

In general, the first dose in man is estimated with reference to the "no observed effect level" (NOEL) or, more usually, the "no observed adverse effect level" (NOAEL) value (usually dose per kilogram) determined in preclinical studies in relevant animal studies. The value determined in the most sensitive animal species is further reduced by a safety factor and converted to an equivalent dose for man on the basis of allometric scaling.

For high-risk medicinal products, the initial dose should be calculated considering the minimal anticipated biological effect level (MABEL). The MABEL approach defines a stricter way of selecting an initial dose for the clinic and was proposed by the *Expert Scientific Group on Phase One Clinical Trials* which was founded in the light of the disastrous effects of TGN1412 (TeGenero AG) in a first-in-man study in London (EMEA/CHMP/SWP/28367/2007). TGN1412 is a superagonistic monoclonal antibody that binds to CD28 and can activate immune cells independently of antigen presentation. All patients that received the test product suffered from fever, vomiting, strong pain, symptoms of a severe inflammation and even organ deficiency. In retrospect, the severe immune reaction most likely arose from a cytokine release storm. Polyclonal activation of immune cells including naïve T cells leads to a massive release of cytokines. The enormous amount of cytokines in the patients' blood circulation put their bodies in a shock-like state and all of them were subsequently admitted to a hospital in need of intensive care (Suntharalingam et al. 2006).

To avoid endangering first-in-man patients, the novel MABEL approach is now to be used for high-risk medicinal products. It is not solely based on therapeutic or adverse effects but rather on biological effects of any kind. The value for MABEL is calculated taking into account all available data from pharmacodynamic and pharmacokinetic experiments. Relevant information includes (1) receptor binding and occupancy data from *in vitro* and *in vivo* studies, (2) concentration response curves determined *in vitro* and *in vivo* and (3) exposures at pharmacological doses in relevant species. Safety factors are also applied in the MABEL calculation of the first dose in man.

An antibody is defined as high-risk (1) when its mode of action is not fully understood or known details give reason to assume that serious adverse reactions could occur in man, (2) when its structure was chemically or genetically modified, (3) when the nature of its target is cause for concern (tissue distribution, cell specificity, disease specificity) and (4) when a relevant animal species is lacking.

With respect to the BiTE antibodies, several of these categories apply and accordingly they can be considered high-risk medicinal products. To find a safe starting dose for the first clinical studies with MT110, the MABEL was determined.

1.10 Objective of this thesis

Bispecific antibodies constitute a novel way of treating cancer patients either to prevent recurrence of tumor after surgical removal of malignant tissue or even as a novel anti-cancer therapy in itself. With their specificity for the CD3ε invariant chain, bispecific antibodies of the BiTE platform recruit the T cells of the host immune system as effector cells. Their second binding arm allows them to detect antigens that are specific for or over-expressed on the respective tumor tissue. Series of *in vitro* experiments and data from phase I clinical studies of MT103 provide evidence for the high potency and effectiveness of BiTE molecules. Inspite of the knowledge that an immunological synapse forms between effector and target cell and that activation markers are up-regulated on T cells (Offner et al. 2006; Brischwein et al. 2007), the mechanism used by BiTE molecules to eradicate cancerous cells is largely unknown.

The primary objective of this thesis was to investigate the mode of action by which BiTE molecules activate T cells to eliminate tumor cells. Besides these mechanistical studies, further objectives included examination of the influence of TGF-β on BiTE-mediated tumor cell lysis, and the effect of glucocorticoid co-medication on T cell activation, tumor cell lysis and concomitant cytokine release. Finally, the minimal anticipated biological effect level of MT110 was to be determined.

2 Materials

2.1 BiTE molecules

MT110 was constructed by standard DNA technologies and produced in chinese hamster ovary (CHO) cells as described in (Brischwein et al. 2006). MT110 was formulated in 25 mM citrate, 200 mM lysine hydrochloride, 0.1 % Tween80 and 15 % trehalose.

Construction and production of MT103 is described in (Loffler et al. 2000). MT103 monomer was formulated at a concentration of 1 µg/ml in PBS/0.1 % HSA.

Control BiTE molecules: Mec 14 was formulated in 10 mM citrate, 75 mM lysine hydrochloride, 0.03 % Tween80 and 4 % trehalose. 5-10xKT3 was formulated in 5 mM citrate, 40 mM lysine hydrochloride, 0.02 % Tween80 and 3 % trehalose.

2.2 Chemicals

Ammonium Chloride (NH_4Cl)	Sigma-Aldrich, Taufkirchen
Basal Iscove's Medium	Biochrom, Berlin
Biocoll Separating Solution	Biochrom, Berlin
BSA	Paesel + Lorei, Duisburg
Dexamethasone	Sigma-Aldrich, Taufkirchen
Dimethylsulphoxide (DMSO)	Sigma-Aldrich, Taufkirchen
EGTA	Sigma-Aldrich, Taufkirchen
EDTA	Sigma-Aldrich, Taufkirchen
Ethanol	Merck, Darmstadt
FCS	Invitrogen, Karlsruhe
Formaldehyde	Merck, Darmstadt
General Caspase Inhibitor Z-VAD-FMK	R&D Systems, Minneapolis MN, USA
Granzyme B Inhibitor IV	Merck, Darmstadt
Human TGF-β1	R&D Systems, Minneapolis MN, USA
Insect Cell Lysis Buffer	BD Biosciences, Heidelberg
Potassium Hydrogen Carbonate ($KHCO_3$)	Merck, Darmstadt
Magnesium Chloride ($MgCl_2$)	Merck, Darmstadt
Methylprednisolone	Sigma-Aldrich, Taufkirchen
PBS	Invitrogen, Karlsruhe
Propidium Iodide	Sigma-Aldrich, Taufkirchen
Protease Inhibitor Cocktail: Complete, EDTA-free	Roche, Mannheim

RPMI 1640	Invitrogen, Karlsruhe
RPMI 1640 (containing 25 mM HEPES and 2 mM L-Glutamine)	Invitrogen, Karlsruhe
Sodium Azide (NaN_3)	Merck, Darmstadt
Staurosporine	Sigma-Aldrich, Taufkirchen
Sulfuric Acid (H_2SO_4)	Merck, Darmstadt
Tetramethylbenzidine (TMB) Liquid Substrate System	Sigma-Aldrich, Taufkirchen
5 % Trypsin-EDTA	Invitrogen, Karlsruhe
Tumor Necrosis Factor-alpha (TNF-α)	NatuTec, Frankfurt am Main
Tween 20	Sigma-Aldrich, Taufkirchen

Erythrocyte Lysis Buffer	dH_2O	1 l
	Ammonium Chloride (NH_4Cl)	8.29 g
	Potassium Hydrogen Carbonate ($KHCO_3$)	1 g
	EDTA	0.037 g
Buffer for CD3 T cell enrichment	PBS	
	BSA	0.5 %
	EDTA	2 mM
FACS Buffer	PBS	
	FCS	1 %
	Sodium Azide (NaN_3)	0.05 %
ELISA Wash Buffer	PBS	
	Tween 20	0.05 %

2.3 Antibodies

Specificity	Conjugation	Clone	Species	Supplier
anti-CD4	FITC	SK3	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-CD4	PE-Cy5	RPA-T4	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-CD8	PE-Cy5	RPA-T8	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-CD8	APC-Cy7	SK1	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-CD2	FITC	RPA-2.10	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-CD11a/ anti-LFA-1	FITC	HI111	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-CD25	PE	M-A251	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-CD25	APC	CD25-3G10	Mouse IgG_1	Invitrogen, Karlsruhe
anti-CD69	PE	FN50	Mouse IgG_1, κ	BD Biosciences, Heidelberg
anti-granzyme B	FITC	GB11	Mouse IgG_1, κ	BD Biosciences, Heidelberg

anti-perforin	FITC	δG9	Mouse IgG_{2b}	BD Biosciences, Heidelberg
anti-foxp3	PE	PCH101	Rat IgG_{2a}, κ	eBioscience, San Diego CA, USA
Isotype control	FITC	MOPC-21	Mouse IgG_1, κ	BD Biosciences, Heidelberg
Isotype control	FITC	27-35	Mouse IgG_{2b}	BD Biosciences, Heidelberg
Isotype control	PE	MOPC-21	Mouse IgG_1, κ	BD Biosciences, Heidelberg
Isotype control	PE	R35-95	Rat IgG_{2a}, κ	BD Biosciences, Heidelberg
anti-CD3 (Ortho-clone OKT3)	-		Mouse IgG_{2a}	Janssen-Cilag GmbH, Neuss
anti-CD28	-	L293	Mouse IgG_1, κ	BD Biosciences, Heidelberg

2.4 Analytical kits

Pan T Cell Isolation Kit II	Miltenyi Biotec, Bergisch Gladbach
PKH26 Red Fluorescent Cell Linker Kit	Sigma-Aldrich, Taufkirchen
BD Cytofix/Cytoperm™ Kit	BD Biosciences, Heidelberg
Caspase-Glo™ 3/7 Assay	Promega, Madison WI, USA
ToxiLight® BioAssay Kit	Cambrex, Rockland ME, USA
Human Cleaved PARP ELISA Set	BD Biosciences, Heidelberg
Apoptosis Detection Kit (APO-Direct)	BD Biosciences, Heidelberg
Human Th1/Th2 Cytokine Kit II	BD Biosciences, Heidelberg

2.5 Eucaryotic cell lines

$EpCAM^+$ Kato III	human gastric carcinoma	European Collection of Cell Cultures (ECACC, Salisbury, UK)
$EpCAM^+$ SW-480	human colon adenocarcinoma	Deutsche Sammlung von Mikroorganismen und Zelllinien (DSMZ, Braunschweig, Germany)
$CD19^+$ Nalm-6	human B cell precursor leukemia	Deutsche Sammlung von Mikroorganismen und Zelllinien (DSMZ, Braunschweig, Germany)
$CD19^+$ Mec-1	human chronic B cell leukemia	Deutsche Sammlung von Mikroorganismen und Zelllinien (DSMZ, Braunschweig, Germany)

2.6 Laboratory consumables

Flasks for cell culture: 80 cm^2 and 175 cm^2	Nunc, Roskilde, Denmark
Conical Tubes: 15 ml and 50 ml	BD Biosciences, Heidelberg
High-Speed Centrifuge Tubes	Nalgene, Rochester NY, USA
Safe-Lock Tubes	Eppendorf, Hamburg
U-tubes: 1.4 ml	Micronic, Lelystad, The Netherlands

Cryogenic Vials	Nunc, Roskilde, Denmark
Serological pipets	BD Biosciences, Heidelberg
Pipette Tips	Gilson International, Limburg-Offheim
MS columns	Miltenyi Biotec, Bergisch Gladbach
Petri Dishes (150 mm x 15 mm)	BD Biosciences, Heidelberg
6-well plates, clear, Flat Bottom	BD Biosciences, Heidelberg
96-well microplates	
Clear, U Bottom	Greiner Bio-One, Frickenhausen
Clear, V Bottom	A. Hartenstein, Würzburg
White, Flat Bottom	Nunc, Roskilde, Denmark
Clear, Flat Bottom, Maxisorp	Nunc, Roskilde, Denmark

2.7 Equipment

Freezing Container	Thermo Fisher Scientific, Waltham MA, USA
Biological Safety Cabinet	Kendro Laboratory Products, Langenselbold
Heraeus HERAsafe HS 12	
Centrifuge Mikro 20	Hettich, Tuttlingen
Centrifuge Rotina 35 R	Hettich, Tuttlingen
Centrifuge Rotanta 46 RS	Hettich, Tuttlingen
Centrifuge Sorvall RC-5C PLUS	Thermo Fisher Scientific, Waltham MA, USA
CO_2 Incubator Heraeus HERAcell	Kendro Laboratory Products, Langenselbold
ELISA Reader PowerWave X Select	BioTek Instruments, Bad Friedrichshall
ELISA Washer Skan Washer 400	Molecular Devices, Sunnyvale CA, USA
Flow Cytometer FACSCalibur	BD Biosciences, Heidelberg
Flow Cytometer FACSCanto II	BD Biosciences, Heidelberg
Hemocytometer	Paul Marienfeld, Lauda-Königshofen
Ice Machine AF 80	Scotsman, Vernon Hills IL, USA
Luminometer SpectraFluor Plus	Tecan, Crailsheim
Microscope Axiovert 25 C	Carl Zeiss, Oberkochen
MiniMACS™ Separator	Miltenyi Biotec, Bergisch Gladbach
Pipettes	Gilson International, Limburg-Offheim
Finnpipettes ®	Thermo LabSystems, Waltham MA, USA
Pipette Multipette pro	Eppendorf, Hamburg
Pipetboy acu	Integra Biociences (IBS), Fernwald

Vortex Mixer REAX top	Heidolph Instruments, Schwabach
Vortex Mixer MS2	IKA®-Werke, Staufen
Water Bath Typ 1008	GFL Gesellschaft für Labortechnik, Burgwedel

2.8 Software for data analysis, and data base

Adobe Reader 8.0

BD CellQuest™ Pro (FACS analysis)

BD FACSDiva™ (FACS analysis)

EndNote

GraphPad Prism 5

KC4 (ELISA analysis)

Microsoft Excel

Microsoft Word

XFluor (Luminometer analysis)

National Center for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/

3 Methods

3.1 Cell culture

All cells were grown in an incubator at 37 °C and a constant CO_2 level of 5 % for maintenance culture and for experiments.

Tumour cells were cultured in 30 ml RPMI 1640 supplemented with 10 % FCS in 175 cm^2 cell culture flasks and passaged every second to third day. For Kato III cells, RPMI 1640 + 10 % FCS containing 25 mM HEPES and 2 mM L-Glutamine was used. SW-480 cells and Nalm-6 were grown in RPMI 1640 + 10 % FCS without any supplements. Mec-1 cells were cultured in Basal Iscove's Medium + 10 % FCS. Every 2 to 3 days cells were split and a portion thereof transferred into fresh media to ensure proper nutrition supply. For the cytotoxicity assays, all target cells were co-cultured with effector cells in RPMI 1640 medium supplemented with 10 % FCS.

3.2 Passaging of cells

Adherent cells in culture flasks were looked at under a microscope. Depending on colour of media and number of cells, a split factor was determined to reduce the density of cells. The whole content of the cell culture flask was transferred into a 50 ml conical tube and the flask rinsed with PBS to remove all remains of media. Five ml PBS + 2.5 % Trypsin-EDTA were added to de-attach the cells from the inner surface of the flask. The flask was incubated at 37 °C for 2 to 4 minutes and then rinsed with 10 ml from the cell suspension in the tube to stop the cleaving reaction of trypsinase. The PBS-media solution containing the bulk of cells was added to the tube and cells centrifuged at 480 g for 4 minutes. A large percentage of the supernatant was discarded and cells resuspended in the remaining volume. A portion of this volume referring to the split factor was transferred into a new cell culture flask and filled up to 30 ml with medium.

Suspension cells do not adhere to the bottom of the cell culture flask. They were passaged as described above but a treatment with PBS + 2.5 % Trypsin-EDTA was unnecessary. For suspension cells, the culture flask could be re-used for up to 8 passages.

Centrifugation of cells was performed at 480 g for 4 min unless indicated differently. Washing means that cells were spun (supernatant discarded), resuspended in a large volume (150 µl for wells in a 96-well microplate, 15 ml for a 50 ml conical tube) of the indicated solution and respun (supernatant discarded).

3.3 Cryoconservation of cells

Cells were stored long-term in liquid nitrogen at - 196 °C. To protect them from the crystals that form when watery solutions are frozen, a cryoprotectant was used. DMSO inhibits the formation of crystals but is toxic for cells when present in unfrozen media.

Cells were trypsinized as necessary and washed to remove traces of media. They were resuspended in medium containing 20 % FCS and 10 % DMSO at approximately 2 x 10^6 cells/ml and stored in a freezing container overnight. The freezing container was filled with isopropanol to ensure freezing of cells at a constant speed of - 1 °C per minute. The next day the cells were transferred to a liquid nitrogen tank.

When thawing the cells for bringing them back into culture, one has to make sure that they are exposed to DMSO for as short a time as possible. Therefore cryoconserved vials were thawed in a 37 °C water bath while being constantly agitated. Immediately upon completion of thawing, cells were transferred into a conical tube with 8 ml medium and centrifuged. The pellet was resuspended in medium and cultured in 15 ml in an 80 cm^2 cell culture flask.

3.4 Effector cell preparation

Peripheral blood mononuclear cells (PBMC) were obtained from leukocyte filters from a local blood donor service. The filters contained blood cells from 500 ml blood. They were rinsed with 3 x 50 ml PBS + 20 % Biocoll. The PBS solution containing all blood cells, such as lymphocytes, erythrocytes and granulocytes, was poured on top of 15 ml Biocoll in 50 ml conical tubes and centrifuged at 400 g for 30 min. During this density gradient centrifugation, erythrocytes and granulocytes pellet in the bottom of the tubes whereas lymphocytes are collected in the interphases. Centrifugation was accelerated and stopped in the slow mode to preserve phase separation. The interphases of several tubes were combined in a new tube, washed with PBS and centrifuged at 600 g for 5 min. Resuspension of pellets in Erythrocyte Lysis Buffer eliminated residual erythrocytes. To remove the lysis buffer from the cells, tubes were filled up with PBS and centrifuged at 600 g for 5 min. PBMC were cultured in 40 ml RPMI 1640 + 10 % FCS, checked for proportional distribution of distinct blood cell populations and used for up to one week.

3.5 Stimulation of effector cells

PBMC were co-cultured for 4 days with $EpCAM^+$ SW-480 cells at an effector-to-target cell ratio of 5:1 in the presence of 10 ng/ml MT110. After 3 days of stimulation, an identical

amount of new target cells were added to the cell suspension to ensure the presence of live target cells until the end of stimulation.

As controls, PBMC were cultured in RPMI 1640 + 10 % FCS for 4 days in the absence or presence of MT110 (10 ng/ml).

Activation that included a co-stimulus was used as a positive control: a plastic petri dish was coated with 1 µg/ml anti-CD3 and 1 µg/ml anti-CD28 in 30 ml PBS for 1 h at 37 °C. The plate was rinsed with PBS before whole PBMC were added. After 3 days of stimulation at 37 °C and 5 % CO_2, PBMC were transferred to an uncoated petri dish and incubated for another 24 h. This step allowed internalized T cell receptor to be redelivered to the cell surface.

3.6 CD3 T cell enrichment

$CD3^+$ human T cells were isolated from peripheral blood mononuclear cells by depletion of non-T cells using the Pan T Cell Isolation Kit II. PBMC were counted and a volume containing the desired amount of T cells centrifuged at 300 g for 10 min. Pellets were resuspended in 40 µl buffer per 10^7 total cells before adding 10 µl biotin antibody cocktail per 10^7 total cells. The cocktail contained antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and glycophorin A, all of which were conjugated to biotin. Non-T cells in the suspension were labeled for 10 min at 4 °C. Ten µl of buffer per 10^7 total cells were added to the suspension, followed by addition of 20 µl magnetic anti-biotin MicroBeads per 10^7 total cells. Anti-biotin antibodies were attached to MicroBeads and enabled a stable connection between non-T cells and MicroBeads. Cells were incubated for 15 min at 4 °C. After incubation, 10 - 20 x labeling volume of buffer was added to the suspension and cells centrifuged at 300 g for 10 min. The pellet was resuspended in 500 µl buffer and applied to an MS column (rinsed beforehand with 500 µl buffer) on a MACS Separator. Labeled non-T cells were held back in the matrix of the column due to magnetic attraction whereas unlabeled T cells passed through and could be collected as effluent. Enriched T cells were assayed for purity by flow cytometry.

3.7 Fluorescent labeling of cells

In order to distinguish cell populations from each other upon FACS analysis, they were labeled fluorescently. Cells can be labeled with a fluorescent membrane dye before being included in the assay or with fluorescence-conjugated antibodies after the assay run.

If the antibody recognizes a pan marker, a cell population can be identified as a whole. If the antibody is specific for an epitope that can be up- and down-regulated then activation status, level of expression of adhesion molecules, etc. can be analysed.

When labeling the cells for flow cytometry, one has to carefully choose fluorochromes that are detected in different channels in order to distinguish the various signals. However, there are exceptions. Antibodies against CD4 and CD8 (both conjugated to PE-Cy5) proved to have distinct emission properties and could be used together in the same setup.

3.8 Membrane-labeling of target cells

As target cells were solely analysed for survival, the complete target cell population was labeled with the fluorescent membrane dye PKH26 (PKH26 Red Fluorescent Cell Linker Kit). The long aliphatic tails of this dye are stably incorporated into the lipid regions of the target cell membrane, and, based on this discrete red fluorescence, target cells can be distinguished. Target cells were washed twice with PBS to remove any residual media and resuspended in 250 µl solution C per 10^7 cells. In a separate vial, 7 µl PKH26 dye per 10^7 cells were diluted in 250 µl solution C and the mix added to the cells. After a two-minute incubation at room temperature, the reaction was stopped by adding 500 µl of FCS. Cells were washed twice with cell culture medium and counted using a hemocytometer.

3.9 Surface labeling of cells with fluorescence-conjugated antibodies

Cells were washed once in FACS Buffer and resuspended in 50 µl FACS Buffer containing the diluted antibody or mixture of antibodies. Dilution of antibodies depended on the specific binding affinity. A titration assay was performed for each antibody beforehand to find the optimal working concentration, usually ranging between 5 and 20 µg/ml. Cells labeled for 30 min at 4 °C. They were washed once with FACS Buffer and resuspended in 50 µl or 100 µl of FACS Buffer for analysis in a flow cytometer.

When cell survival was measured, pellets were resuspended in 50 µl. Another 50 µl FACS Buffer containing Propidium Iodide (final concentration of 1 µg/ml) were added. In all other cases when cell survival was not of interest, pellets were resuspended in 100 µl FACS Buffer alone.

3.10 Intracellular staining of cells with fluorescence-conjugated antibodies

In order to analyse expression of intracellular proteins (granzyme B, perforin, foxp3) cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Kit to allow for antibodies

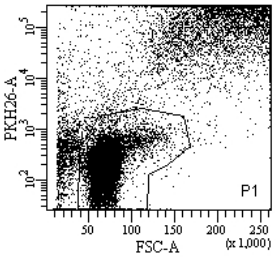
to penetrate the cells. Additional surface labeling was performed as a first step as described above. Cells were then washed, resuspended in 100 µl of Cytofix/Cytoperm solution and incubated for 20 min at 4 °C. A washing step with Perm/Wash solution ensured that any remaining Cytofix/Cytoperm solution was removed while cell membranes remained permeabilized.

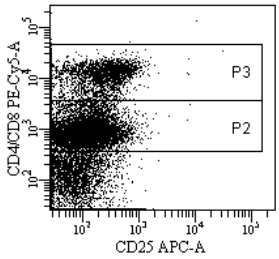
Cells were resuspended in Perm/Wash solution containing antibodies for intracellular staining and incubated for another 30 min at 4 °C. After a final washing step in Perm/Wash solution, cells were resuspended in 100 µl of FACS Buffer and analysed in a flow cytometer.

For analysis, lymphocytes were gated in a dot plot (Plot 1) showing all cells. Cells in P1 were displayed in Plot 2 showing CD25 expression (activation status) of $CD4^+$ and $CD8^+$ cells. A gate was drawn around $CD4^+$ (P2) and $CD8^+$ cells (P3) and each of both individually displayed in Plots 3 to 6. Plots 3 and 4 display intensity of granzyme B staining. Plots 5 and 6 show the basal signal using an isotype antibody. It is obvious that the basal fluorescent signal increases over time upon stimulation probably due to an increase in size of the cells. The threshold for granzyme B^{high} status was set according to the basal signal as determined by isotype staining.

Percentage of granzyme B^{high} cells was calculated by dividing number of granzyme B^{high} (P4) cells by number of $CD4^+$ (P2) or number of $CD8^+$ (P3) cells (granzyme B^{high} cells [%]).

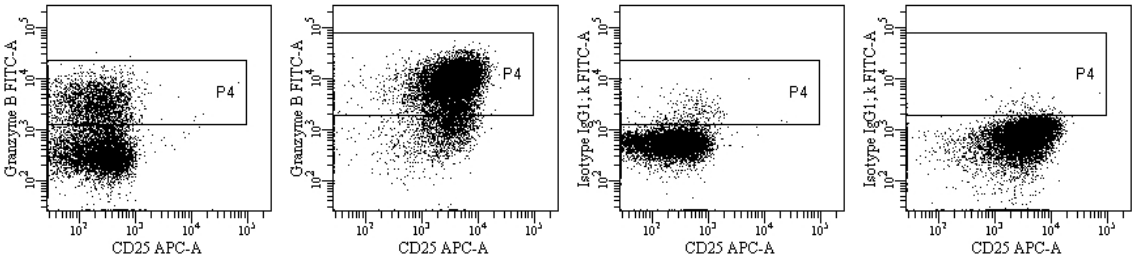
All cells[CHa1] (Plot 1) Cells in P1 (Plot 2)





Cells in P2 or P3 respectively

6 h (Plot 3) 33 h (Plot 4) 6 h (Plot 5) 33 h (Plot 6)



$\frac{P4}{P2}$ = granzyme B^{high} $CD4^+$ cells [%] $\frac{P4}{P3}$ = granzyme B^{high} $CD8^+$ cells [%]

3.11 Cytotoxicity assay

The main set-up to examine the mode of action of BiTE antibodies was a cytotoxicity assay. It was mostly performed in 96-well microplates (clear, U bottom).

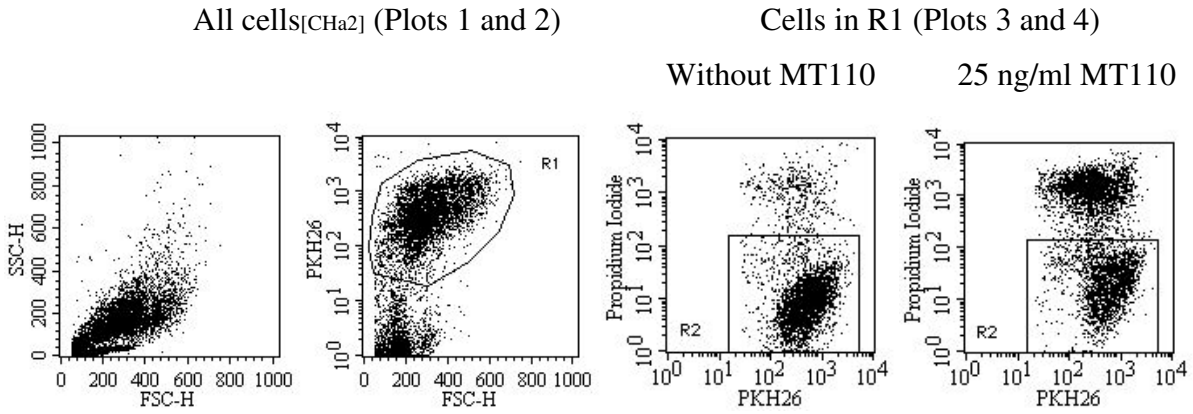
Effector cells were washed, counted and brought to a concentration of 1.5 x 10^6 cells per ml in RPMI 1640 + 10 % FCS. Target cells were also washed, dyed with PKH26 if necessary, and diluted to a concentration of 3.75 x 10^5 cells per ml in the same media. In each well, 100 µl (150,000 cells) of effector cell suspension were mixed with 80 µl (30,000 cells) of target cell suspension resulting in an effector cell to target cell (E:T) ratio of 5:1. When other E:T ratios were tested, the volumes and number of target cells remained the same, while the concentration and, accordingly, number of effector cells were adapted. A dilution of BiTE antibody in RPMI 1640 + 10 % FCS (20 µl total volume) was added. The BiTE antibodies were tested in serial dilutions. Concentrations ranged from 2.4 pg/ml to 100 ng/ml for MT103 and from 0.3 pg/ml to 500 ng/ml for MT110. Total volume of the samples equalled 200 µl.

When supplements like dexamethasone, methylprednisolone, EGTA + $MgCl_2$, caspase inhibitor, staurosporine, granzyme B inhibitor or TGF-β were added, the total volume of 200 µl and numbers of cells were kept constant while volumes of cell suspensions were changed.

After the reaction, cell suspensions were transferred to a 96-well microplate (clear, V bottom) and centrifuged. After washing with FACS Buffer, they were stained with antibodies and/or Propidium Iodide (final concentration of 1 µg/ml). Propidium Iodide is a membrane impermeable dye that enters cells via holes in the cell membrane and intercalates irreversibly in the DNA helices. As live cells do not have perforated membranes, a Propidium Iodide$^+$ cell can be considered dead. Numbers of live and dead target cells were detected by FACS analysis.

PKH26 stain separates target cells from effector cells. PKH26$^+$ cells were gated (R1) and displayed in plots 3 and 4. Propidium Iodide signal distinguishes PI$^+$ (dead) from PI$^-$ (alive) cells (Plots 3 and 4).

Number of PI$^-$ alive cells in R2 was divided by number of PKH26$^+$ target cells yielding percent of living target cells. Percent of living target cells was subtracted from 100 resulting in percentage of lysis.



R1 = number of all target cells

R2 = number of living target cells

$$\frac{R2}{R1} \times 100 = \frac{n_{\text{living target cells}}}{n_{\text{target cells}}} \times 100 = \text{Living Target Cells [\%]}$$

$$100 - \text{Living Target Cells [\%]} = \text{Lysis [\%]}$$

Using the software GraphPad Prism 5, the percentage of lysis was plotted against BiTE concentration or E:T ratio. Error bars indicate standard error of the mean (SEM). In some instances, dose response curves were analyzed with the four parametric logistic regression model for evaluation of sigmoid dose response curves with variable hill slope. EC_{50} values could be determined from dose response curves.

3.12 Analysis of caspase 3/7 activation

Activity of caspases 3 and 7 was detected using the Caspase-Glo™ 3/7 Assay. Effector and target cells were washed, counted and seeded in a 96-well microplate (white, flat bottom) at the cell numbers described above but with only half the volumes resulting in a sample size of 100 µl. After the cytotoxicity reaction, 100 µl of Caspase-Glo™ 3/7 reagent were added to each well.

The reagent contains a lysing compound, a substrate for caspases and luciferase. Active caspases are set free from the lysed cells and cleave the pro-luminescent caspase 3/7 substrate. Cleaved substrate is detected by luciferase generating a colour reaction. Samples were measured in a luminometer.

3.13 Release of adenylate kinase

Adenylate kinase is present in all living cells and catalyzes the conversion of ADP to ATP. It is released from the cytosol into the surrounding medium when cells enter apoptosis and their membranes become leaky. Release of this enzyme was determined using the ToxiLight® BioAssay Kit.

Effector cells (1.5×10^5) and target cells (3×10^4) were co-cultured with a BiTE solution in a 96-well microplate (white, flat bottom) in a total volume of 100 µl. After the reaction completed, 50 µl of ToxiLight 100 % Lysis Reagent were added to control wells and incubated for 10 minutes to yield the maximal signal. To compensate for the difference in volume, 50 µl of Tris Acetat Buffer were added to all other sample wells. Lyophilized adenylate kinase detection reagent was reconstituted in assay buffer and added to sample wells in 100 µl aliquots. The reagent provides ADP, luciferin and luciferase, which react to generate a bioluminescent signal. Adenylate kinase converts ADP to ATP. Luciferase catalyzes the generation of light from ATP and luciferin. After 5 minutes of incubation, the plate was measured in a luminometer.

3.14 Analysis of PARP cleavage

Poly (ADP-ribose) polymerase (PARP) is a 116 kDa nuclear protein, which is strongly activated by DNA strand breaks. During apoptosis, active caspases like caspase 3 and 7 cleave PARP to yield an 85 kDa and a 25 kDa fragment. Samples from a cytotoxicity assay were checked for presence of the cleavage products using the commercial Human Cleaved PARP ELISA Set.

Effector cells (2.5×10^7) and target cells (5×10^6) at an E:T ratio of 5:1 were co-cultured in 6 ml RPMI 1640 + 10 % FCS in each well of a 6-well plate. BiTE antibody (12.5, 50 and 200 ng/ml), staurosporine (10 µM), caspase inhibitor (20 µM) or granzyme B inhibitor (10 nM, 10 µM) were added to individual wells. After 1 day, cells were lysed on ice for 45 min in insect cell lysis buffer + protease inhibitor cocktail. Samples were centrifuged at 40,000 g for 45 min to remove cellular debris. Cleared lysates were filled in wells of a 96-well microplate (clear, flat bottom, Maxisorp) that had been coated with anti-human PARP antibody. The enzyme-linked immunosorbent assay (ELISA) was performed as described below.

3.15 ELISA for cleaved PARP

A 96-well microplate (clear, flat bottom, Maxisorp) was coated with 100 µl per well of anti-human PARP antibody diluted 1:250 in PBS overnight at 4 °C. Wells were washed three times with ELISA Wash Buffer in an ELISA washer and blocked with 200 µl PBS + 10 % FCS per well. After incubation at room temperature for 1 h, wells were again washed three times in an ELISA Washer. Lyophilized standard was reconstituted with PBS + 10 % FCS to yield a stock standard of 250 Units/ml and diluted 1:2 in a series of 6 tubes. Standard samples and cleared lysates were transferred to the wells and incubated for 2 h at room temperature.

After five washes, biotinylated anti-human PARP mixed with streptavidin-horseradish peroxidase conjugate was added to the wells and incubated at room temperature for 1 h. Seven final washes were performed including soaking the wells in ELISA Wash Buffer for 30 seconds. A hundred µl of TMB Liquid Substrate were added to each well and the plate incubated at room temperature for 30 minutes in the dark. After addition of 50 µl 2 N H_2SO_4 to each well, absorbance was analysed in an ELISA Reader at 450 nm.

3.16 Analysis of DNA fragmentation

DNA in apoptotic cells is not stable. Activated endonucleases degrade the DNA gradually leading to double- and single-strand breaks.

Damaged DNA can be analysed by flow cytometry using the Apoptosis Detection Kit (APO-Direct). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of FITC-conjugated deoxyuridine triphosphates (FITC-dUTP) to the 3'-hydroxyl (-OH) ends of double- and single-stranded DNA. After dUTP incorporation, DNA break sites are identified by flow cytometry. This method is referred to as terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL).

A cytotoxicity assay with 4 x 10^6 effector cells and 8 x 10^5 target cells (E:T 5:1) in 4 ml RPMI 1640 + 10 % FCS in each well of a 6-well plate was performed for 1 day. Suspensions were transferred to 15 ml conical tubes and centrifuged at 500 g for 4 minutes. Pellets were resuspended in 2.5 ml 1 % formaldehyde in PBS and fixed on ice for 60 minutes. After repeated washing in PBS, 70 % ice-cold ethanol was added to the cells and tubes incubated at - 20 °C overnight.

Ethanol was removed by centrifugation and cells washed in wash buffer twice. Fifty µl of a staining solution (prepared as described below) were added to each sample:

Reaction buffer	10.00 µl
TdT Enzyme	0.75 µl
FITC-dUTP	8.00 µl
Distilled H_2O	32.25 µl for one sample

Cells were stained for 5 h at 37 °C before addition of 1 ml of rinse buffer to each tube for washing. The cell pellets were resuspended in 300 µl PI/RNase staining buffer and incubated at room temperature for 30 minutes in the dark. Cells could be kept in the staining buffer for analysis in a flow cytometer.

3.17 Analysis of cytokine production

The levels of certain cytokines in a sample could be quantitatively analyzed via the beads of the Human Th1/Th2 Cytokine Kit II. Each of six bead populations is coated with antibodies specific for one cytokine out of IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ. A mixture of the beads is incubated with the supernatants of a cytotoxicity reaction. Cytokines in the supernatants bind to the beads. Bound cytokines are detected by a mix of PE-conjugated anti-human IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ antibodies. The bead-cytokine-antibody complexes are measured in a flow cytometer and can be quantified in comparison to a standard of recombinant human cytokine proteins.

Lyophilized standard containing recombinant human IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ was reconstituted in assay diluent and allowed to equilibrate for 15 minutes before being diluted in a 1:2 series.

The amount of beads needed for an experiment is calculated per test well. Five µl from each bead population were combined in a tube, mixed and added to a 96-well microplate (clear, V bottom) in 25 µl aliquots. Equal volumes of PE Detection Reagent and samples or standards, respectively were added, and the plate incubated for 3 h at room temperature in the dark. Samples were washed twice in wash buffer and analyzed by flow cytometry.

3.18 Estimation of the minimal anticipated biological effect level

To find a safe starting dose for first-in-man clinical trials according to the MABEL approach, data from the following assay systems were used:

- T cell activation (up-regulation of the T cell activation marker CD25 on PBMC)
- redirected tumor cell lysis (uptake of the viability stain Propidium Iodide (PI) in tumor cells)
- release of cytokines
- calorimeter-based cytotoxicity (release of adenylate kinase)
- calculation of fractional receptor occupancy

Methods for MABEL calculation from cytokine release, calorimeter-based cytotoxicity and fractional receptor occupancy will not be further explained as they were not conducted by the author. Only the methodology to calculate MABEL from T cell activation and redirected tumor cell lysis is presented. The effective MT110 concentrations that induced 20 % of the maximal effect (EC_{20} values) were determined as a measure for MABEL.

Cytotoxicity assays were performed, stained and analyzed by flow cytometry as described above. 'Percent of living target cells' was calculated as in 3.11.

$$\text{Living Target Cells [\%]} = \frac{n_{\text{living target cells}}}{n_{\text{target cells}}} \text{ x } 100$$

As PBMC from different donors were to be compared, specific lysis was calculated for each individual curve according to following equation:

$$\text{Specific Lysis [\%]} = \left(1 - \frac{\text{living targets}[\%]_{\text{sample}}}{\text{living targets}[\%]_{\text{blank}}}\right) \text{ x } 100$$

Using GraphPad Prism 5 software, the percentages of activated T cells and of specific lysis were plotted against BiTE concentration. Dose response curves of each donor were analyzed with the four parametric logistic regression model for evaluation of sigmoid dose response curves with variable hill slope, and EC_{50} values were evaluated. EC_{20} values were then calculated according to the following formula.

$$EC_{20} = EC_{50} \times 0.25^{\frac{1}{\text{hillslope}}}$$

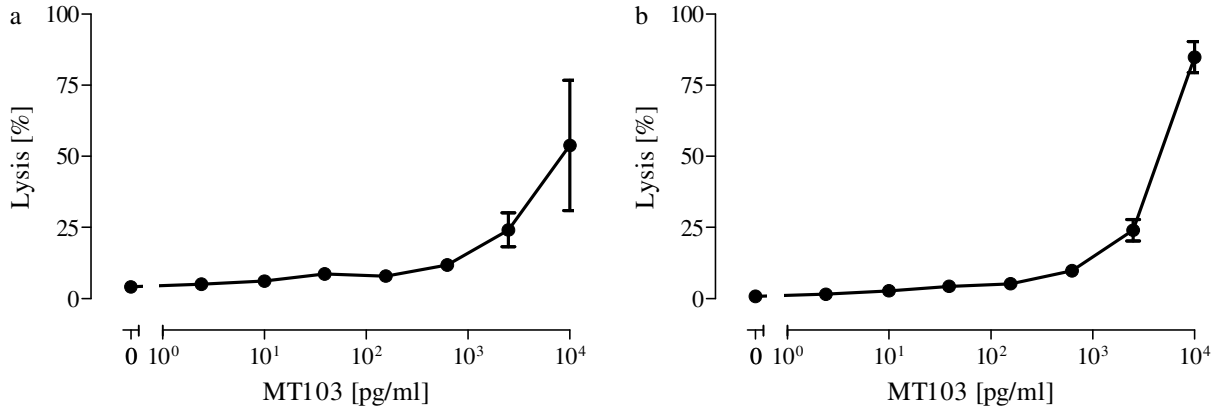
4 Results

4.1 Effects of the BiTE molecule MT103

4.1.1 Lysis of tumor cells by PBMC

With its two different binding arms the bispecific antibody MT103 binds CD3 and CD19 simultaneously. CD3 is associated with the T cell receptor on T cells (Frank et al. 1986). Therefore all mature T cells are $CD3^+$. CD19 is a B cell marker (Clark and Lane 1991). B lymphocytes and follicular dendritic cells, but no other cells in the human body express CD19. When MT103 is bound to either one of these cells alone ($CD3^+$ or $CD19^+$ cell) no physiological effect is observable. However, as soon as the second arm of MT103 finds its binding partner, an immunological synapse forms between the two cells. The T cell is activated to start its cytotoxic program resulting in the death of the $CD19^+$ B cell (Loffler et al. 2000; Dreier et al. 2002).

PBMC were mixed with relevant (i.e., CD19 expressing) target cells and the BiTE molecule MT103 in order to test the potential of MT103 to activate T cells into exerting their cytolytic program.



Figure[CHa3] 6 Lysis of tumor cells by PBMC
PBMC were co-cultured with Mec-1 cells (E:T 1:1) and increasing doses of MT103. Reactions were stopped after (a) 24 h and (b) 48 h. Redirected cell lysis was determined by propidium iodide uptake into cell nuclei using flow cytometric analysis.

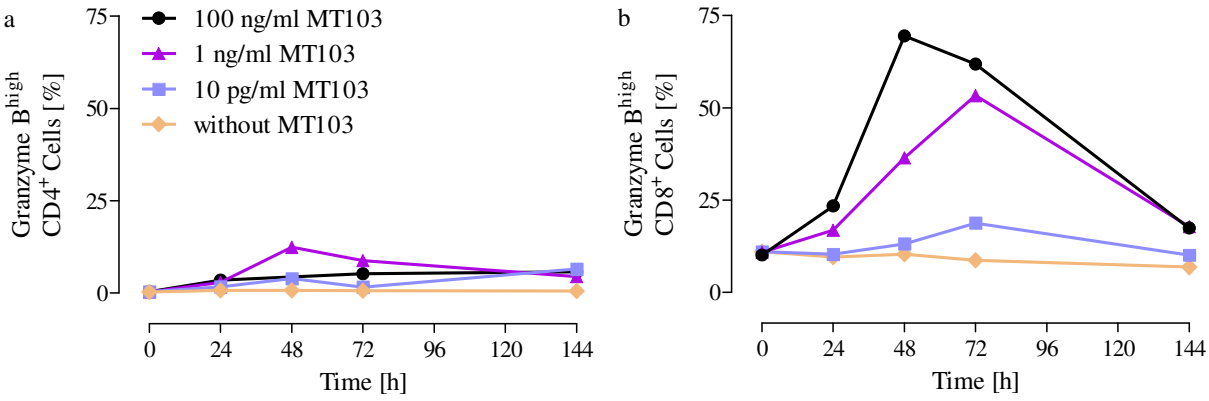
At low concentrations of MT103 (2.4 - 156 pg/ml) almost no reduction of living target cells could be observed after 24 h or 48 h. With concentrations of 1 ng/ml and higher, T cells lysed tumor cells very potently. The lysis rate was 54 % after 24 h (Figure 6 a) and 85 % after 48 h (Figure 6 b) when 10 ng/ml MT103 were present during the reactions.

4.1.2 Granzyme B expression in T cells in response to stimulation

T cells mainly eliminate tumor and infected cells via effector molecules like granzyme B and perforin (de Vries et al. 2007). Upon stimulation, cytotoxic T lymphocytes produce such effector proteins in large amounts and store them in cytotoxic granules before they are exocytosed towards the immunological synapse. From there, granzyme B and perforin can enter the target cell and exert their lytic functions.

Inside the target cell, granzyme B activates pro-caspase 3, cleaves the heterodimer CAD/ICAD setting free the caspase-activated DNAse CAD and cleaves Bid to tBid which inserts into the mitochondrial outer membrane and causes release of cytochrome *c* from mitochondria (Lieberman 2003).

The granzyme B expression of T cells in response to stimulation with MT103 in presence of target cells was tested.



Figure[CHa4] 7 Granzyme B expression in $CD4^+$ and $CD8^+$ cells in response to stimulation
PBMC were cultured without MT103, or with 10 pg/ml, 1 ng/ml or 100 ng/ml MT103. Endogenous B cells functioned as target cells for T cells. Samples were stained with (a) anti-CD4, (b) anti-CD8 and anti-granzyme B or isotype antibody respectively after 0, 1, 2, 3 and 6 days and analyzed by flow cytometry.

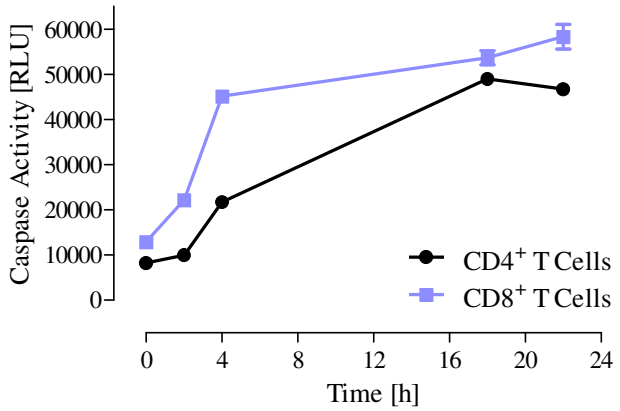
In $CD4^+$ cells an increase of granzyme B expression could not be observed over a time course of 6 days (Figure 7 a). In $CD8^+$ cells, granzyme B was strongly up-regulated upon stimulation with MT103 in the presence of target cells. Speed and level of up-regulation were dependent on the concentration of MT103. Already, at the very beginning of the reaction, 11 % of $CD8^+$ T cells were granzyme B^{high}. This population likely consisted mostly of effector memory T (T_{EM}) cells. T_{EM} cells show an elevated level of granzyme B content due to a previous encounter with their relevant antigen. With this stock of effector molecules, T_{EM} cells are quickly ready to eliminate target cells presenting their cognate antigen.

After 2 days, 69 % of $CD8^+$ cells were granzyme B^{high} when 100 ng/ml MT103 had been administered. On day 3, the content of granzyme B^{high} $CD8^+$ cells was slightly lower (62 %)

and had fallen to 17 % by day 6. $CD8^+$ cells exposed to 1 ng/ml MT103 reached a peak value of 53 % granzyme B^{high} cells after 72 h and ended on the same level of 18 % as samples treated with the higher MT103 concentration (Figure 7 b).

4.1.3 Activation of caspases

As a next step, activation of caspases, an event that occurs downstream of granzyme B function, was tested. Caspases are proteases with a cysteine residue in the active site. They cleave specifically at aspartic acid residues and are responsible for the breakdown of the cytoskeleton and the nuclear membrane as well as fragmentation of DNA during apoptosis (Russell and Ley 2002).



Figure[CHa5] 8 Activation of caspases by $CD4^+$ and $CD8^+$ cells
$CD4^+$ and $CD8^+$ T cells were isolated from human PBMC and mixed with Mec-1 cells (E:T 5:1) and 1 ng/ml MT103. Content of active caspases in the cells was measured using the Caspase-Glo™ 3/7 Assay after 0 h, 2 h, 4 h, 18 h and 22 h.

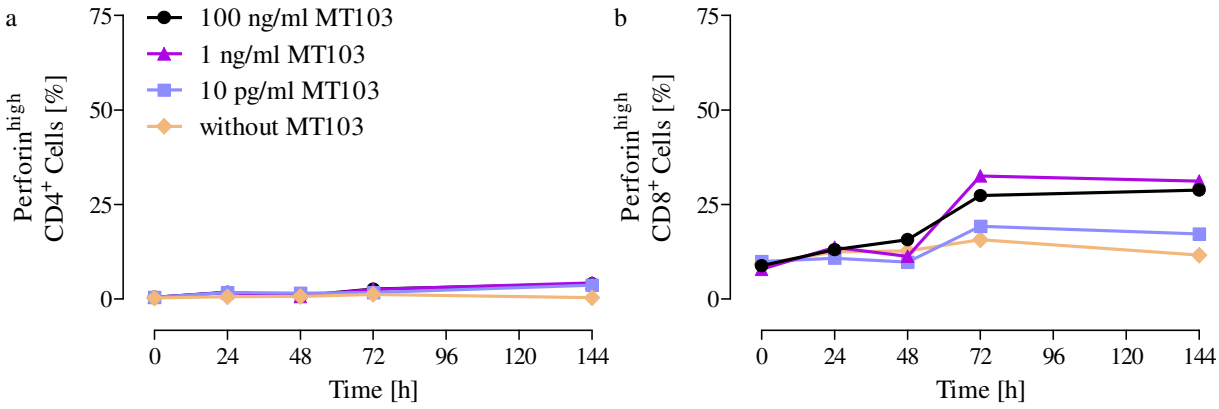
Stimulation of T cells with MT103 in the presence of target cells resulted in an enormous increase in the content of active caspases in the reaction samples. Within 4 h, the signal rose by 2.5-fold in samples with $CD4^+$ cells, and 3.5-fold in samples with $CD8^+$ cells. After 22 h, samples with $CD4^+$ cells as effector cells contained 6 times as many active caspases as at the start of the reaction. In samples with $CD8^+$ cells, 5 times as many active caspases were detected (Figure 8).

4.1.4 Perforin expression in T cells in response to stimulation

Perforin is a component of the cytotoxic granules in T cells. Together with granzyme B, it is produced in large amounts in T cells that encountered stimulation. The role of perforin during the process of target cell elimination is not quite clear. It has been shown that perforin is taken

up by target cells and polymerizes in the target cell membrane to form ring-like structures with a central pore (Millard et al. 1984; Tschopp et al. 1986; Sauer et al. 1991).

The perforin expression of T cells in response to activation with MT103 was tested in the presence of target cells.



Figure[CHa6] 9 Perforin expression in $CD4^+$ and $CD8^+$ cells in response to stimulation
PBMC were cultured without MT103, or with 10 pg/ml, 1 ng/ml or 100 ng/ml MT103. Endogenous B cells functioned as target cells for T cells. Samples were stained with (a) anti-CD4, (b) anti-CD8 and anti-perforin or isotype antibody, respectively after 0, 1, 2, 3 and 6 days and analyzed by flow cytometry.

Perforin could not be detected in $CD4^+$ cells at any time (Figure 9 a). $CD8^+$ cells showed a basal level of 9 % $perforin^{high}$ cells. When activated with MT103 in presence of target cells up to 33 % of $CD8^+$ cells gained a $perforin^{high}$ status, which persisted among $CD8^+$ cells for an extended period of time. Expression levels on day 6 were very similar to expression levels on day 3 (Figure 9 b).

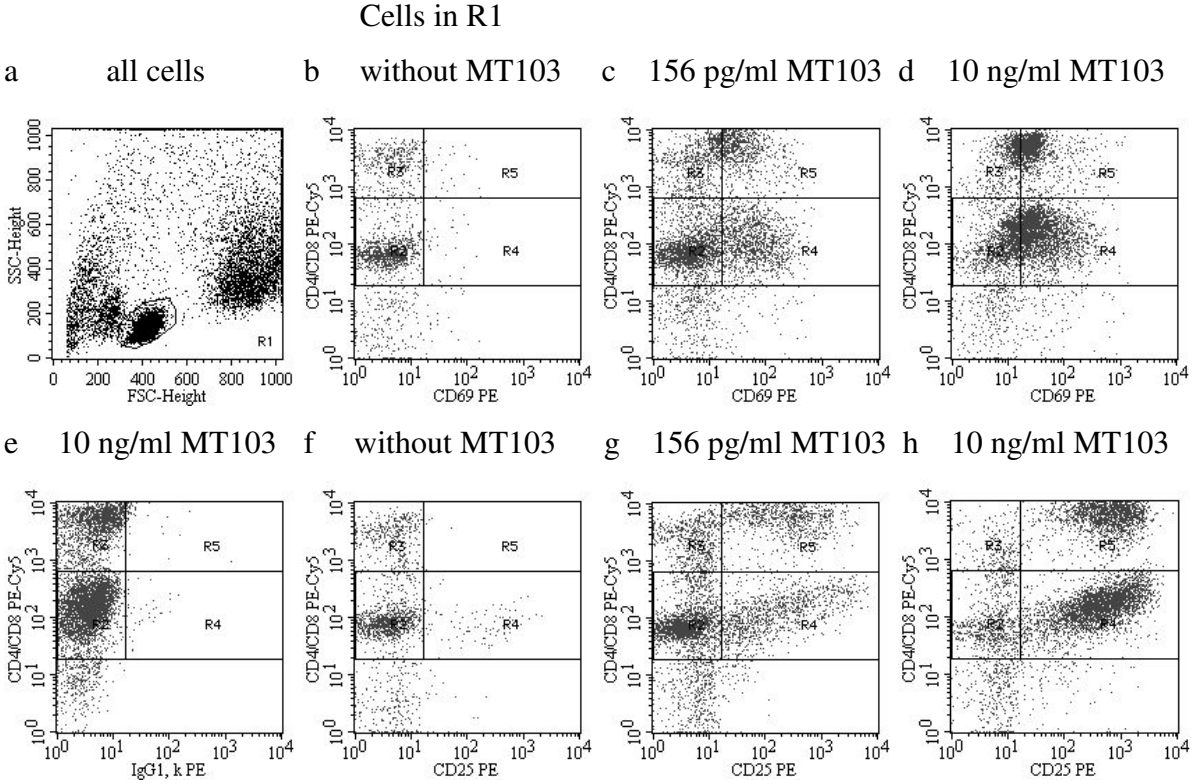
4.1.5 Expression of activation markers CD69 and CD25

Activation markers are absent or expressed at very low levels on resting T cells. But their expression is induced or up-regulated following T cell activation (Yokoyama et al. 1989). CD69 and CD25 are well-examined representatives of the class of activation markers. CD69 is the earliest inducible cell surface glycoprotein aquired during activation of T cells. Expression of CD69 is apparent after 1 h of T cell activation and reaches a maximum after 20 h (Yokoyama et al. 1989). CD69 is a member of the C-type lectin superfamily and acts as a co-stimulatory molecule for T cell activation and proliferation (Ziegler et al. 1994). CD25, the alpha chain of the IL-2 receptor, reaches its peak level of expression after 2 to 3 days (Ortega et al. 1984). Activated T cells express a 10 - 20-fold excess of alpha chains compared to the βγ chains of the IL-2 receptor (Saito et al. 1988). The main physiological roles of the IL-2 receptor are to promote the proliferation of T cells upon activation, to deliver survival

signals to antigen-activated cells, and also to promote the return of activated cells to a quiescent state (Hoyer et al. 2008). Albeit being an activation marker, CD25 is constitutively found on $CD4^+$ regulatory T (T_{reg}) cells (Fowell et al. 1991; Itoh et al. 1999).

Neither CD69 nor CD25 are expressed on unstimulated T cells (with the exception of $CD25^+$ $CD4^+$ T_{reg} cells). When T cells are activated with BiTE in the presence of target cells, the signal for CD25 and CD69 turns from a clearly negative into a distinct positive one as can be seen in the dot plots of a FACS analysis (Figure 10).

Figure 10 describes how the percentages of $CD69^+$ and $CD25^+$ cells in the T cell population were determined.



$\frac{R4}{R2+R4}$ = % $CD4^+$ $CD69^+$ T cells resp. $\frac{R4}{R2+R4}$ = % $CD4^+$ $CD25^+$ T cells

$\frac{R5}{R3+R5}$ = % $CD8^+$ $CD69^+$ T cells resp. $\frac{R5}{R3+R5}$ = % $CD8^+$ $CD25^+$ T cells

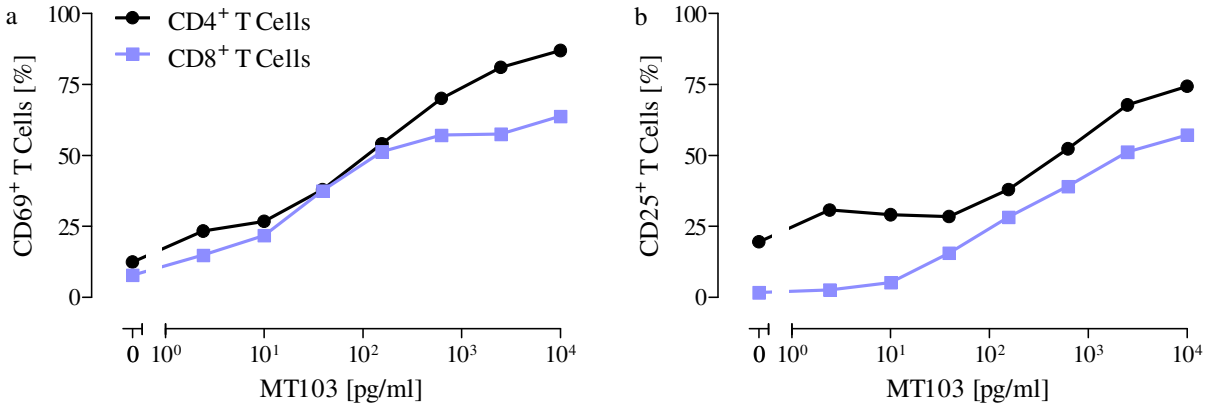
Figure**[CHa7]** 10 FACS analysis of expression of activation markers CD69 and CD25 on $CD4^+$ and $CD8^+$ cells PBMC were co-cultured with Nalm-6 cells (E:T 5:1), without MT103, or with 156 pg/ml or 10 ng/ml MT103. After 72 h, the reactions were stopped and suspensions stained with anti-CD4, anti-CD8 and (b - d) anti-CD69 or (f - h) anti-CD25 or (e) isotype antibody respectively.

For analysis of FACS data, a gate was drawn around the lymphocytes (R1) in an FSC/SSC-dot plot (Figure 10 a). Cells from this gate were displayed in the following dot plots. In $CD4^+$ and $CD8^+$ T cells, separate $CD69^+$ (Figure 10 b) and $CD25^+$ (Figure 10 f) populations could

clearly be distinguished from $CD69^-$ and $CD25^-$ cells. Gates were created around $CD69^-$ and $CD25^-$ populations and further gates put adjacent to the negative populations. Cells switching from the negative to the positive gates with the onset of expression were characterized as $CD69^+$ or $CD25^+$ (Figure 10 c, d, g, h).

Isotype staining reveals unspecific binding of the analytical antibodies to any other antigen present in the cell suspensions. The control antibody used was the same isotype as the anti-CD69 and anti-CD25 antibodies but did not recognize any human antigen. Staining of cells with an isotype control antibody did not result in a fluorescence signal (Figure 10 e). It can be concluded that staining with anti-CD69 and anti-CD25 was highly specific.

The up-regulation of CD69 and CD25 on the surface of T cells in response to stimulation with MT103 in the presence of target cells was investigated over a range of MT103 concentrations.



Figure[CHa8] 11 Expression of activation markers CD69 and CD25 on $CD4^+$ and $CD8^+$ cells
PBMC were co-cultured with Nalm-6 cells (E:T 5:1) and increasing doses of MT103. Cell lysis was allowed to proceed for 24 h. Cells were stained with anti-CD4, anti-CD8 and (a) anti-CD69 or (b) anti-CD25, respectively and analyzed by flow cytometry.

$CD4^+$ and $CD8^+$ cells up-regulated CD69 with respect to MT103 concentration in the presence of target cells. Eighty seven % of $CD4^+$ and 64 % of $CD8^+$ cells were $CD69^+$ at 10 ng/ml MT103 after 24 h (Figure 11 a). These results correlated with the expression pattern of CD25. Increasing doses of MT103 resulted in an increased expression of CD25. Maximal levels were 74 % for $CD4^+$ and 57 % for $CD8^+$ cells.

$CD4^+$ T cells showed a basal level of CD25 expression of 20 % (Figure 11 b). In accordance with the CD69 and CD25 expression pattern described in the literature (Ortega et al. 1984; Yokoyama et al. 1989), CD25 response to BiTE stimulation on $CD4^+$ and $CD8^+$ cells was slower than the corresponding CD69 response. It has been shown that $CD4^+$ regulatory T cells constitutively express CD25 (Fowell et al. 1991; Itoh et al. 1999). Regulatory T cells are a constitutive component of the T cell population. They modulate the immune response and

prevent an overflowing growth of cytotoxic T lymphocytes (Sakaguchi 2000; Shevach 2002). The unstimulated $CD25^+$ $CD4^+$ T cells described above could therefore be regulatory T cells.

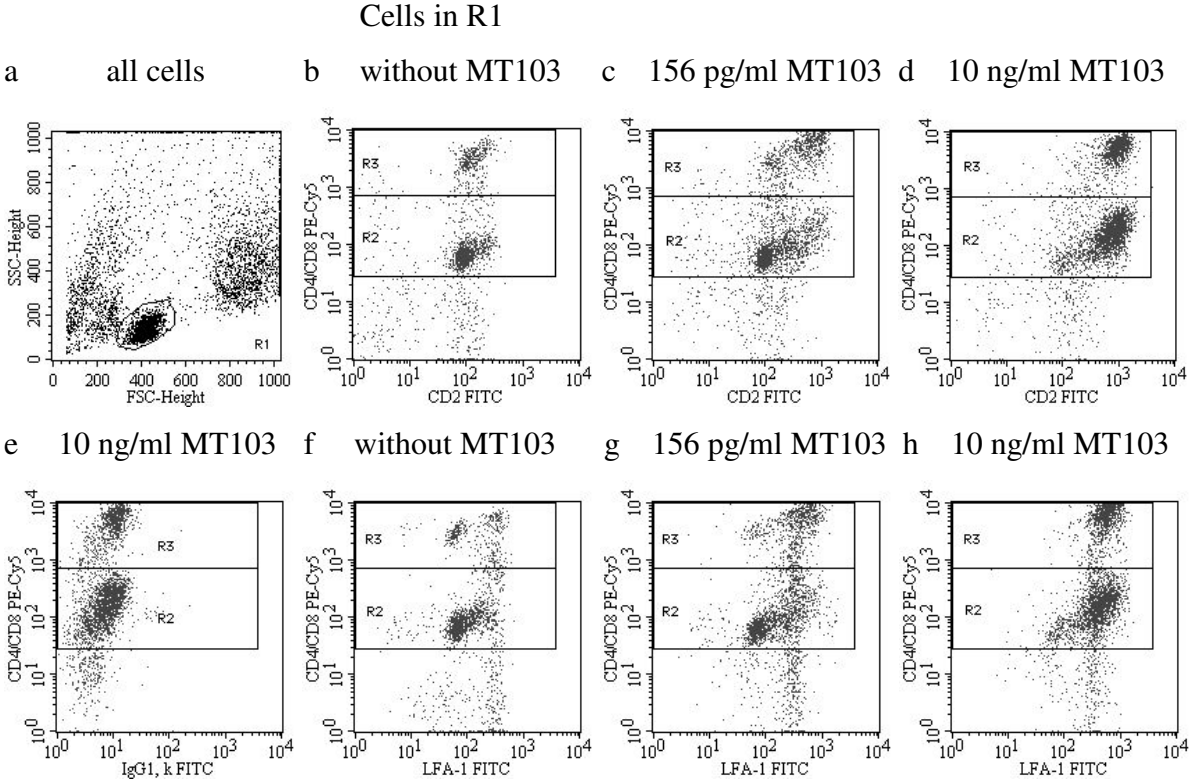
4.1.6 Expression of adhesion molecules CD2 and LFA-1

The CD2 molecule on T lymphocytes is a transmembrane surface glycoprotein that mediates cell-cell contact by binding to LFA-3 (CD58), a cell surface glycoprotein widely expressed on various cell types including hematopoietic and epithelial cells. CD2 holds important functions for cognate recognition of antigen presenting cells and for the cytolytic effector function of cytotoxic T lymphocytes (Hunig 1985; Shaw et al. 1986; Selvaraj et al. 1987).

Like CD2, LFA-1 is an adhesion molecule. It binds to ICAM-1 and is particularly important for the formation of the immunological synapse (Hogg et al. 2003).

Both molecules are constitutively expressed on the surface of T cells and up-regulated upon stimulation.

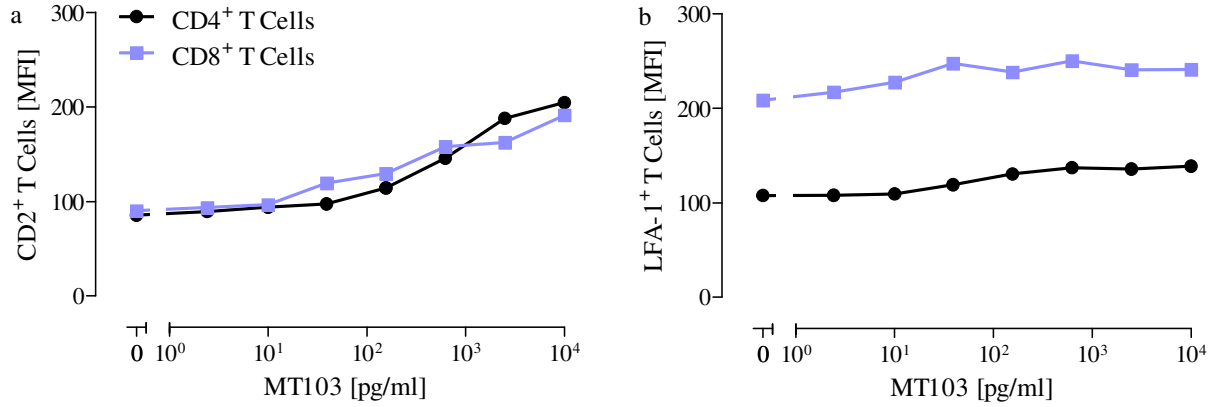
Figure 12 shows alterations in the CD2 and LFA-1 signals detected by flow cytometry when effector cells were activated with MT103 in the presence of target cells.



Figure[CHa9] 12 FACS analysis of expression of adhesion molecules CD2 and LFA-1 on $CD4^+$ and $CD8^+$ cells PBMC were co-cultured with Nalm-6 cells (E:T 5:1), without MT103, or with 156 pg/ml or 10 ng/ml MT103. After 72 h the reactions were stopped and suspensions stained with anti-CD4, anti-CD8 and (b - d) anti-CD2 or (f - h) anti-LFA-1 or (e) isotype antibody respectively.

Unstimulated as well as stimulated T cells stained positive for CD2 and LFA-1 in accordance with constitutive expression of these adhesion molecules on T cells. An increase in expression of CD2 and LFA-1 in a population of stimulated T cells was quantified by an increase of the mean fluorescence intensity (MFI), a signal for the expression level of the observed molecule. Staining with an isotype control antibody showed that binding of anti-CD2 and anti-LFA-1 antibodies was specific (Figure 12).

The alteration in expression of CD2 and LFA-1 on the surface of T cells in response to stimulation with MT103 in the presence of target cells was investigated.



Figure[CHa10] 13 Expression of adhesion molecules CD2 and LFA-1 on $CD4^+$ and $CD8^+$ cells
PBMC were co-cultured with Nalm-6 cells (E:T 5:1) and increasing doses of MT103. Cell lysis was allowed to proceed for 24 h. Cells were stained with anti-CD4, anti-CD8 and (a) anti-CD2 or (b) anti-LFA-1 respectively and analyzed by flow cytometry.

Expression of CD2 was responsive to T cell stimulation with MT103 in the presence of target cells. The more BiTE was present in the samples, the more CD2 was detected on $CD4^+$ and $CD8^+$ cells. With the maximal concentration of MT103 (10 ng/ml), expression levels of CD2 had doubled from initial levels. Both T cell subsets showed identical expressive behaviour (Figure 13 a).

Increase of LFA-1 expression on $CD4^+$ or $CD8^+$ cells in response to stimulation with MT103 in the presence of target cells was small in comparison to CD2 signals. LFA-1 signal in the $CD4^+$ cell subset increased by 29 % with 10 ng/ml MT103. For $CD8^+$ cells, the increase was 15 %. Throughout all samples, $CD8^+$ cells expressed two times as much LFA-1 on their surface as $CD4^+$ cells (Figure 13 b).

4.1.7 Dexamethasone as a co-medication in cancer therapy

Activated T cells synthesize and secrete pro-inflammatory cytokines as IFN-γ and TNF-α. Medical compounds activating the immune system have often been shown to increase

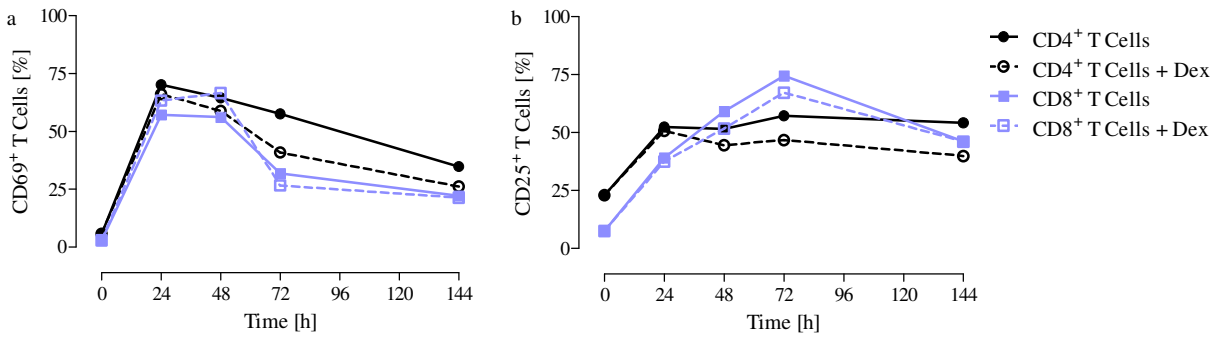
expression of cytokines in patients. An over-production of cytokines can lead to fever, inflammation, hypotension or even death in the most severe cases (cytokine storm). In order to keep cytokine production at a safe level, patients often receive dexamethasone as co-medication. Dexamethasone is a highly potent synthetic glucocorticoid specimen. Being active via the glucocorticoid receptor, dexamethasone alters the transcription pattern of cells resulting in a reduction of cytokine expression (Adcock and Caramori 2001). In the clinical oncological setting, it is used to prevent a harmful over-production of cytokines by T cells.

To test the effect of dexamethasone on the activation of T cells, their capacity to express activation markers and adhesion molecules, and, most importantly, their cytokine production, effector cells were pre-incubated in the presence or absence of 3 x 10^{-7} M dexamethasone for 24 h and used as effector cells in regular cytotoxicity assays.

4.1.7.1 Influence of dexamethasone on expression of activation markers

By pre-incubating effector cells with dexamethasone, cytokine expression can be reduced maximally without negatively influencing the cytotoxic potential of the effector T cells. One hallmark of potent cytotoxic T cells is their expression of activation markers. Activation markers CD69 and CD25 are predominantly expressed on stimulated T cells. The lowest dexamethasone concentration that has maximal effects on cytokine secretion was determined in dose-effect experiments using PBMC effector cells of seven different donors (Brandl et al. 2007).

A kinetic of CD69 and CD25 response to stimulation with MT103 in the presence of target cells is shown in Figure 14. Effector cells had been pre-incubated in the presence or absence of 3 x 10^{-7} M dexamethasone for 24 h.



Figure[CHa11] 14 Influence of dexamethasone on expression of activation markers
PBMC were incubated in the absence or presence of 3 x 10^{-7} M dexamethasone for 24 h. After pre-incubation, PBMC were centrifuged, resuspended in culture medium and subsequently incubated with Nalm-6 cells as target cells (E:T 5:1) in the presence of 625 pg/ml MT103. Cell lysis was allowed to proceed for 24 h, 48 h, 72 h and 6 days. Cells were stained with anti-CD4, anti-CD8 and (a) anti-CD69 or (b) anti-CD25, respectively and analyzed by flow cytometry.

CD69 is an early activation marker and accordingly the highest expression level of CD69 on T cells was observed after one day of BiTE stimulation. At this time, CD69 could possibly already have been in the process of down-regulation as peak values of CD69 are often measured after 20 h of activation (Yokoyama et al. 1989). This would provide an explanation for a lower CD69 level on $CD8^+$ than on $CD4^+$ cells. $CD8^+$ cells are more susceptible to activation than $CD4^+$ cells, starting expression, and likewise down-regulation, of CD69 earlier. Expression of CD69 reached 70 % on $CD4^+$ cells and 57 % on $CD8^+$ cells after one day. On day 6, CD69 expression had fallen to a level of 35 % on $CD4^+$ cells and 22 % on $CD8^+$ cells. Dexamethasone-treated and -untreated $CD4^+$ cells reached the same peak value of CD69 expression after one day. But down-regulation of CD69 on day 3 was stronger in dexamethasone-treated (41 % $CD69^+$ cells) than in -untreated $CD4^+$ cells (58 % $CD69^+$ cells). Dexamethasone only slightly altered the expression of CD69 on $CD8^+$ cells (Figure 14 a).

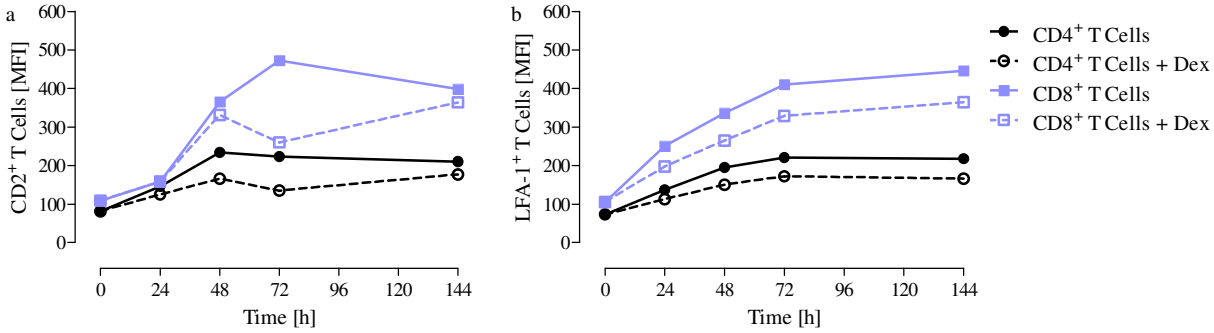
The activation marker CD25 was gradually up-regulated over several days upon stimulation. After 3 days, 75 % of $CD8^+$ cells expressed CD25. $CD4^+$ cells started at a high basal level of 23 % (as described above these cells could be $CD25^+$ $CD4^+$ T_{reg} cells), reached 51 % CD25 expression after one day and remained at that level for the whole period of investigation (6 days). Dexamethasone caused a slight reduction (about 7 %) of CD25 on $CD8^+$ cells. Reduction of CD25 expression on $CD4^+$ cells was more pronounced. On day 6, 40 % of dexamethasone-treated $CD4^+$ cells expressed CD25 in comparison to 54 % of -untreated $CD4^+$ cells (Figure 14 b).

In summary, an influence of dexamethasone on the expression of activation markers was visible but did not seem to cause a detrimental effect.

4.1.7.2 Influence of dexamethasone on expression of adhesion molecules

Other molecules important for T cell-mediated elimination of target cells are CD2 and LFA-1, which are necessary for formation of a stable immunological synapse between the T cell and target cell.

Figure 15 shows the time course of CD2 and LFA-1 response to stimulation with MT103 in the presence of target cells. Effector cells had been pre-incubated in the presence or absence of 3×10^{-7} M dexamethasone for 24 h.



Figure[CHa12] 15 Influence of dexamethasone on the expression of adhesion molecules
PBMC were incubated in the absence or presence of 3 x 10^{-7} M dexamethasone for 24 h. After pre-incubation, PBMC were centrifuged, resuspended in culture medium and subsequently incubated with Nalm-6 cells as target cells (E:T 5:1) in the presence of 625 pg/ml MT103. Cell lysis was allowed to proceed for 24 h, 48 h, 72 h and 6 days. Cells were stained with anti-CD4, anti-CD8 and (a) anti-CD2 or (b) anti-LFA-1, respectively and analyzed by flow cytometry.

$CD4^+$ cells up-regulated CD2 expression by 3-fold from the initial expression level after two days of stimulation. When effectors had been pre-incubated with dexamethasone, up-regulation on day 2 was only doubled. The expression of CD2 on $CD8^+$ cells was increased by 4.5-fold upon stimulation. In dexamethasone-treated $CD8^+$ cells, the MFI signal for CD2 only reached 55 % of the signal for -untreated cells on day 3 (Figure 15 a).

The expression of LFA-1 on $CD4^+$ and $CD8^+$ cells was three and four times as high, respectively as initial values in a cytotoxicity assay after three days. Dexamethasone pre-treatment resulted in an MFI signal for LFA-1 that was constantly 20 - 30 % lower in both $CD4^+$ and $CD8^+$ cells than in untreated cells (Figure 15 b).

Thus, dexamethasone slightly decreased expression of CD2 and LFA-1.

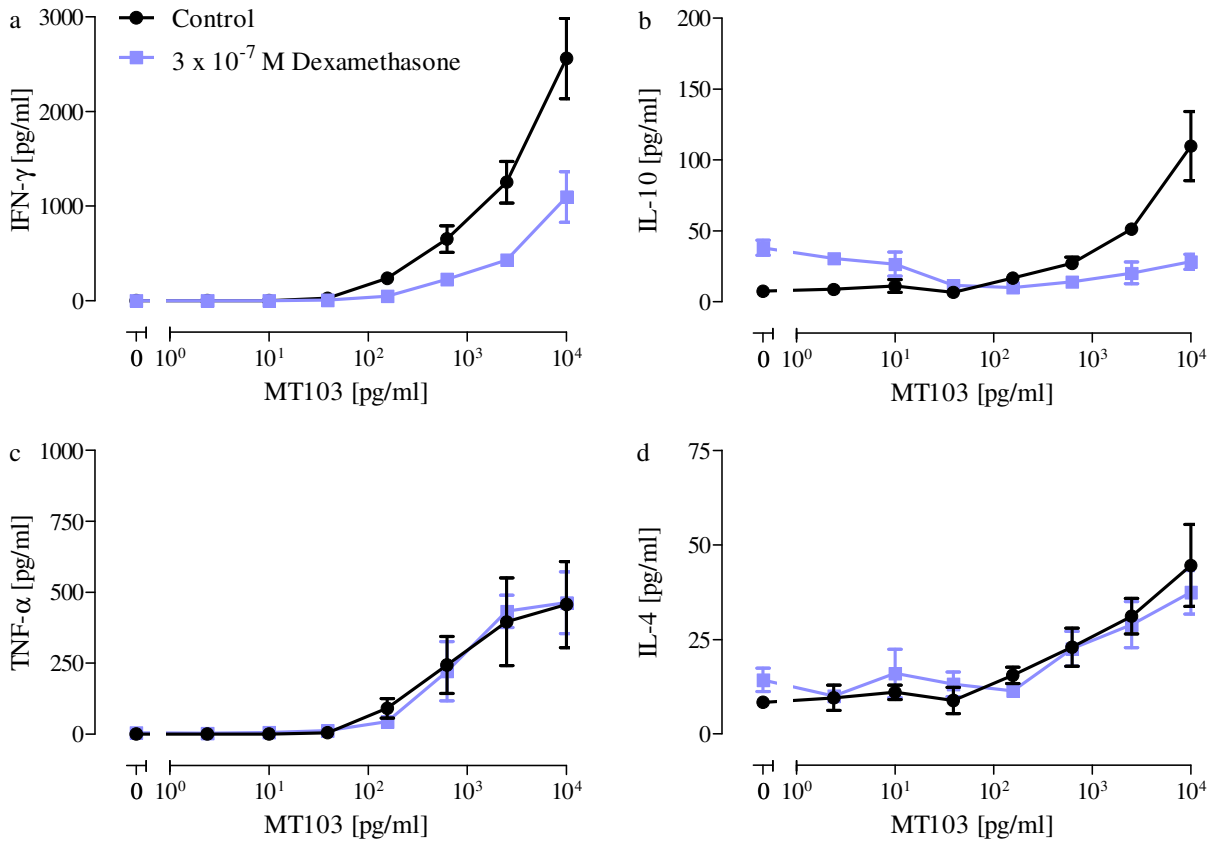
4.1.7.3 Influence of dexamethasone on expression of cytokines

Cytokines are messenger molecules (proteins, peptides or glycoproteins) that bind to cell surface receptors on their target cells. Upon binding of cytokines to their receptors, cascades of intracellular signaling eventually lead to up- or down-regulation of target genes.

An over-reaction of the immune system may involve a cytokine storm. Such a massive production of cytokines often follows administration of an immunostimulatory medicinal compound. A cytokine storm can lead to severe consequences for the human body, ranging from fever and inflammatory reactions to systemic collapse and finally death.

Effector cells were pre-incubated with 3 x 10^{-7} M dexamethasone and compared to untreated T cells in their capacity to produce cytokines upon activation with MT103 in the presence of target cells.

The cytokines examined included IFN-γ, TNF-α, IL-10 and IL-4. IFN-γ promotes NK cell activity (Carnaud et al. 1999) and up-regulates MHC class II molecules in B cells, DCs, and cells of the monocyte-macrophage lineage (professional APCs) (Mach et al. 1996). TNF-α is thought to be the controlling element in the 'cytokines network' and is responsible for the production of other cytokines (e.g., IL-1, IL-6 and IL-8) (Brennan et al. 1992). IL-10 is a strong immunosuppressor of T cells (Maeda et al. 1995). IL-4 is an anti-inflammatory cytokine important for homeostasis of the immune system (Bot et al. 2004).



Figure[CHa13] 16 Influence of dexamethasone on cytokine expression
PBMC were incubated in the absence or presence of 3 x 10^{-7} M dexamethasone for 24 h. After pre-incubation, PBMC were centrifuged, resuspended in culture medium and subsequently incubated with Nalm-6 cells as target cells (E:T 5:1) in the presence of increasing concentrations of MT103. After 24 h, supernatants from reaction samples were analyzed for content of cytokines using the Human Th1/Th2 Cytokine Kit II and flow cytometry.

The production of IFN-γ was reduced from 2.500 to 1.000 pg/ml at the highest BiTE concentration when effector cells had been pre-treated with dexamethasone (Figure 16 a). IL-10 release was decreased from 100 to 28 pg/ml (Figure 16 b). The production of TNF-α or IL-4 was not influenced by pre-incubation of effector cells with dexamethasone (Figure 16 c and d). The 24 h incubation period may explain the unresponsiveness of TNF-α and IL-4 expression towards dexamethasone treatment. These cytokines were shown to reach their

peak of expression after 8 to 12 h of stimulation (Brandl et al. 2007). After 24 h, induction of these cytokines and accordingly, a possible regulative effect of dexamethasone, may no longer be achievable.

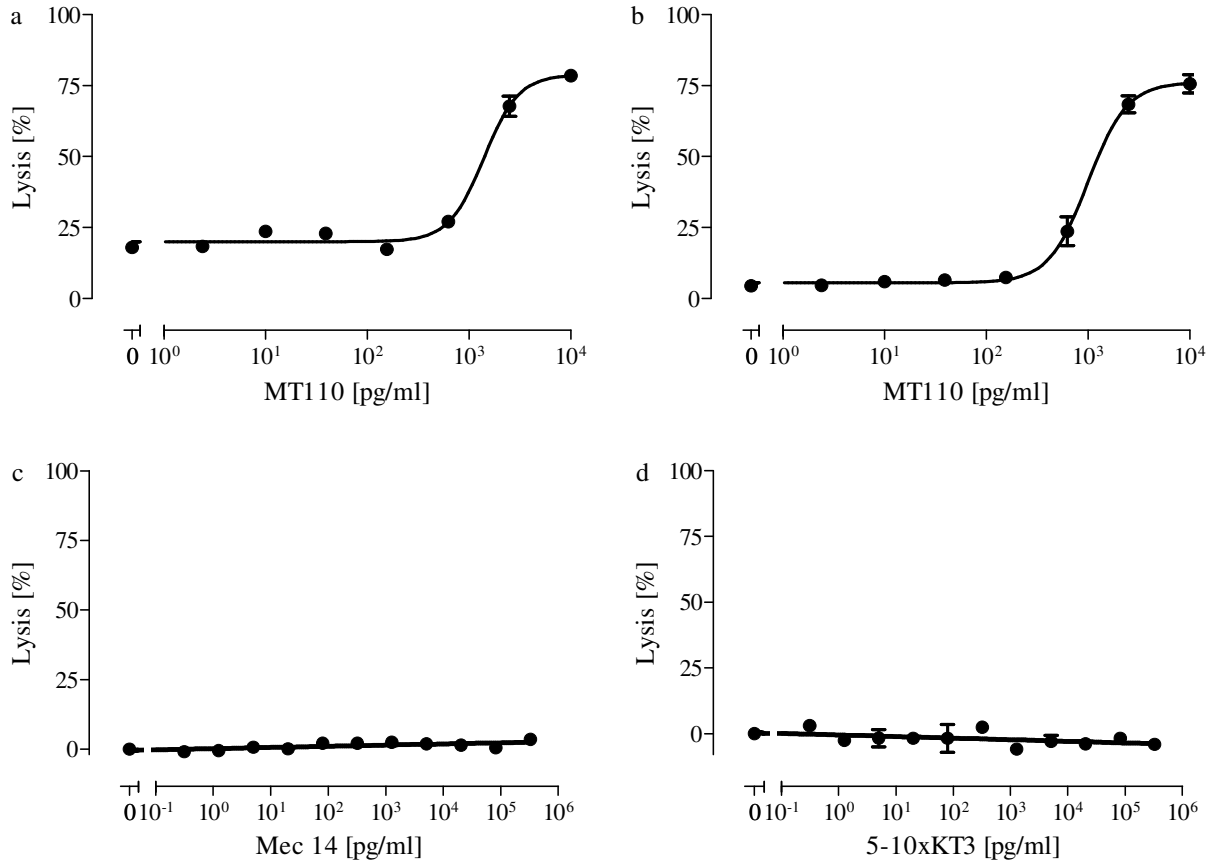
A pre-incubation with dexamethasone showed a remarkable effect on the expression of some cytokines. The production of other cytokines was hardly altered in response to dexamethasone after 24 h of stimulation.

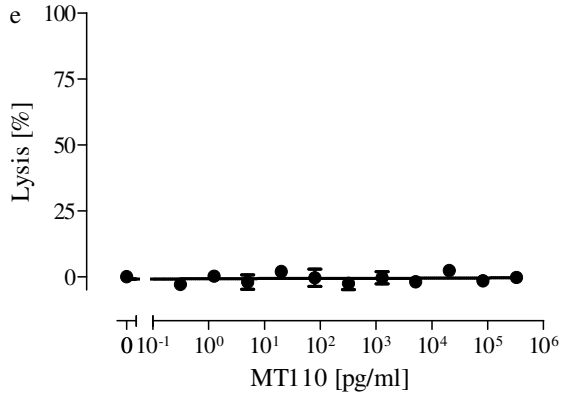
4.2 Mode of action of the BiTE molecule MT110

4.2.1 Lysis of tumor cells by PBMC

The bispecific single chain antibody construct MT110 is a construct that consists of two different binding domains linked together by a short peptide sequence. With its binding property for the invariant CD3ε chain of the TCR complex on T cells on the one side, and a binding region for human EpCAM on the other, it can attract T cells to $EpCAM^+$ tumor cells and allow for interaction with and ultimately lysis of target cells (Brischwein et al. 2006).

Cytolytic potential of T cells stimulated with MT110 in presence of target cells was tested in a cytotoxicity assay.





Figure[CHa14] 17 Lysis of tumor cells by PBMC

PBMC were co-cultured with Kato III cells (E:T 1:1) and increasing doses of MT110. Reactions were stopped after (a) 24 h and (b) 48 h. MT110 was replaced by (c) Mec 14 control BiTE or (d) 5-10xKT3 control BiTE at an E:T ratio of 10:1, and samples were analysed after 24 h. (e) Kato III cells were incubated with increasing doses of MT110 in the absence of effector cells and analyzed after 24 h. Redirected cell lysis was determined by propidium iodide uptake into cell nuclei using flow cytometric analysis.

At low concentrations of MT110 between 2.4 - 156 pg/ml, lysis of tumor cells was minimal after 24 h and 48 h. At a concentration of 625 pg/ml, the lysis rate started to increase and reached 76 % with 10 ng/ml MT110 (Figure 17 a and b).

The use of bi-specific single-chain antibody constructs Mec 14 and 5-10xKT3 demonstrated the restrictions of MT110 activity. The Mec 14 control construct shares the same CD3-binding arm with MT110, but has a different target-binding arm recognizing a small molecule herbicide. In contrast, the 5-10xKT3 control construct shares the same EpCAM-binding arm with MT110, but has a different CD3-binding arm reacting with murine but not human CD3.

When mixtures of PBMC and tumor cells were incubated in the presence of serial dilutions of these control BiTE molecules, no tumor cell lysis was observed (Figure 17 c and d). Simultaneous engagement of both binding arms seems to be required for the biological activities of MT110.

MT110 had virtually no effect on tumor cell lysis in the absence of effector T cells even above a concentration of 100 ng/ml, demonstrating that the anti-tumor activity is entirely mediated by redirected T cells (Figure 17 e).

4.2.2 Lytic potential of pre-stimulated T cells

To test the lytic performance of T cells in response to a several day-incubation period under stimulatory conditions, PBMC were subjected to two different activation procedures and two control conditions that lasted for 4 days:

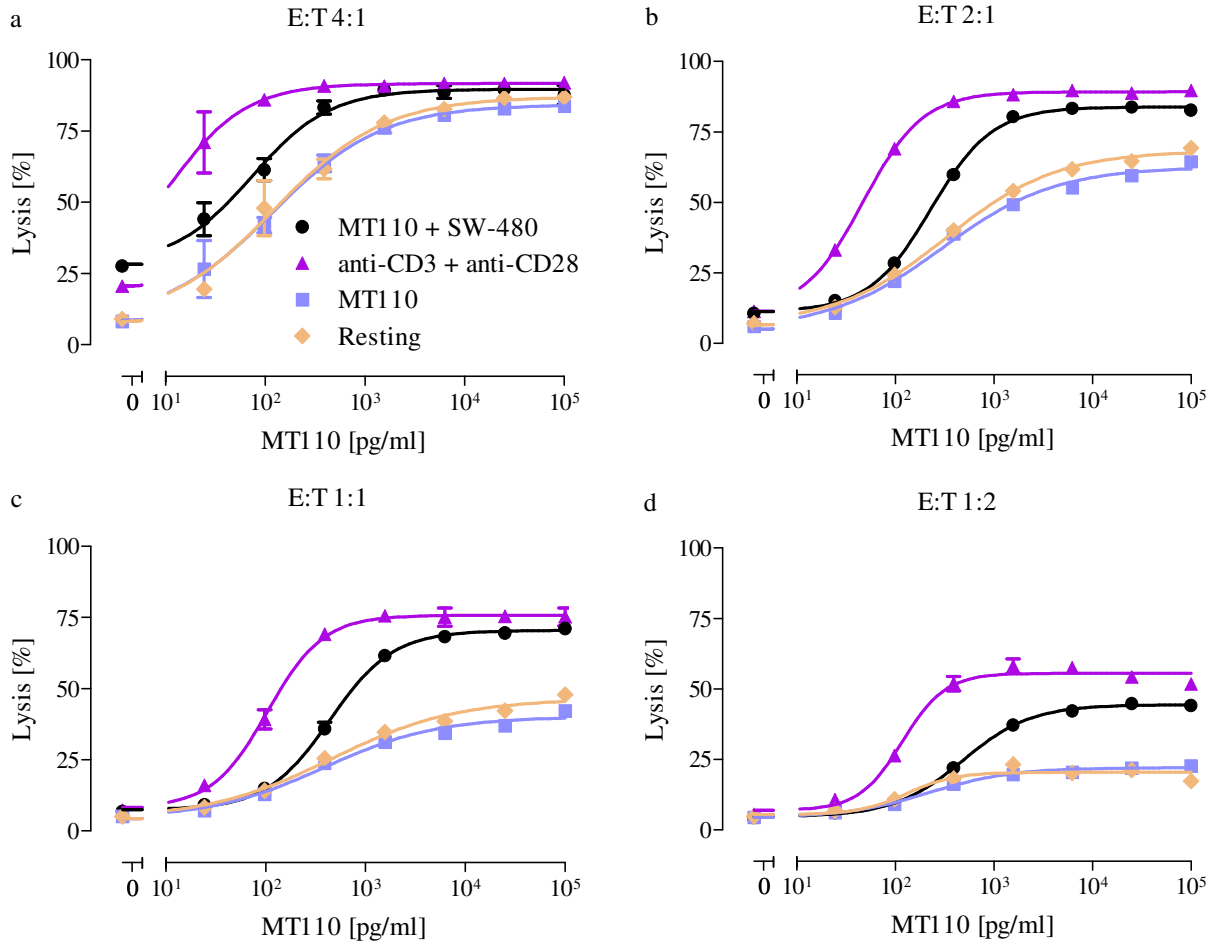
I. PBMC + SW-480 cells (E:T 5:1) + 10 ng/ml MT110. After 3 days, fresh SW-480 cells were added and the cell suspension cultured for another 24 h.

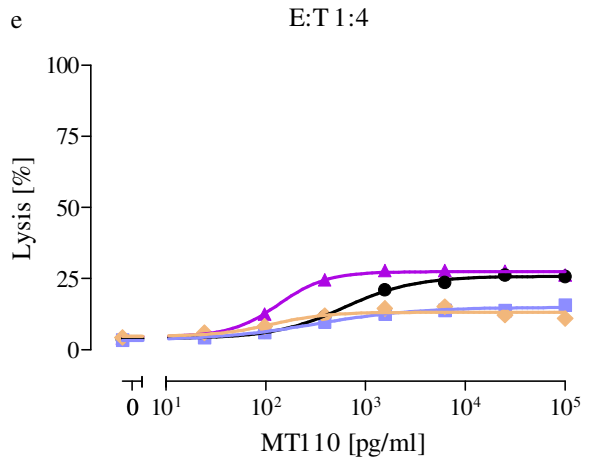
II. PBMC in a petri dish coated with anti-CD3 and anti-CD28. After 3 days, the cells were transferred to an uncoated plate and cultured for another 24 h. This step should allow for internalized T cell receptor to be re-expressed on the cell surface. As had been shown in previous experiments, a recovery phase was not necessary for BiTE-stimulated T cells.

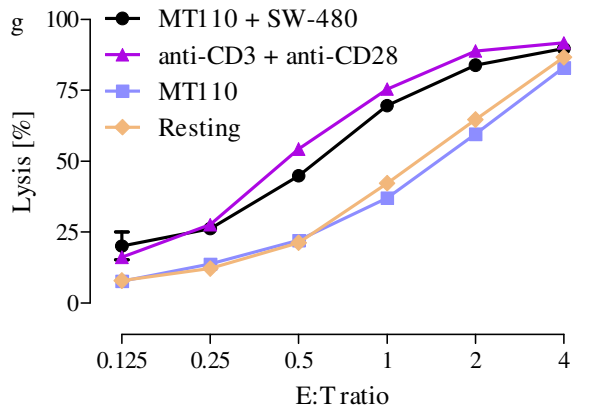
III. PBMC + 10 ng/ml MT110

IV. PBMC alone

After the pre-incubation period, $CD3^+$ cells were negatively isolated from the suspensions using magnetic beads. SW-480 cells were also removed during this process. Isolated $CD3^+$ cells were mixed with Kato III cells at different E:T ratios of 4:1, 2:1, 1:1, 1:2, 1:4 and 1:8. Cytotoxicity assays were performed with increasing doses of MT110 for 24 h.







h

E:T	MT110 + SW-480	anti-CD3 + anti-CD28	MT110	Resting
	EC_{50} MT110 [ng/ml]			
4:1	0.073	0.011	0.119	0.125
2:1	0.229	0.047	0.299	0.327
1:1	0.440	0.105	0.384	0.460
1:2	0.480	0.117	0.223	0.131
1:4	0.615	0.135	0.325	0.116
1:8	0.747	0.273	3.119	0.196

Figure[CHa15] 18 Lytic potential of pre-stimulated T cells
PBMC were co-cultured with SW-480 cells (E:T 5:1) and 10 ng/ml MT110, incubated with plate-bound anti-CD3 and anti-CD28 or with 10 ng/ml MT110 alone or left untreated for four days. After pre-treatments, $CD3^+$ T cells were purified from the suspensions and mixed with Kato III cells at the indicated E:T ratios and increasing doses of MT110. (a - f) After 24 h, cell lysis was monitored by nuclear uptake of propidium iodide using flow cytometric analysis. (g) Lysis values of all pre-incubation conditions were plotted against E:T ratios (25 ng/ml MT110). (h) Concentrations of MT110 for half maximal lysis of target cells (EC_{50} values) at all tested E:T ratios.

Pre-activated T cells lysed target cells more potently at all E:T ratios tested. Pre-stimulation of effector cells with anti-CD3/anti-CD28 resulted in the highest lysis rates. BiTE-pre-activated T cells could reach a similar level of target cell elimination with an MT110 concentration above 1.6 ng/ml. At an E:T ratio of 4:1, BiTE-pre-activated T cells were almost equally efficient as those pre-activated with anti-CD3/anti-CD28 (Figure 18 a). The difference in maximal lysis levels between anti-CD3/anti-CD28- and BiTE-pre-stimulated T cells grew larger with decreasing E:T ratios (Figure 18 b - f). However, under the same conditions, the relative differences between EC_{50} values of both effector cell populations showed a tendency to decrease (Figure 18 h).

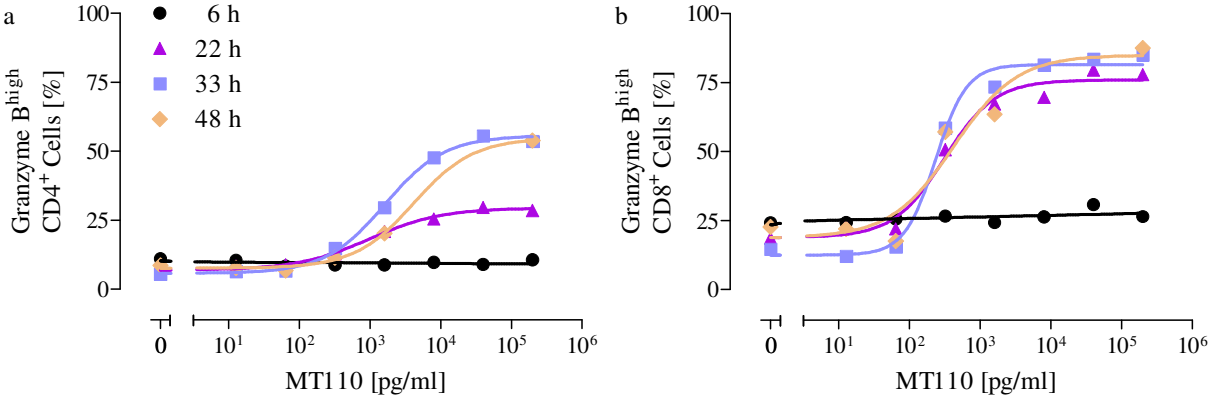
T cells from control incubations became nearly as potent as pre-activated T cells at an E:T ratio of 4:1 and high MT110 concentrations (Figure 18 a). With decreasing E:T ratios,

cytolytic potential of control T cells dropped below the curves of pre-stimulated T cells (Figure 18 b - f). With low E:T ratios, control curves were rather flat (Figure 18 d - f). The EC_{50} values of control incubations calculated for E:T 1:2, 1:4 and 1:8 are therefore not reliable (Figure 18 h).

4.2.3 Granzyme B expression in T cells in response to stimulation

Granzyme B is an effector molecule of major importance for the elimination of tumor and infected cells. Activated T cells produce high levels of granzyme B (Kanavaros et al. 2000). Granzyme B enters target cells through an as yet not fully understood mechanism. Inside the target cells, granzyme B activates various caspases and causes release of cytochrome *c* from mitochondria (Lieberman 2003). Both pathways result in the death of the target cells.

The expression level of granzyme B in T cells activated with MT110 in the presence of target cells was examined over the course of two days.



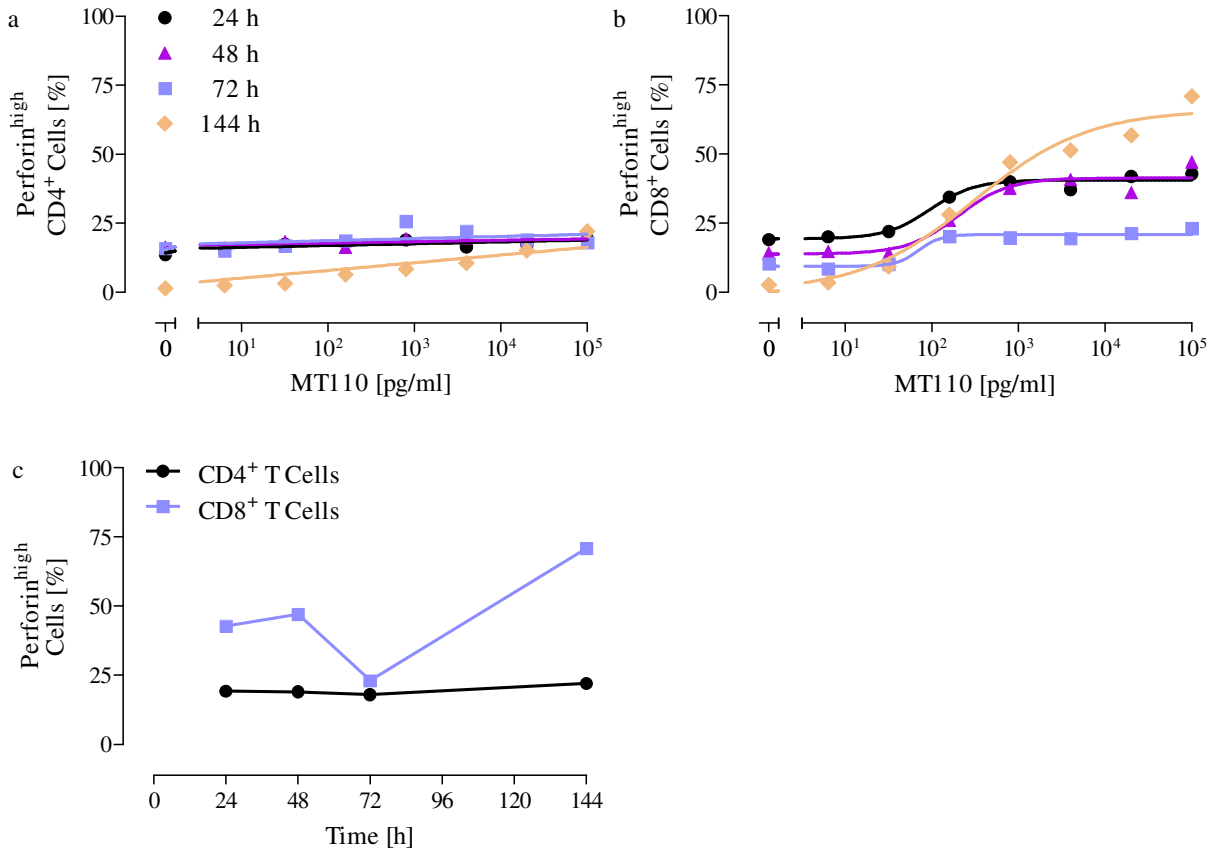
Figure[CHa16] 19 Granzyme B expression in T cells in response to stimulation
PBMC were co-cultured with Kato III (E:T 5:1) and increasing doses of MT110. After 6 h, 22 h, 33 h and 48 h reaction samples were stained for cell surface (a) CD4, (b) CD8, CD69 and intracellular granzyme B. Staining of cells was measured by FACS analysis.

Both $CD4^+$ and $CD8^+$ T cells required more than 6 h for the onset of up-regulation of granzyme B in response to stimulation. Granzyme B was produced by T cells in correlation to concentration of MT110. After 22 h, granzyme B expression was half maximal (29 %) in $CD4^+$ cells whereas the maximal level was already reached in $CD8^+$ cells at that time. A maximal percentage of 54 % of $CD4^+$ cells became granzyme B^{high} after 33 h (Figure 19 a). In the $CD8^+$ T cell subset, 78 % of cells had converted to granzyme B^{high} status after 22 h and remained on that level for the duration of investigation (two days) (Figure 19 b).

4.2.4 Perforin expression in T cells in response to stimulation

Perforin is another effector molecule produced by T cells that is necessary for the elimination of infected or malignant cells. T cells secrete perforin together with granzyme B and other cytotoxic components into the immunological synapse. Perforin has the capacity to polymerize in the target cell membrane forming holes of 5 - 20 nm diameter (Millard et al. 1984; Tschopp et al. 1986; Sauer et al. 1991). Whether Ca^{2+}-efflux from the cytoplasm is the only immediate consequence or if other molecules are also able to passage through the channel has not yet been established.

The expression level of perforin in T cells stimulated with MT110 in the presence of target cells was examined over the course of six days.



Figure[CHa17] 20 Perforin expression in T cells in response to stimulation
PBMC were co-incubated with Kato III cells (E:T 5:1) and increasing doses of MT110. After 24 h, 48 h, 72 h and 144 h, reaction samples were stained for cell surface (a) CD4, (b) CD8 and intracellular perforin. Staining of cells was measured by FACS analysis. (c) Time course of perforin expression in $CD4^+$ and $CD8^+$ cells (100 ng/ml MT110).

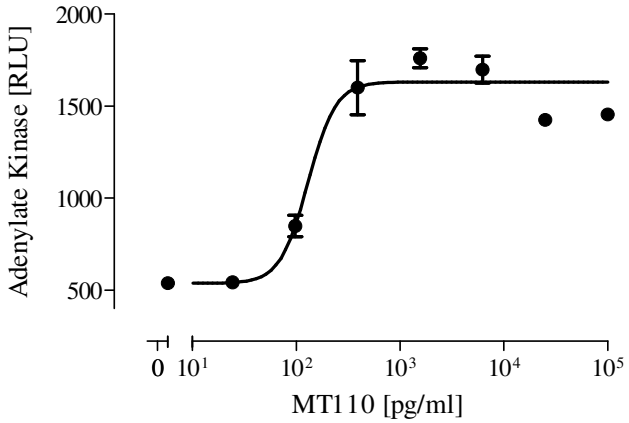
Twenty % of unstimulated $CD4^+$ cells were found to be $perforin^{high}$ but this value could not be increased by stimulation of T cells with MT110 in the presence of target cells (Figure 20 a). $CD8^+$ cells showed a dose-dependent response towards BiTE stimulation. While 20 % of unstimulated $CD8^+$ cells expressed high levels of perforin, 47 % of $CD8^+$ cells had gained a

perforin^{high} status after 2 days of stimulation with 1 ng/ml MT110. On day 3, perforin content in $CD8^+$ cells was temporarily exhausted; only 23 % of cells showed a high perforin expression. But the value went up to 71 % after 6 days (Figure 20 b). Figure 20 c clearly shows the low level of perforin in $CD8^+$ cells on day 3.

4.2.5 Release of adenylate kinase during lysis of tumor cells

Adenylate kinase is a phosphotransferase enzyme that catalyzes the interconversion of adenine nucleotides and plays an important role in homeostasis of the cellular energy (Randak and Welsh 2005).

Adenylate kinase is not released from the cytosol of a healthy cell at any time. Content of adenylate kinase in the cell culture medium provides information on the integrity of target cell membranes.



Figure[CHa18] 21 Release of adenylate kinase during lysis of tumor cells
Isolated $CD3^+$ T cells were mixed with Kato III cells (E:T 5:1) and increasing doses of MT110. After 44 h, release of adenylate kinase was measured using the ToxiLight® BioAssay Kit.

When T cells were activated with MT110 in the presence of target cells, the level of released adenylate kinase tripled over the concentration range of MT110. MT110 at a concentration of 24.4 pg/ml did not cause any changes in the release pattern of adenylate kinase but with 391 pg/ml MT110, a three-fold increase from the basal value was reached (Figure 21).

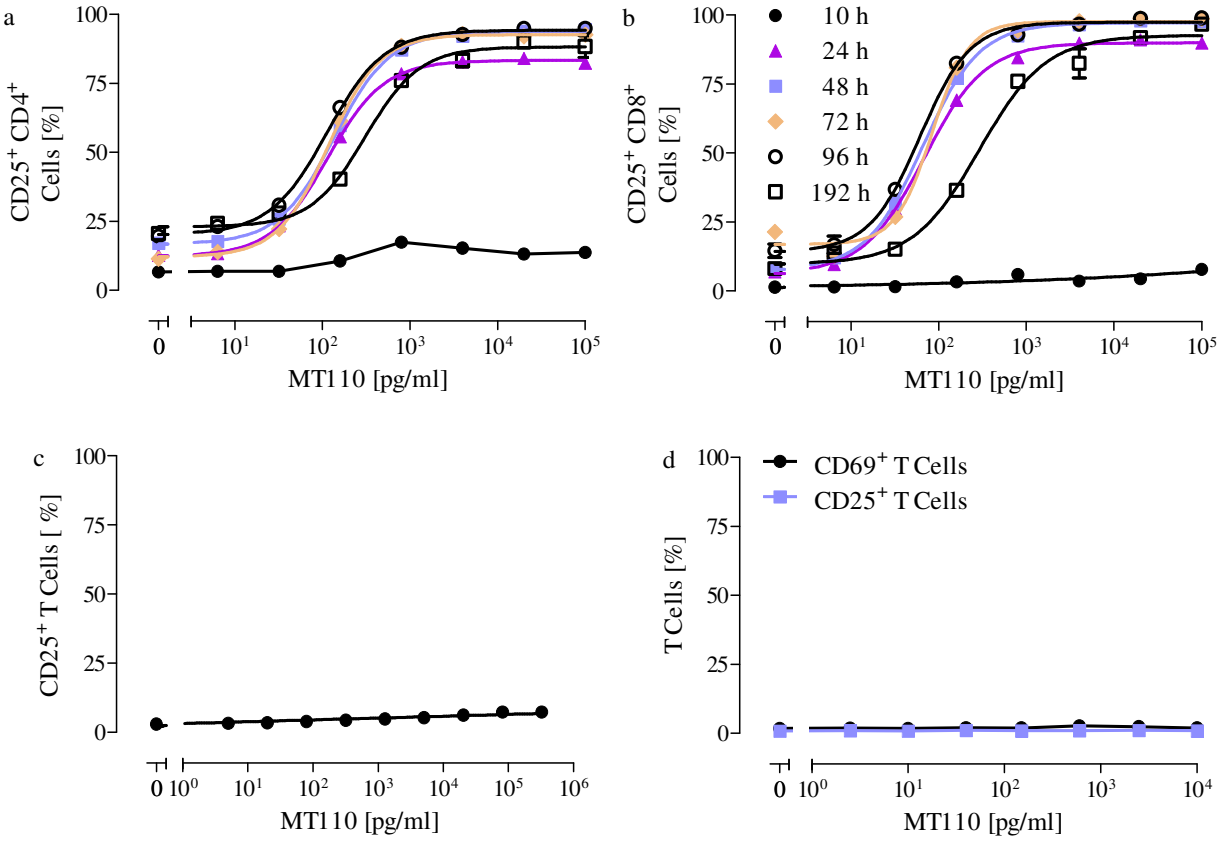
The membranes of the cells involved in this assay were clearly damaged during the cytotoxicity assay with MT110.

4.2.6 Expression of CD25, CD2 and LFA-1

When primary T cells receive stimulatory signals through their T cell receptor, new cell surface glycoproteins are synthesized and expressed (Yokoyama et al. 1988; Testi et al. 1989). Cell surface antigens inducible upon activation are therefore called activation markers.

Activation marker CD25 is a type I transmembrane protein and part of the IL-2 receptor. The interaction between IL-2 and IL-2 receptor is responsible for distinct cellular changes within the target cells: IL-2 signaling exerts important roles in cell cycle progression (Cantrell and Smith 1984), delivers survival signals to the cell (Gillis et al. 1978), activates cellular differentiation programs (Le Gros et al. 1990; Seder et al. 1994; Swain 1994) and primes the cell for activation-induced cell death (Lenardo 1991; Singer and Abbas 1994; Zheng et al. 1995).

The expression pattern of CD25 upon stimulation with MT110 in the presence of target cells was examined over a period of eight days.

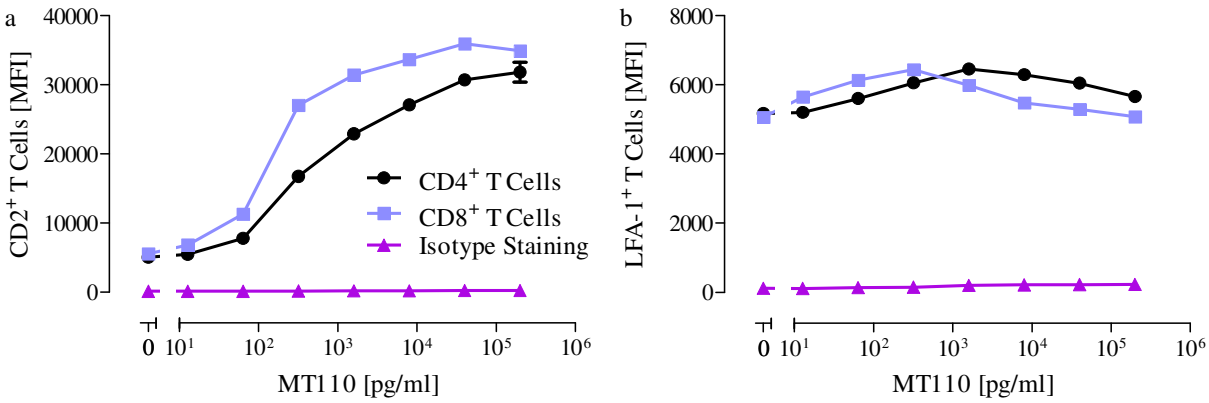


Figure[CHa19] 22 Expression of CD25 on $CD4^+$ and $CD8^+$ cells
PBMC were mixed with Kato III cells (E:T 5:1) and increasing doses of MT110. Expression of CD25 on the cell surface of (a) $CD4^+$ and (b) $CD8^+$ cells was analyzed by cell surface staining and FACS analysis after 10 h, 1, 2, 3, 4 and 8 days. (c) PBMC were incubated with increasing doses of MT110 in the absence of target cells. CD25 expression was analyzed after 24 h by flow cytometry. (d) PBMC were incubated with $EpCAM^-$ Nalm-6 cells (irrelevant target cells) and increasing doses of MT110. Expression of CD25 and CD69 was analyzed after 24 h by flow cytometry.

An increase in expression of activation marker CD25 could be observed on both $CD4^+$ and $CD8^+$ cells after 1 day and lasted over the duration of the experiment (8 days). The up-regulation of expression was dependent on the concentration of MT110 (Figure 22 a and b).

MT110 had no detectable effect on T cell activation in the absence of target cells (Figure 22 c) or in the presence of EpCAM$^-$ tumor cells (Figure 22 d). This demonstrates that simultaneous binding of the EpCAM and CD3 binding arms of MT110 is required for T cell activation and that MT110 is highly specific for the EpCAM target antigen.

As mentioned in 4.1.6, CD2 is a glycoprotein on the surface of T cells. It is found on approximately 90 % of mature peripheral T cells and mediates adhesion between T lymphocytes and their cognate cellular partners that express the specific ligand LFA-3 (Hunig 1985; Shaw et al. 1986; Selvaraj et al. 1987). In addition, CD2 by itself or in conjunction with T cell receptor stimulation, transduces signals resulting in T lymphocyte activation (Yang et al. 1986; Hunig et al. 1987). LFA-1 is an adhesion molecule that plays an important role in T cell trafficking and T cell adhesion to APCs (Hogg et al. 2003).
T cells were activated with MT110 in the presence of target cells and assayed for their cell surface expression of CD2 and LFA-1.



Figure[CHa20] 23 Expression of CD2 and LFA-1 on CD4$^+$ and CD8$^+$ cells
PBMC were mixed with Kato III cells (E:T 5:1) and increasing doses of MT110. Expression levels of (a) CD2 and (b) LFA-1 on CD4$^+$ and CD8$^+$ cells were analyzed by cell surface staining and flow cytometry after 24 h. An antibody of the same isotype as the detection antibodies but without specificity for a relevant antigen was used as a control.

MT110 caused an increase in CD2 expression in a dose-dependent manner on both CD4$^+$ and CD8$^+$ cells. When 200 ng/ml MT110 had been added to samples, the increase was as high as 7-fold (Figure 23 a), whereas LFA-1 could be up-regulated neither on CD4$^+$ cells nor on CD8$^+$ cells (Figure 23 b).

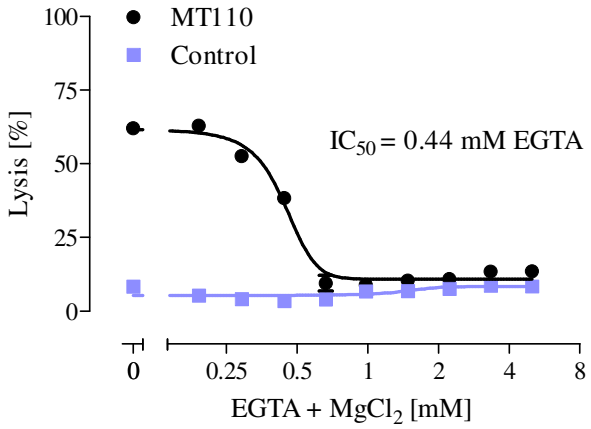
4.2.7 Importance of Ca^{2+} ions for lysis of tumor cells

As shown in 4.1.4 and 4.2.4, BiTE-mediated stimulation of PBMC increases the level of perforin inside T cells. The pore-forming protein perforin multimerizes in the plasma

membrane of a target cell in a Ca^{2+}-dependent manner (Pipkin and Lieberman 2007). In order to find out about the importance of Ca^{2+} for MT110-dependent elimination of target cells, EGTA was added in increasing concentrations to a standard cytotoxicity assay with PBMC and Kato III cells. EGTA acts as a chelator for divalent cations and depletes the medium of free Ca^{2+} ions. EGTA preferentially sequesters Ca^{2+} ions but would also chelate Mg^{2+} ions. Therefore $MgCl_2$ was added together with EGTA to sample wells. Magnesium is an important component for stabilization of the ATP molecule, oxidative phosphorylation in mitochondria and biosynthesis of nucleic acid. Furthermore, LFA-1 dependent cell adhesion requires magnesium (Shaw et al. 1986).

4.2.7.1 Influence of EGTA on lysis of tumor cells

As a complexant for divalent cations, EGTA chelates free Ca^{2+} ions in the culture medium. Cytotoxicity of BiTE-stimulated T cells towards target cells was tested in the presence of increasing concentrations of EGTA.



Figure[CHa21] 24 Influence of EGTA on lysis of tumor cells
Isolated $CD3^+$ cells were co-cultured with Kato III cells (E:T 4:1) in the absence or presence of 500 ng/ml MT110. Increasing doses from 0.19 mM to 5 mM EGTA + $MgCl_2$ were added to the samples. The reaction was stopped after 20 h and redirected cell lysis was determined by propidium iodide uptake into cell nuclei using flow cytometric analysis.

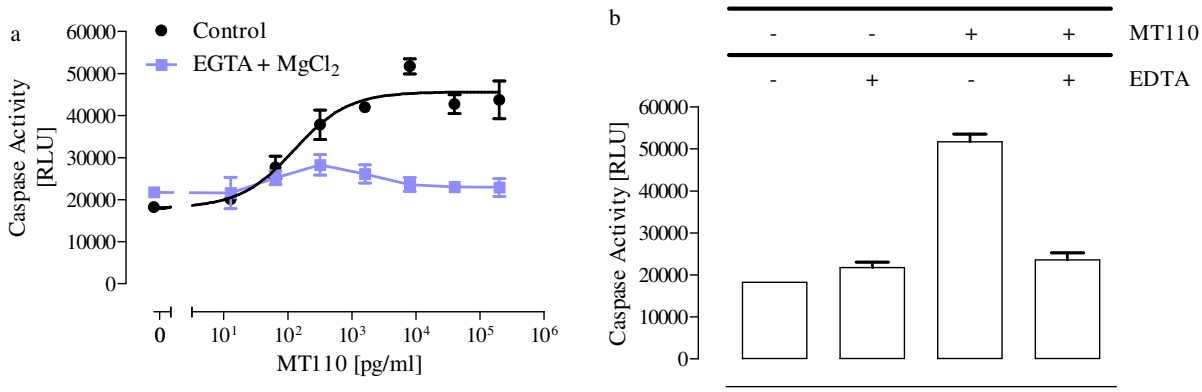
Sixty-two % of target cells were killed in the presence of 500 ng/ml MT110 and the absence of EGTA after 20 h. At a concentration of 0.29 mM EGTA, lysis started to drop and reached base level (9 %) at 0.66 mM EGTA. The EGTA concentration that reduced lysis of target cells by 50 % (inhibitory concentration IC_{50}) was 0.44 mM (Figure 24).

EGTA inhibited lysis of tumor cells in a standard cytotoxicity assay.

4.2.7.2 Influence of EGTA on activation of caspases

As EGTA obviously inhibited target cell lysis, BiTE-induced activation of caspases in the presence of EGTA was tested.

Caspases are a family of cysteine proteases that work together in the proteolytic caspase cascade activated by apoptotic stimuli (Nunez et al. 1998). Caspase 3 and caspase 7 are thought to be preferentially activated during apoptosis (Harris et al. 1998). As shown in 4.1.3 BiTE molecules are capable of activating caspases 3 and 7 in a properly setup cytotoxicity assay.



Figure[CHa22] 25 Influence of EGTA on activation of caspases
PBMC were mixed with Kato III cells (E:T 5:1) and increasing doses of MT110. 2 mM EGTA + $MgCl_2$ were added to one set of reaction wells. (a) After 6 h, content of active caspases in the cells was analyzed using the Caspase-Glo™ 3/7 Assay. (b) Column bar graph comparing the values without MT110 and with 8 ng/ml MT110.

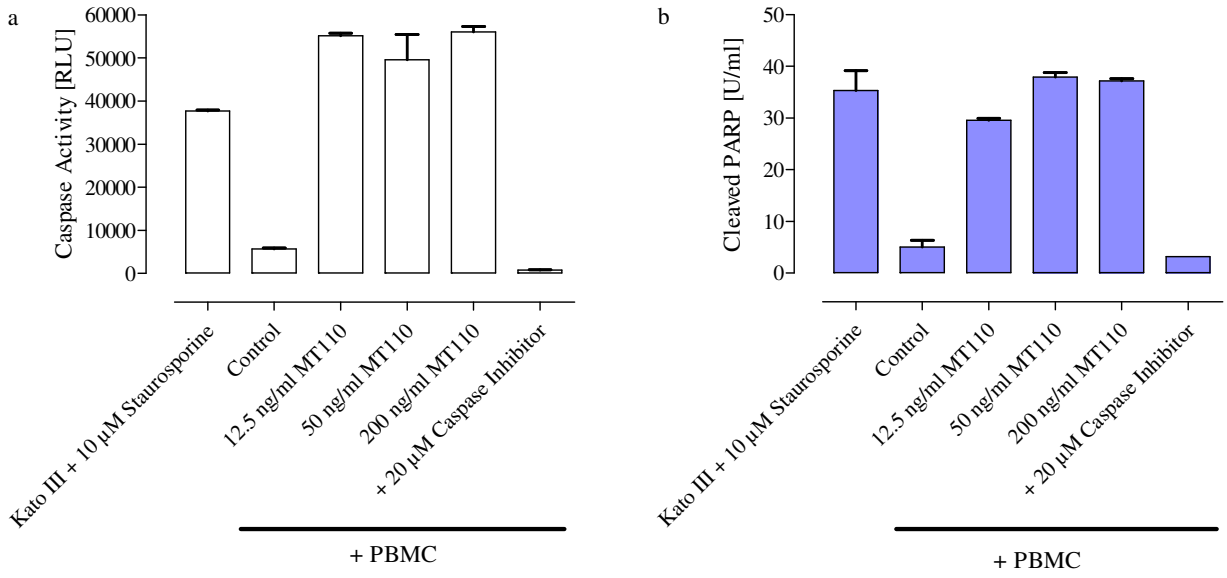
After 6 h, MT110 dose dependently created a signal between 18.000 and 44.000 relative light units (RLU), which serves as a direct measure for activity of caspases 3 and 7. When 2 mM EGTA + $MgCl_2$ were present in the medium, the signal mostly remained on the basal level around 25.000 RLU (Figure 25).

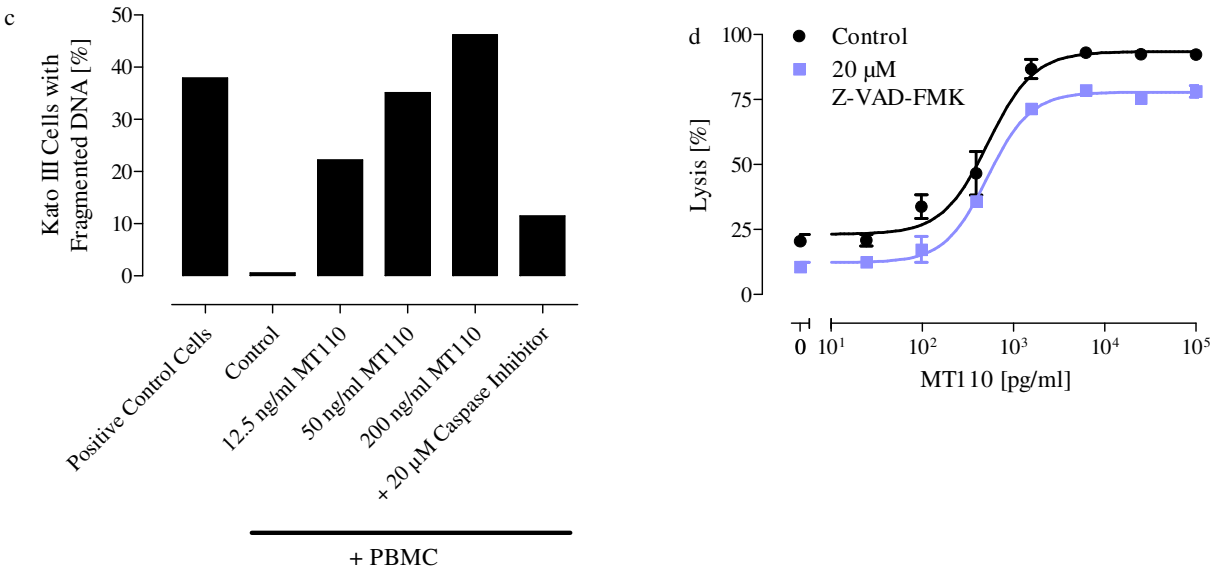
The addition of EGTA to a standard cytotoxicity assay inhibited activation of caspases 3 and 7.

4.2.8 Importance of caspases for lysis of tumor cells

When a cell receives apoptotic stimuli from outside (radiation, hormones, viral infection) or inside the cell, the inactive precursors of the caspases (a family of death proteases) are cleaved to become active enzymes. Functional caspases destroy a number of structural and housekeeping proteins (e.g., the nuclear lamins and the proteins of the cytoskeleton) and they contribute to fragmentation of DNA eventually resulting in apoptosis (Russell and Ley 2002). Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme involved in numerous nuclear processes such as DNA repair and transcription (Oei et al. 1997), acts as a substrate for caspases. Cleavage of PARP is a valuable indicator of apoptosis (Cohen 1997).

The ability of MT110 to induce classical apoptosis events (cleavage of PARP and DNA fragmentation) and uptake of propidium iodide into nuclei of target cells was tested. At the same time the influence of the cell-permeable irreversible caspase inhibitor Z-VAD-FMK upon the reactions was examined.





Figure[CHa23] 26 Importance of caspases for lysis of tumor cells

PBMC were co-cultured with Kato III cells (E:T 5:1), without MT110, with 12.5 ng/ml, 50 ng/ml or 200 ng/ml MT110. An additional sample with 200 ng/ml MT110 was supplemented with 20 µM caspase inhibitor Z-VAD-FMK. Samples with Kato III cells and 10 µM staurosporine served as positive control. (a) Content of active caspases in the cells was analyzed after 29 h using the Caspase-Glo™ 3/7 Assay. (b) Cleavage of PARP was measured with a sandwich ELISA after 23 h of redirected T cell lysis. (c) DNA fragmentation in Kato III cells was determined after 26 h via fluorescent labeling of double strand breaks (TUNEL assay). (d) Isolated $CD3^+$ cells were mixed with Kato III cells (E:T 5:1) and increasing doses of MT110 for 44 h. One set of samples was supplemented with 20 µM caspase inhibitor. Lysis of Kato III cells was monitored by nuclear uptake of propidium iodide using flow cytometric analysis.

Stimulation of PBMC with MT110 in the presence of target cells resulted in a 10-fold increase in activation of pro-caspases. Already the lowest concentration of MT110 (12.5 ng/ml) caused a maximal luminescence signal. Addition of 20 µM caspase inhibitor Z-VAD-FMK reduced the signal below the control value (Figure 26 a).

More PARP molecules were cleaved in MT110-supplemented cytotoxicity assays compared to control assays. With 50 ng/ml MT110, the amount of cleaved PARP in cell lysates was seven times as high as the basal level. Twenty µM caspase inhibitor Z-VAD-FMK completely inhibited the effect of MT110; cleavage of PARP was as rare as in the control sample (Figure 26 b).

DNA was fragmented resulting in 22 % apoptotic cells when 12.5 ng/ml MT110 were present in the medium of a cytotoxicity reaction. When higher concentrations of MT110 were added, the percentage of apoptotic cells rose accordingly. But when 20 µM caspase inhibitor Z-VAD-FMK were added to samples with 200 ng/ml MT110, fragmentation of DNA was reduced to a low level of only 12 % apoptotic cells (Figure 26 c).

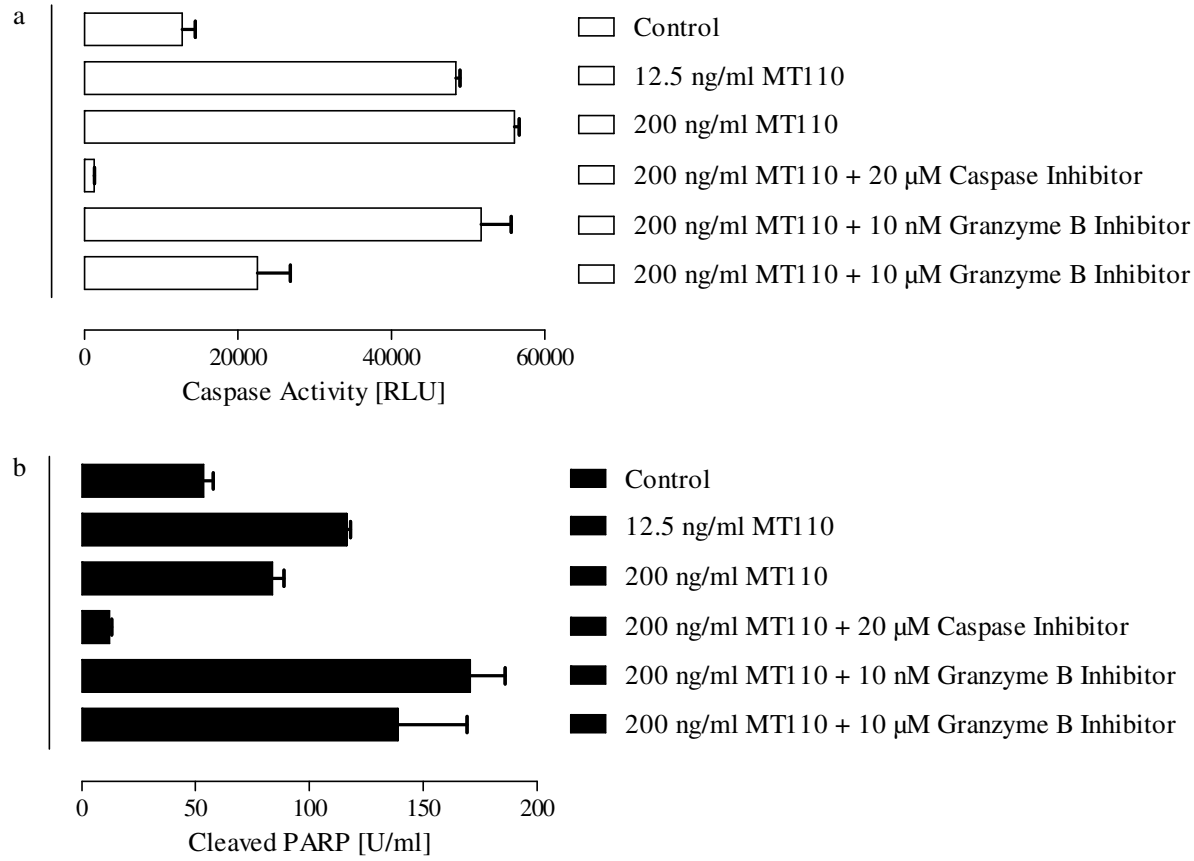
However, caspase inhibitor Z-VAD-FMK did not influence BiTE-induced tumor cell death. The curves in Figure 26 d start at different basal values but display the same EC_{50} value. The

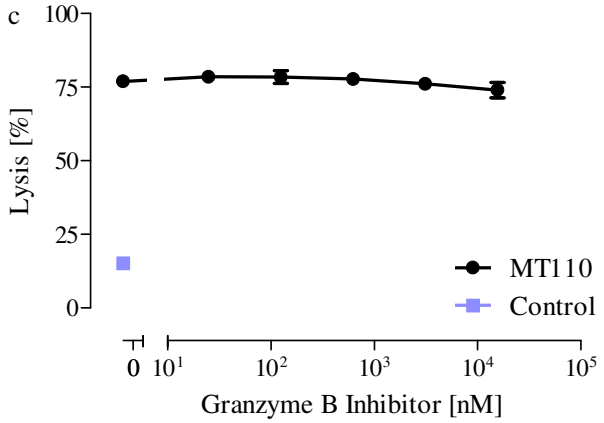
caspases seem to exert crucial functions in cleavage of PARP and fragmentation of DNA but are not indispensable for BiTE-mediated tumor cell death.

4.2.9 Importance of granzyme B for lysis of tumor cells

Granzyme B holds a central position in initiating the apoptosis cascade. The protease activates caspases, cleaves PARP directly or indirectly through the activation of caspases (Harris et al. 1998) and induces fragmentation of DNA.

Content of active caspases and cleaved PARP in regular cytotoxicity assays was compared to content in samples with caspase inhibitor or granzyme B inhibitor.





Figure[CHa24] 27 Importance of granzyme B for lysis of tumor cells
PBMC were mixed with Kato III cells (E:T 5:1) and various supplements: 12.5 ng/ml MT110, 200 ng/ml MT110, 200 ng/ml MT110 + 20 µM caspase inhibitor, 200 ng/ml MT110 + 10 nM granzyme B inhibitor or 200 ng/ml MT110 + 10 µM granzyme B inhibitor. (a) After 26 h, the cells were analyzed for content of active caspases using the Caspase-Glo™ 3/7 Assay. (b) After 23 h, another set of the same reactions was analyzed for content of cleaved PARP in an ELISA assay. (c) Co-cultures of PBMC and Kato III cells (E:T 5:1) were incubated with 100 ng/ml MT110 and increasing concentrations of granzyme B inhibitor. After 27 h, redirected cell lysis was determined by propidium iodide uptake into cell nuclei using flow cytometric analysis.

Already 12.5 ng/ml MT110 in a cytotoxicity reaction increased the content of active caspases by more than 3-fold compared to the control value without MT110. A higher MT110 concentration (200 ng/ml) further elevated the level of active caspases. Addition of caspase inhibitor to the cell culture medium with 200 ng/ml MT110 reduced the activation of caspases to a level far below the control value without MT110. Ten nM of granzyme B inhibitor were not enough to have an influence on the activation of caspases induced by MT110. An increase in concentration of granzyme B inhibitor by a factor of 1000 (10 µM) reduced MT110-induced caspase activity by more than half but not down to base level (Figure 27 a).

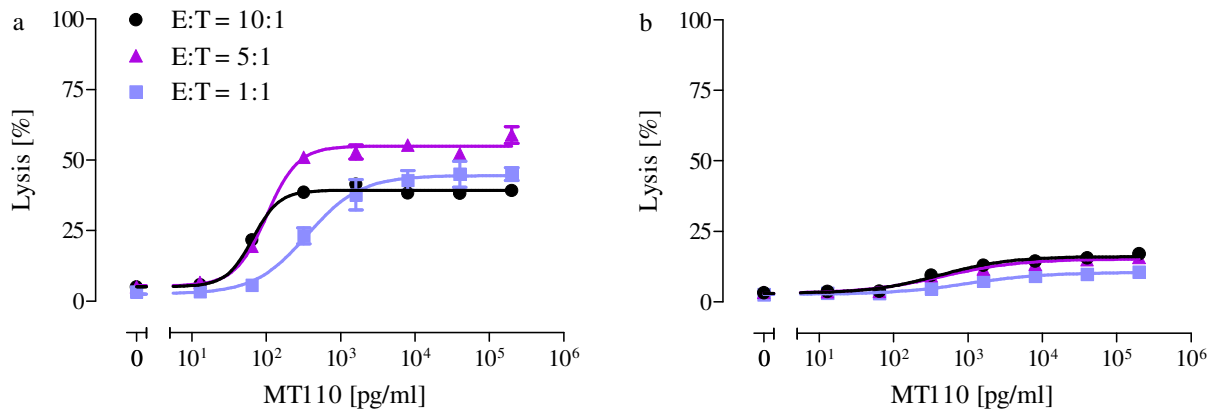
A similar response pattern was observed when cleavage of PARP - a characteristic signal for apoptosis - was examined. Although the sample containing PBMC + target cells + 200 ng/ml MT110 showed a lower cleavage rate for PARP than the one with 12.5 ng/ml MT110. Comparing this result with previous results (Figure 26), with the effects on activation of caspases in the parallel experiment (Figure 27 a) and with the “10 nM granzyme B inhibitor” sample in this experiment, it might be considered an artefact. Incubation with caspase inhibitor resulted in a strongly down-regulated cleavage rate of PARP. Ten nM of granzyme B inhibitor did not prevent cleavage of PARP. Increasing the concentration of granzyme B inhibitor seems to reduce cleavage rate of PARP by 19 % although the error bar does not allow a clear statement (Figure 27 b).

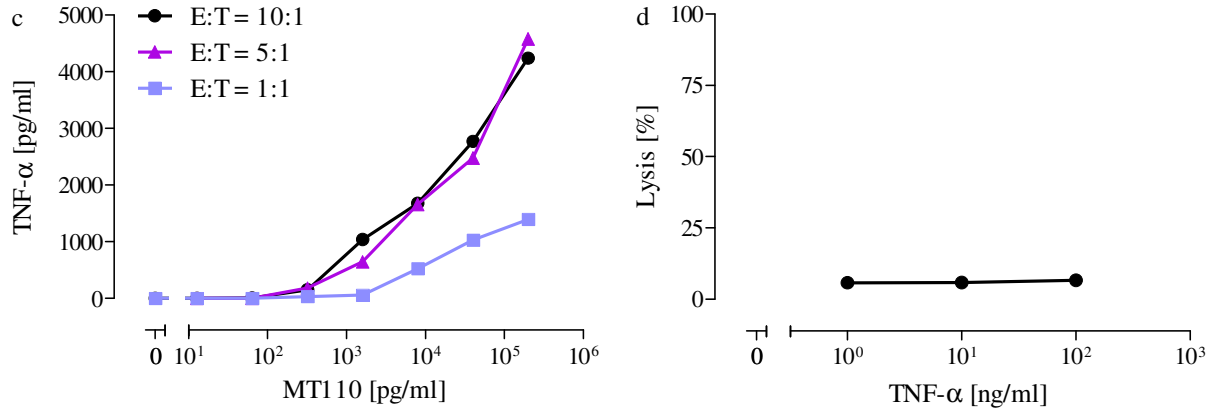
MT110-induced tumor cell death was not compromised by the presence of granzyme B inhibitor (Figure 27 c). Granzyme B inhibitor had a medium to minor effect on apoptotic signals as detected in a cytotoxicity reaction but did not impair MT110-mediated lysis of tumor cells.

4.2.10 Importance of soluble factors for lysis of tumor cells

Activated T cells produce and secrete a broad variety of interleukins, cytokines and effector molecules like granzyme B and perforin. It was evaluated whether or not supernatant from a cytotoxicity assay contained substances that would lead to tumor cell death.

The cytokine TNF-α is known to induce apoptosis in tumor cell lines (Janicke et al. 1998). Additionally, TNF-α showed potent anti-tumor activity in animal models (Carswell et al. 1975). TNF-α is produced by T cells, macrophages and neutrophils and is critical for cell trafficking, inflammation, maintenance of lymphoid organ structure, and host defense against various pathogens, such as *Listeria* and *M. tuberculosis* (Pfeffer et al. 1993; Rothe et al. 1993; Flynn et al. 1995; Pasparakis et al. 1996). Supernatants of cytotoxicity assays were tested for content of TNF-α.





Figure[CHa25] 28 Importance of soluble factors for lysis of tumor cells
PBMC were co-cultured with Kato III cells at E:T ratios of 10:1, 5:1 and 1:1 and increasing doses of MT110. (a) After 27 h cell lysis was examined via PI staining using flow cytometry. (b) Supernatants of reaction samples were centrifuged to remove any residual effector or target cells and added to fresh target cells. Target cells were incubated with supernatants for 28 h and analyzed for cell lysis via PI staining using flow cytometry. (c) A portion of the supernatants from (a) was analyzed for content of TNF-α using the Human Th1/Th2 Cytokine Kit II and flow cytometry. (d) 1 ng/ml, 10 ng/ml and 100 ng/ml of commercially-available TNF-α was added to Kato III cells and vitality of cells analyzed after 27 h via PI staining using flow cytometry.

Soluble factors in reaction sample supernatants did not contain compounds that caused target cell death. When supernatants of a classical cytotoxicity assay (Figure 28 a) were transferred to fresh target cells, almost no lysis could be observed (Figure 28 b). EC_{50} values for the cytotoxicity assays ranged from 65 pg/ml (E:T 10:1) to 100 pg/ml (E:T 5:1) and 358 pg/ml MT110 (E:T 1:1) (Figure 28 a).

Supernatants were investigated for content of the cytokine TNF-α, a pro-inflammatory cytokine. Activation of T cells with MT110 in the presence of target cells induced production of TNF-α. Content of TNF-α in supernatants increased in correlation to concentration of MT110 and E:T ratio. With 200 ng/ml MT110 and an E:T ratio of 1:1, content of the cytokine in the supernatant rose from 0 to 1.4 ng/ml TNF-α. When the E:T ratio was 5:1, TNF-α level reached 4.6 ng/ml (Figure 28 c).

Obviously TNF-α alone can not lyse Kato III cells. This finding was supported by an experiment where commercially available TNF-α was added in three different concentrations to Kato III cells. After 27 h of incubation, death of tumor cells could not be detected (Figure 28 d).

4.3 Correlation between TGF-β and the efficiency of T cells in tumor cell lysis

Production of TGF-β helps progressing tumors to escape from immune surveillance (Akhurst and Derynck 2001; Derynck et al. 2001; Siegel and Massague 2003). A great number of cancerous cell lines are known to produce TGF-β directly. TGF-β might contribute to the inhibition of the anti-tumor immune response by suppressing lymphocyte function e.g., through transcriptional repression of cytolytic genes (Thomas and Massague 2005).

In order to investigate the influence of TGF-β on specific properties of T cells, experiments were performed with activated T cells that had been pre-exposed to TGF-β over the course of 2.5 days. In comparison, activated T cells naïve to TGF-β were tested in the same experiments. Activation of T cells was achieved by co-culture with MT110 and relevant target cells: $EpCAM^+$ SW-480 cells. Stimulation with anti-CD3 and anti-CD28 served as a positive control. As this procedure includes a co-stimulus, it is supposed to be an extremely potent way of activation. Unstimulated T cells incubated in the presence or absence of TGF-β were used as a negative control.

A TGF-β concentration was chosen that was reported in the literature to be effective (Thomas and Massague 2005).

In detail, pre-incubation conditions were as follows:

I. PBMC + SW-480 (E:T 5:1) + 10 ng/ml MT110. After 2 days, an identical amount of SW-480 target cells was added to the cell suspension.
II. PBMC + SW-480 (E:T 5:1) + 10 ng/ml MT110 + 100 pM TGF-β. Addition of fresh target cells after 2 days.
III. PBMC on a petri dish coated with anti-CD3 and anti-CD28. Cells were transferred to an uncoated petri dish after 2 days. Internalized T cell receptor should be redelivered to the T cell surface during the recovery phase. According to previous experiments, this step was not necessary for BiTE-stimulated T cells.
IV. PBMC on a petri dish coated with anti-CD3 and anti-CD28 + 100 pM TGF-β. Transfer to an uncoated petri dish after 2 days.
V. PBMC
VI. PBMC + 100 pM TGF-β

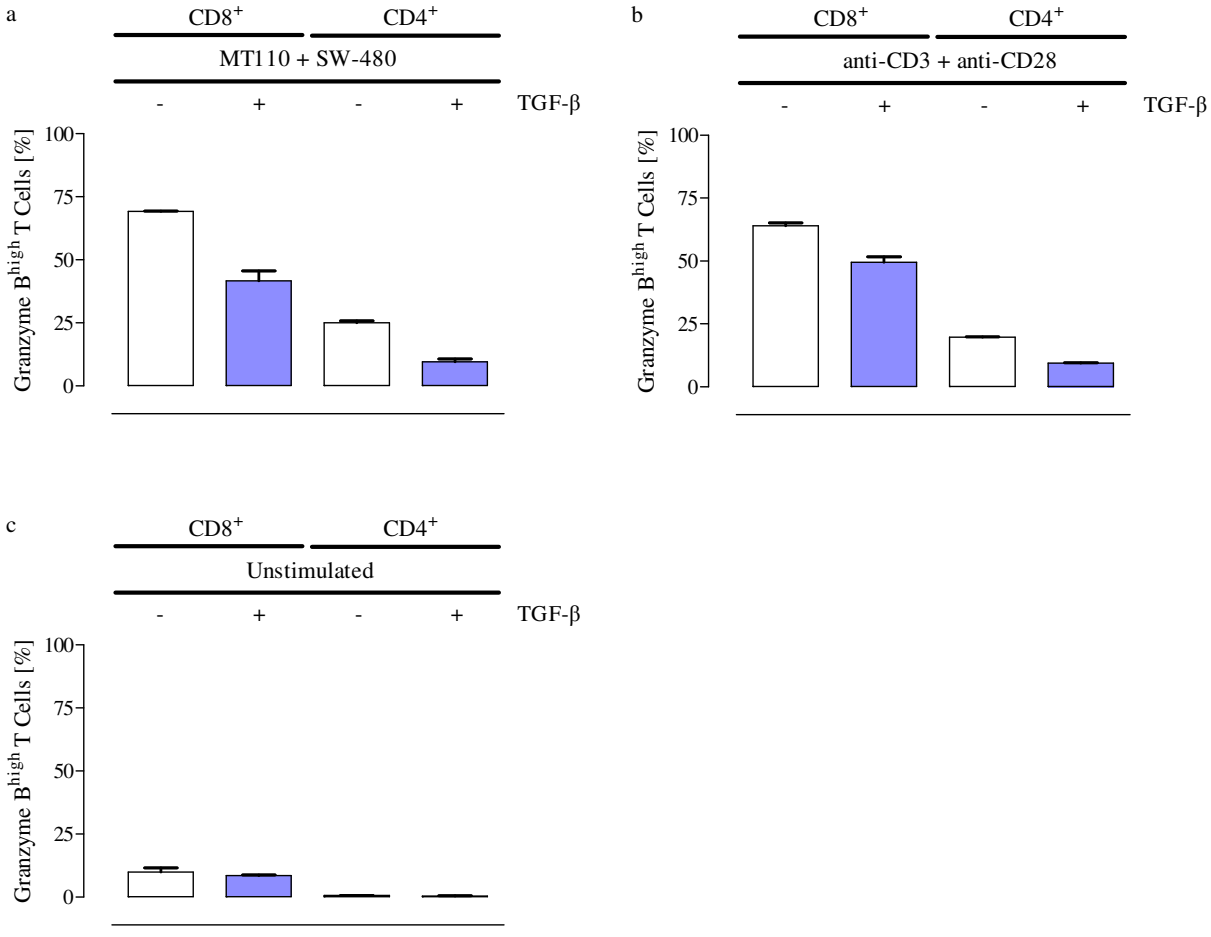
After the pre-incubation period, $CD3^+$ cells were negatively isolated from the suspensions using magnetic beads. SW-480 cells were also removed during this process. Isolated $CD3^+$ cells were analyzed for intracellular markers or subjected to cytotoxicity assays with Kato III

cells as target cells and either increasing doses of MT110 or at various E:T ratios. Lysis of Kato III cells was measured by FACS analysis.

4.3.1 Influence of TGF-β on expression of granzyme B, perforin and foxp3 in stimulated T cells

TGF-β is described as specifically inhibiting the expression of cytolytic gene products including granzyme B and perforin in cytotoxic T lymphocytes (Thomas and Massague 2005). In a Smad-dependent manner, TGF-β decreased both the proportion of $CD8^+$ T cells expressing granzyme B and the individual level of granzyme B expression per cell.

The effect of TGF-β on granzyme B production of T cells in an MT110-induced tumor elimination reaction was tested.



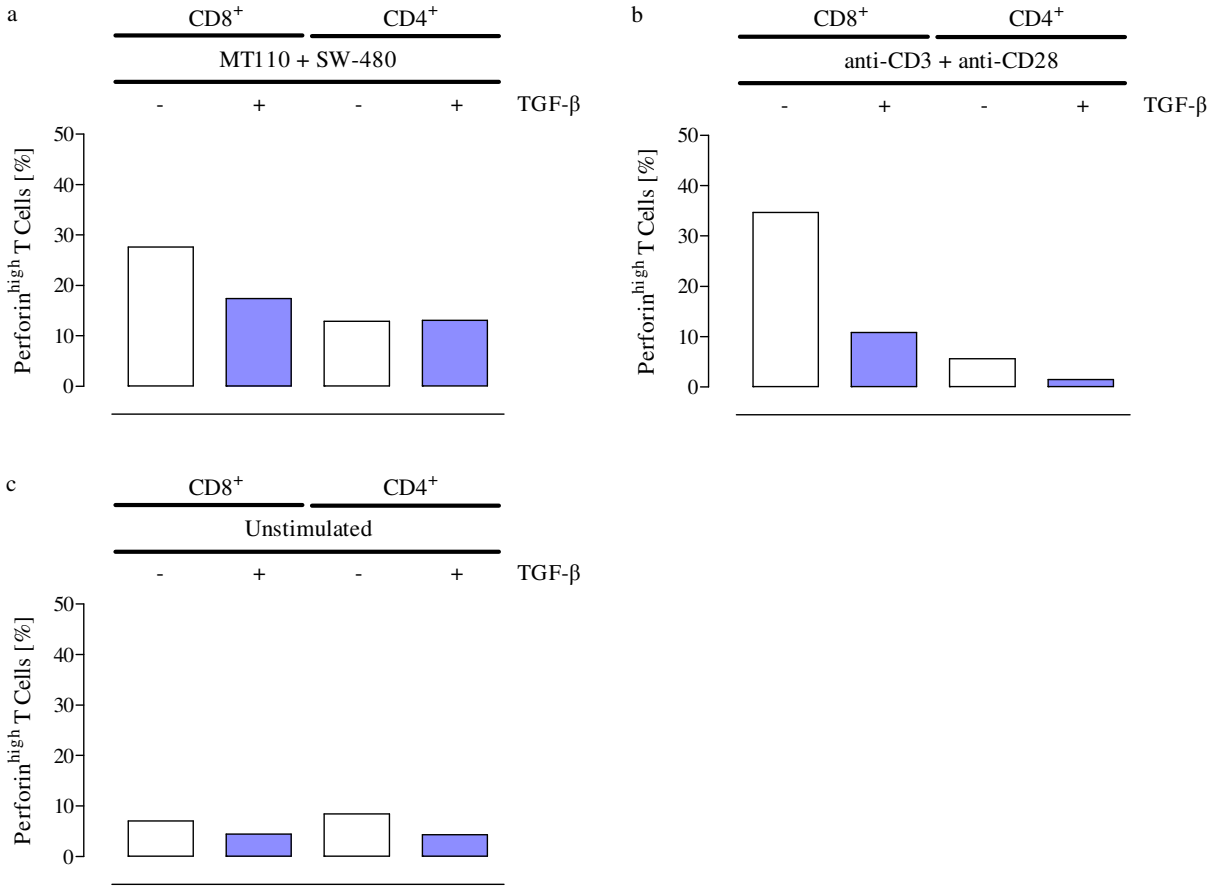
Figure[CHa26] 29 Influence of TGF-β on expression of granzyme B

PBMC were incubated (a) with SW-480 cells (E:T 5:1) and 10 ng/ml MT110 or (b) on a petri dish coated with anti-CD3/anti-CD28 or (c) left unstimulated. Incubation was performed in absence or presence of 100 pM TGF-β. After 2.5 days, reaction samples were stained for cell surface CD4, CD8 and intracellular granzyme B. Staining of cells was measured by FACS analysis.

TGF-β reduced the capacity of T cells to express granzyme B. T cells co-cultured with SW-480 cells and MT110 showed lower expression of granzyme B in both $CD8^+$ and $CD4^+$ T cells when 100 pM TGF-β were present during the incubation period. The percentage of granzyme B^{high} cells dropped from 69 % to 42 % in the $CD8^+$ and from 25 % to 10 % in the $CD4^+$ T cell subset (Figure 29 a).

Under stimulatory conditions that included a co-stimulus, granzyme B expression was reduced from 64 % to 50 % granzyme B^{high} cells in $CD8^+$ T lymphocytes and from 20 % to 10 % granzyme B^{high} cells in $CD4^+$ T lymphocytes (Figure 29 b). In unstimulated T cells, the number of granzyme B^{high} cells was slightly decreased (Figure 29 c).

To further test the influence of TGF-β on protein biosynthesis of effector molecules by T cells, expression levels of perforin were examined in TGF-β-treated cells versus -untreated T cells.



Figure[CHa27] 30 Influence of TGF-β on expression of perforin
PBMC were incubated (a) with SW-480 cells (E:T 5:1) and 10 ng/ml MT110 or (b) on a petri dish coated with anti-CD3/anti-CD28 or (c) left unstimulated. Incubation was performed in the absence or presence of 100 pM TGF-β. After 2.5 days, reaction samples were stained for cell surface CD4, CD8 and intracellular perforin. Staining of cells was measured by FACS analysis.

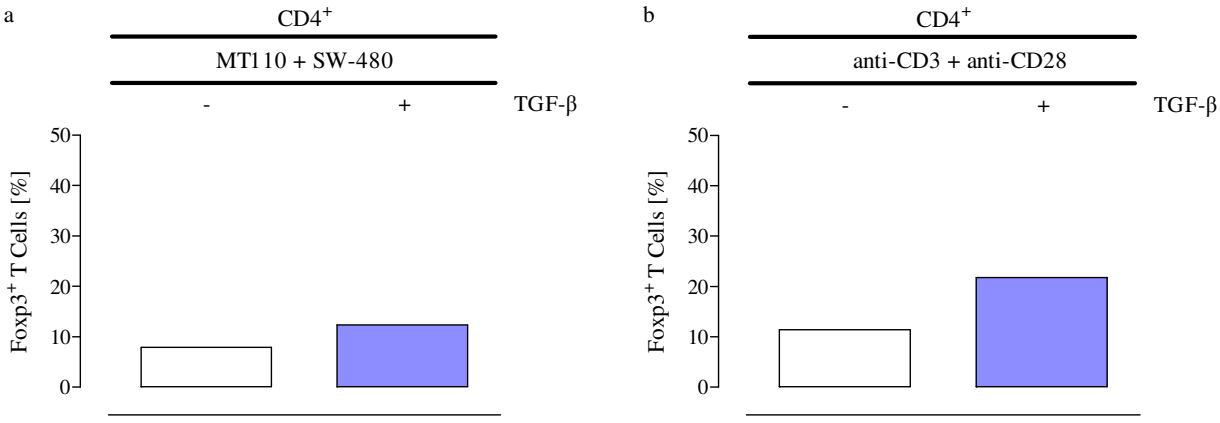
When BiTE-stimulated T cells had been exposed to TGF-β, the percentage of perforin^{high} CD8$^+$ cells was reduced by 11 %. Perforin expression in CD4$^+$ cells did not respond to the presence of TGF-β (Figure 30 a).

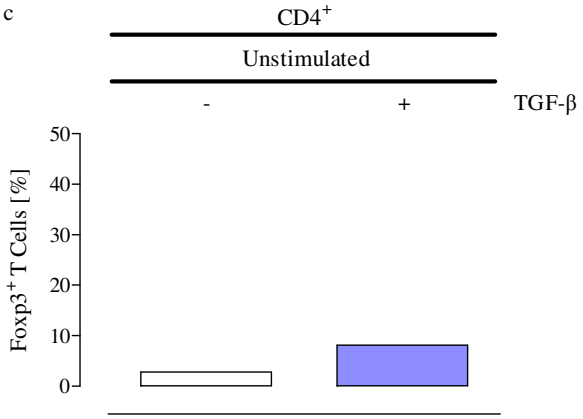
Similar but more pronounced effects were obtained when T cells were stimulated with anti-CD3 and anti-CD28. The influence of TGF-β resulted in a lower expression of perforin in both CD8$^+$ and CD4$^+$ T cells. The percentage of perforin^{high} cells decreased from 35 % to 11 % in the CD8$^+$ T cell subset and from 6 % to 2 % in CD4$^+$ T lymphocytes upon exposure to TGF-β as compared to activation in the absence of TGF-β (Figure 30 b).

A decrease in perforin expression could also be observed for unstimulated CD8$^+$ and CD4$^+$ cells when they were incubated with TGF-β alone although initial values were rather low. In CD8$^+$ cells, the reductive effect of TGF-β on perforin expression was 3 %, while in CD4$^+$ cells it was 4 % (Figure 30 c).

The expression of the forkhead family transcription factor foxp3 is characteristic for regulatory T cells (Ziegler 2006). Regulatory T cells play a central role in the control of autoimmune responses and immunologic tolerance. They regulate proliferation of CTLs that have encountered their relevant antigen in order to prevent unlimited growth of CTLs (Mempel et al. 2006). TGF-β can induce regulatory CD4$^+$ T cells when present in the cell culture supernatant for 72 h (Chen et al. 2003).

The percentage of foxp3$^+$ CD4$^+$ T cells in a cytotoxicity assay supplemented with TGF-β was compared to a cytotoxicity assay without TGF-β.





Figure[CHa28] 31 Influence of TGF-β on expression of foxp3
PBMC were incubated (a) with SW-480 cells (E:T 5:1) and 10 ng/ml MT110 or (b) on a petri dish coated with anti-CD3/anti-CD28 or (c) left unstimulated. Incubation was performed in the absence or presence of 100 pM TGF-β. After 2.5 days, reaction samples were stained for cell surface CD4 and intracellular foxp3. Staining of cells was measured by FACS analysis.

When PBMC were stimulated with MT110 and SW-480 cells for four days, the presence of 100 pM TGF-β in the culture medium increased the percentage of regulatory T cells in the $CD4^+$ T cell subset from 8 % to 12 % (Figure 31 a).

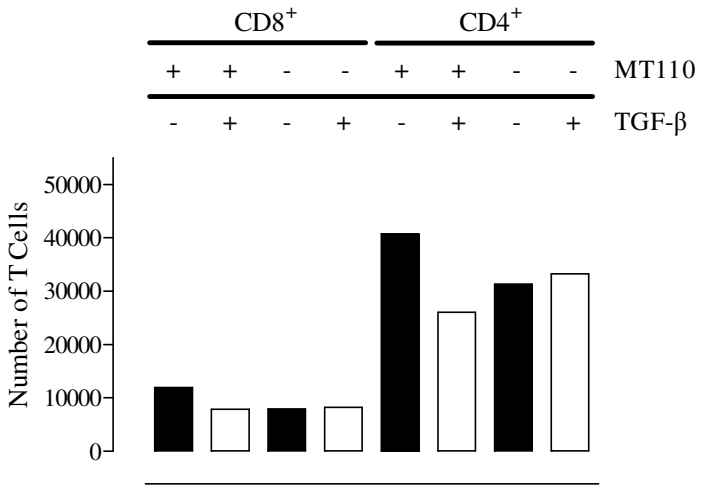
In anti-CD3 and anti-CD28 activated PBMC, the percentage of $foxp3^+$ cells rose from 11 % to 22 % in $CD4^+$ cells (Figure 31 b).

Even unstimulated $CD4^+$ T cells expressed more foxp3 when exposed to TGF-β (increase of 5 %) (Figure 31 c).

4.3.2 Influence of TGF-β on proliferation of stimulated T cells

TGF-β has been shown to inhibit proliferation of various subsets of T cells including primary T cells (Kehrl et al. 1986). Its anti-proliferative effect is attributed to inhibition of IL-2 production (Brabletz et al. 1993), up-regulation of cell cycle inhibitors p15 (Hannon and Beach 1994), p21 (Datto et al. 1995) or p27 (Polyak et al. 1994) as well as down-regulation of expression of c-myc (Coffey et al. 1988).

The numbers of T cells in cytotoxicity reactions with or without TGF-β were determined.

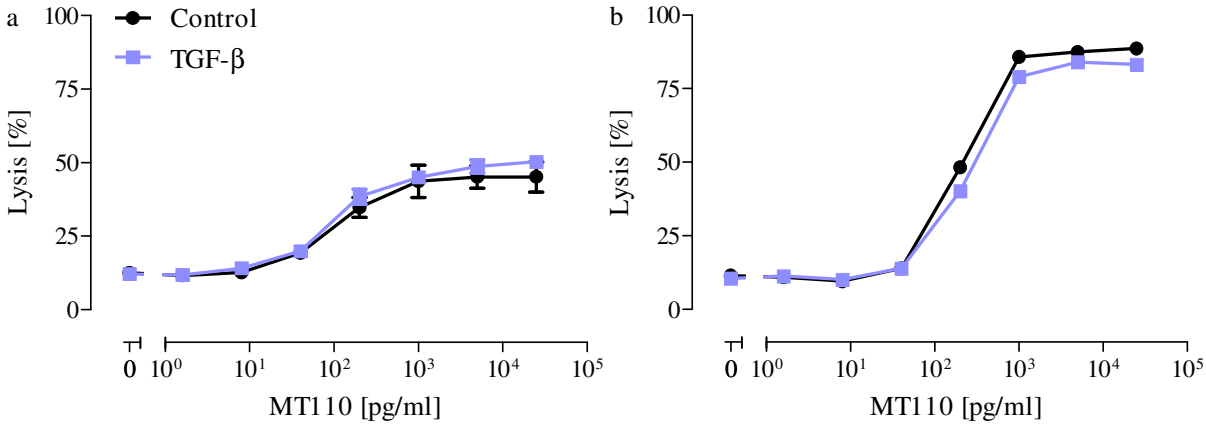


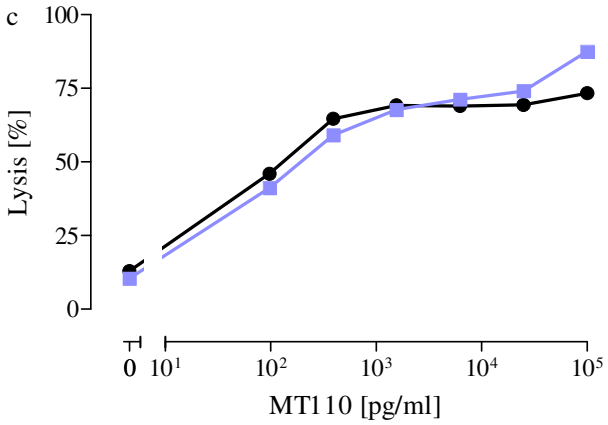
Figure[CHa29] 32 Influence of TGF-β on proliferation of stimulated T cells
PBMC were co-cultured with SW-480 cells (E:T 5:1) in the absence or presence of 10 ng/ml MT110. Incubation was performed in the absence or presence of 100 pM TGF-β. After 4 days, reaction samples were stained for cell surface CD3, CD4 and CD8 and analyzed by flow cytometry.

T cells that were activated with MT110 and target cells for four days showed reduced proliferation when TGF-β was present during the incubation phase. The effect could be observed in both the $CD4^+$ and $CD8^+$ T cell subset. When PBMC were incubated without stimulation, $CD8^+$ T cells did not proliferate but there was a slight increase in the number of $CD4^+$ T cells (Figure 32). The newly generated $CD4^+$ T cells are likely T_{reg} cells as it is well known that TGF-β induces $CD4^+$ T_{reg} cells (compare Figure 31) (Fantini et al. 2004).

4.3.3 Short-term effects of TGF-β on cytolytic activity of T cells

To study acute TGF-β effects on redirected lysis of tumor cells by peripheral T cells, TGF-β was added to regular cytotoxicity assays.





Figure[CHa30] 33 Short-term effects of TGF-β on cytolytic activity of T cells
PBMC were (a) left untreated, (b) co-cultured with SW-480 cells (E:T 5:1) and 10 ng/ml MT110 or (c) incubated with platebound anti-CD3 and anti-CD28 for 2.5 days. After pre-treatments, $CD3^+$ T cells were purified from the suspensions and tested for cytolytic activity in a cytotoxicity assay with Kato III cells (E:T 1:1) as target cells and MT110 in various concentrations in the presence or absence of 100 pM TGF-β. After 25 h, redirected lysis of Kato III cells was determined by propidium iodide uptake into cell nuclei using flow cytometric analysis.

TGF-β did not cause unstimulated T cells to react significantly different in its presence or absence. Maximal lysis could be obtained with the highest MT110 concentrations and averaged around 46 % (Figure 33 a).

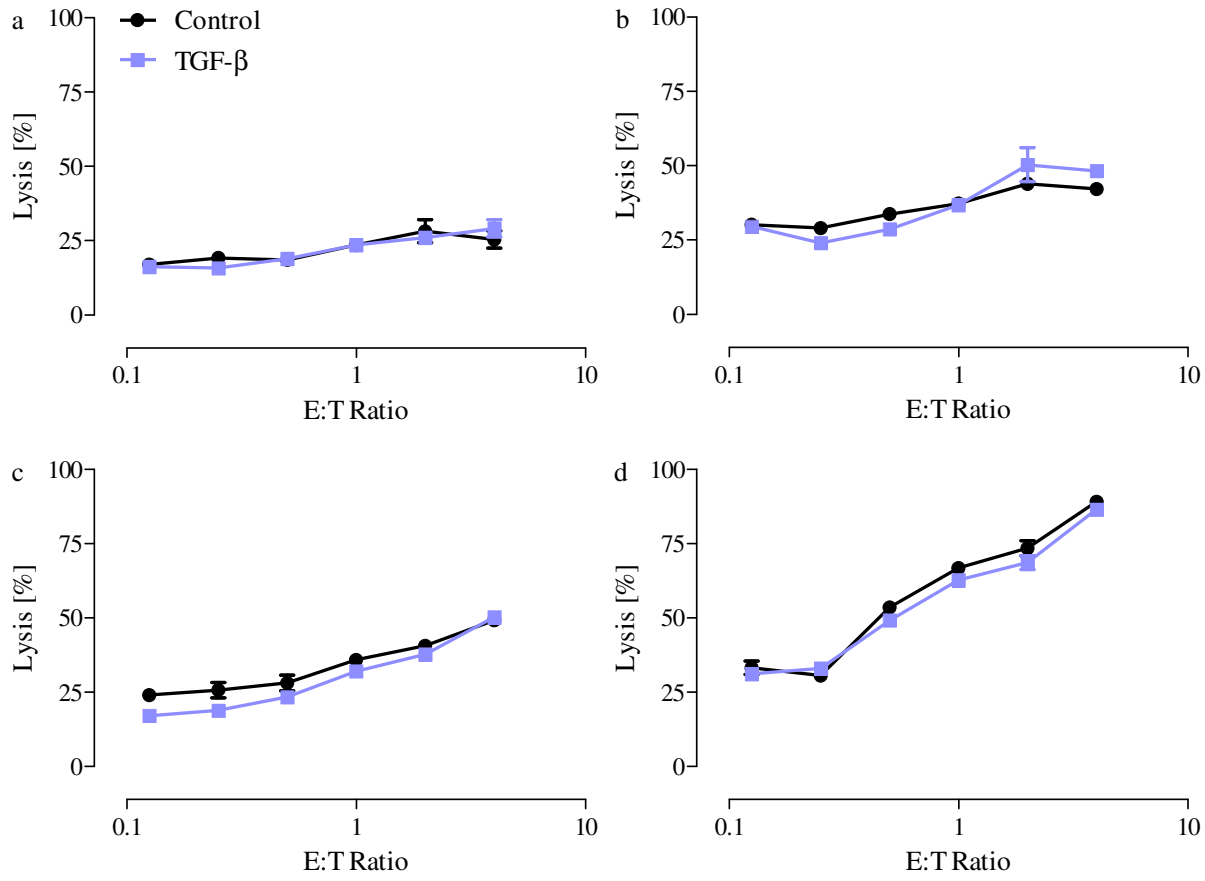
Pre-stimulated T cells did not react towards TGF-β either. Addition of 100 pM TGF-β to sample wells did not show an effect on cytolytic performance of BiTE re-stimulated T cells. Eighty % of target cells were lysed with a concentration of 1 ng/ml MT110 or higher (Figure 33 b).

As a positive control, PBMC were stimulated with platebound anti-CD3 and anti-CD28. Co-cultured with Kato III cells and MT110, they did not respond to the presence of TGF-β during the cytolytic reaction. Lysis of target cells was around 69 % when 1 ng/ml MT110 or more were administered (Figure 33 c).

TGF-β does not seem to exert an observable effect on cytolytic activity of T cells when present in the cell culture supernatant for only 1 day.

4.3.4 Effects of chronic treatment with TGF-β on cytolytic activity of T cells

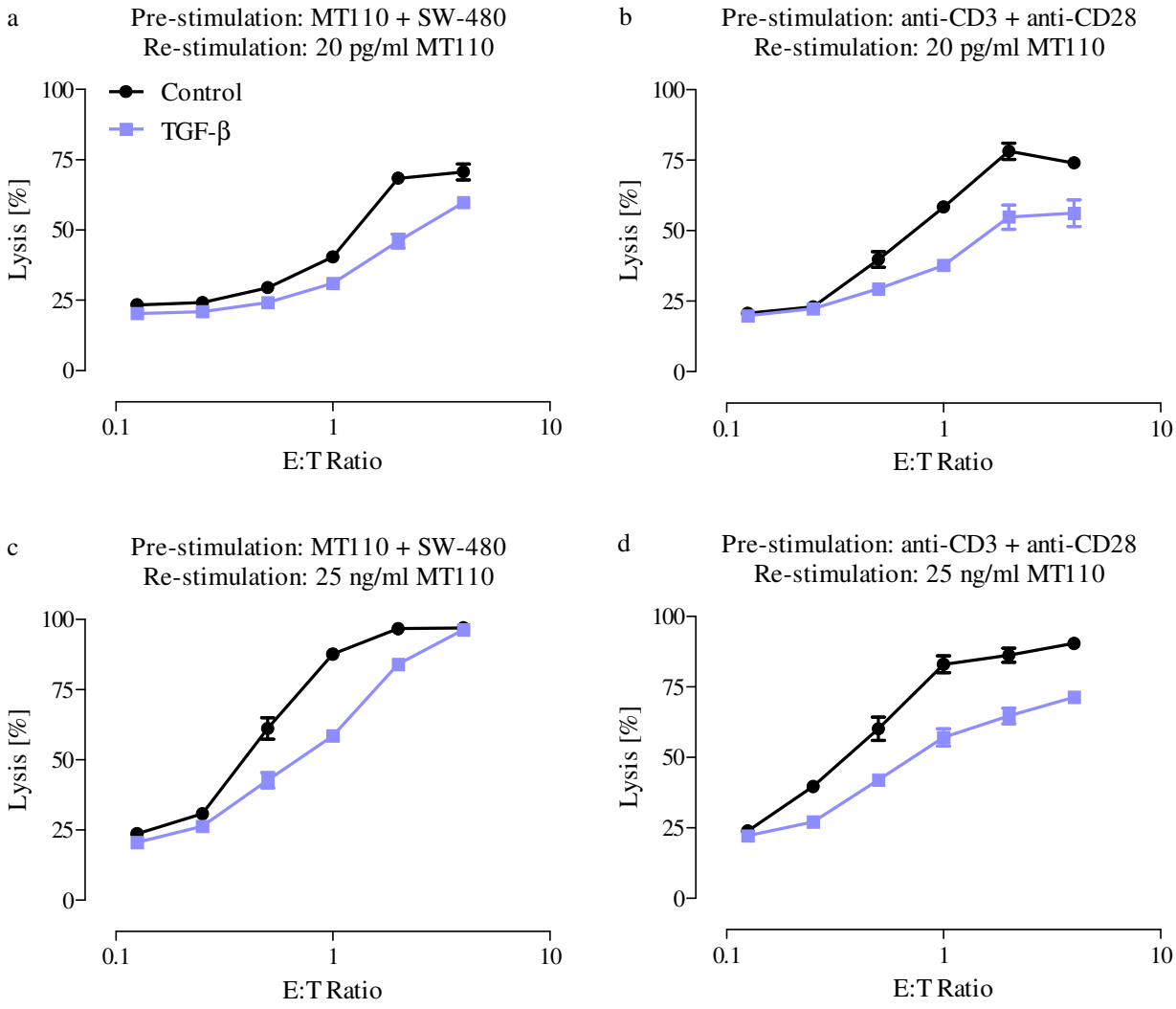
To look at the effects of exposure to TGF-β over an extended period of time, the incubation phase with TGF-β was prolonged. TGF-β, at a concentration of 100 pM, was administered to unstimulated PBMC for 2.5 days.



Figure[CHa31] 34 Effects of chronic treatment with TGF-β on cytolytic activity of unstimulated T cells
After incubation in the presence or absence of 100 pM TGF-β for 2.5 days, $CD3^+$ T cells were purified from whole PBMC, co-cultured with Kato III cells and with (a + b) 20 pg/ml or (c + d) 25 ng/ml MT110. The E:T ratio was titrated within the range of 4:1 to 0.1:1 (= 1:10) to test the cytotoxic potential of T cells on a single-cell level and below. Reactions were stopped after (a + c) 11 h or (b + d) 25 h. Lysis of Kato III cells was monitored by nuclear uptake of propidium iodide using flow cytometric analysis.

Unstimulated T cells exposed to TGF-β for 2.5 days performed almost as well as control cells that had been cultured in medium alone. TGF-β-treated T cells showed a marginally worse lytic activity. A direct dependency between lysis and E:T ratio was more easily observed when the concentration of MT110 was high (Figure 34 c and d). When only 20 pg/ml MT110 were administered, 11 h were too short to discern differences between E:T ratios. Therefore an increase in the number of effector cells was not mirrored by an increase in lysis (Figure 34 a). After 25 h, a slight dependency between lysis and E:T ratio could be observed at 20 pg/ml (Figure 34 b). With 25 ng/ml MT110, this dependency was evident already after 11 h (Figure 34 c) and the range of lytic rates was larger after 25 h: 33 % lysis of target cells for E:T 0.2:1 and 87 % for E:T 10:1 (Figure 34 d).

Previous experiments were repeated with stimulated cells and analyzed 11 h after re-stimulation with MT110 in the presence of target cells.



Figure[CHa32] 35 Effects of chronic treatment with TGF-β on the cytolytic activity of stimulated T cells after 11 h

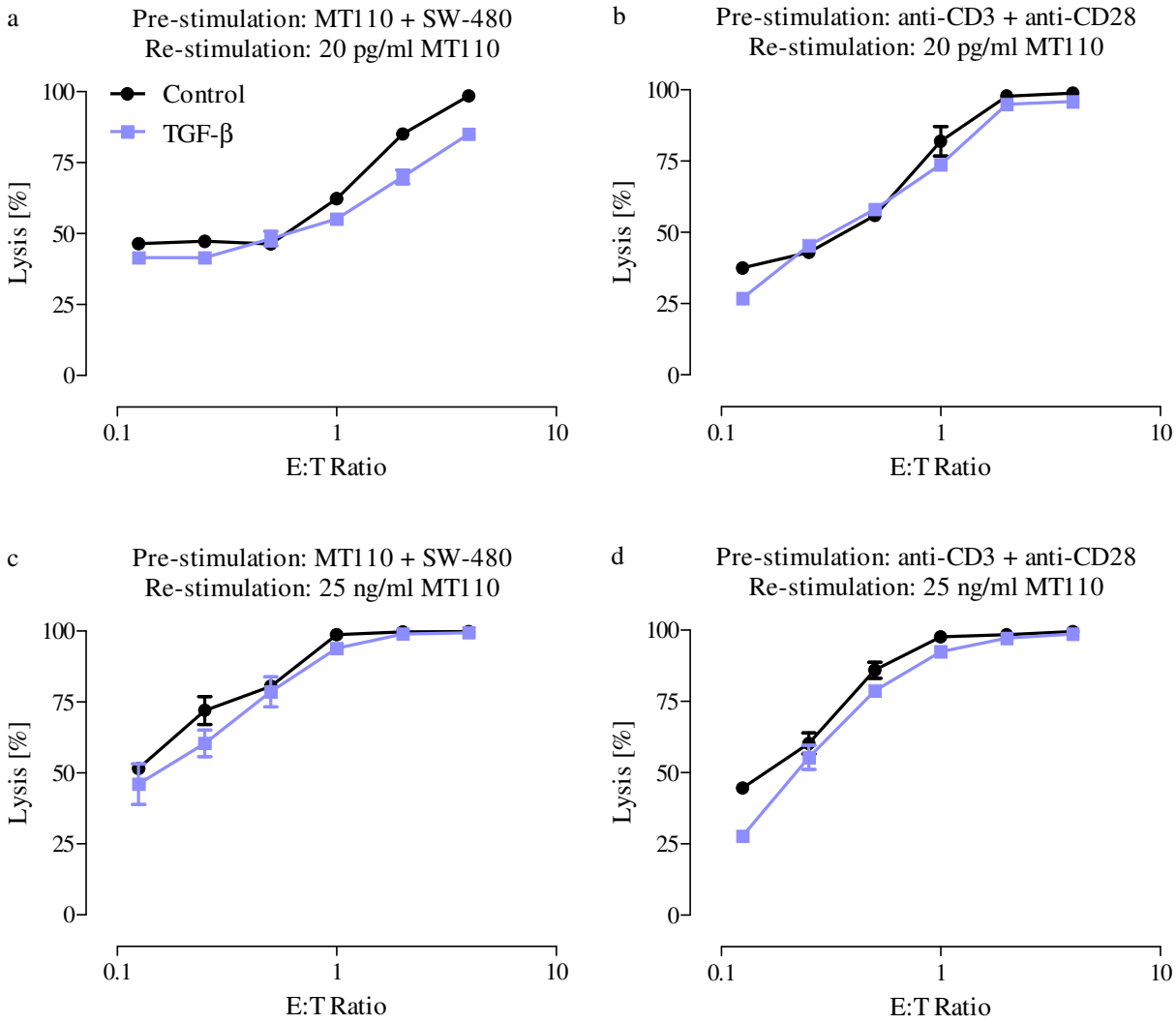
PBMC were incubated (a + c) with SW-480 cells (E:T 5:1) and 10 ng/ml MT110 or (b + d) with platebound anti-CD3/anti-CD28 for two days in the presence or absence of TGF-β. $CD3^+$ T cells were purified from stimulatory conditions and mixed with Kato III cells at E:T ratios described above and with (a + b) 20 pg/ml or (c + d) 25 ng/ml MT110. After 11 h, lysis of Kato III cells was monitored by nuclear uptake of propidium iodide using flow cytometric analysis.

For both the high and low concentration of MT110, a reduced activity was observed for TGF-β-treated BiTE-pre-stimulated effector cells at all E:T ratios, but the effect was very small for low E:T ratios of 0.1:1 and 0.2:1 (Figure 35 a). The effect was stronger at high concentrations of MT110. With a BiTE concentration of 25 ng/ml, the difference in lytic potential was as high as 30 % for an E:T ratio of 1:1, but both effector cell populations reached a lysis level of around 96 % at an E:T ratio of 4:1 (Figure 35 c).

A control experiment using platebound anti-CD3 and anti-CD28 as stimulus in the pre-incubation phase revealed a very similar T cell response pattern towards incubation with TGF-β over several days. The cytokine caused reduced cytotoxicity of T cells (8 - 20 % less lysis) when E:T ratios were above 0.2:1 (Figure 35 b and d).

When effector cells were pre-stimulated and simultaneously exposed to TGF-β for 2.5 days, TGF-β-treated T cells performed less well in BiTE-induced lysis of tumor cells than T cells naïve to TGF-β.

The reaction time for the cytotoxicity assay with TGF-β-treated and -untreated stimulated effector cells was prolonged to 25 h.



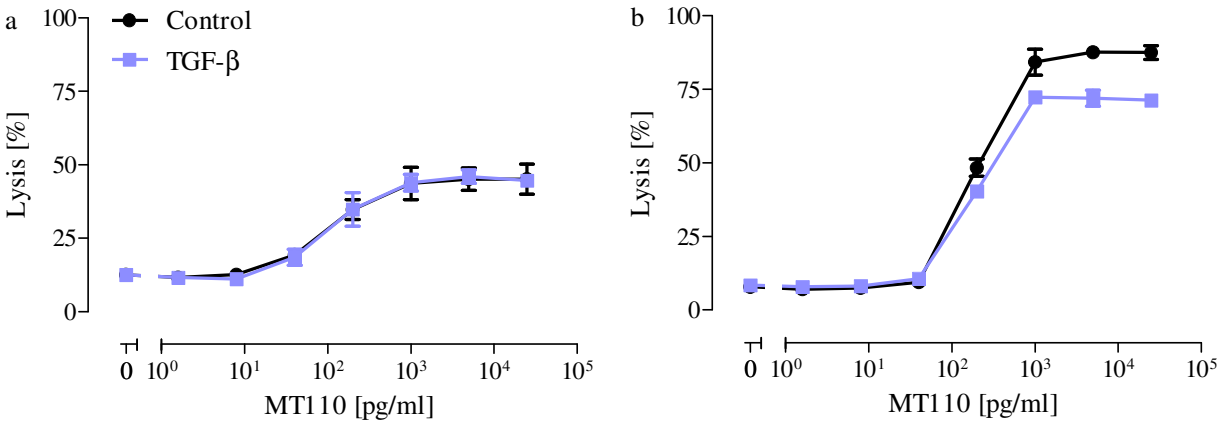
Figure[CHa33] 36 Effects of chronic treatment with TGF-β on cytolytic activity of stimulated T cells after 25 h
PBMC were incubated (a + c) with SW-480 cells (E:T 5:1) and 10 ng/ml MT110 or (b + d) with platebound anti-CD3/anti-CD28 for two days in the presence or absence of TGF-β. $CD3^+$ T cells were purified from stimulatory conditions and mixed with Kato III cells at E:T ratios described above and with (a + b) 20 pg/ml or (c + d) 25 ng/ml MT110. After 25 h, lysis of Kato III cells was monitored by nuclear uptake of propidium iodide using flow cytometric analysis.

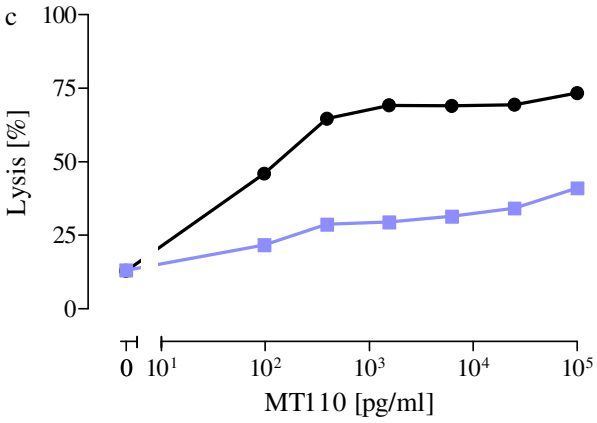
With an MT110 concentration of 25 ng/ml, BiTE-stimulated TGF-β-treated T cells reached the same lysis level as untreated T cells in samples with E:T ratios of 2:1 or higher (Figure 36 c). With a low MT110 concentration (20 pg/ml), TGF-β-treated T cells still acted less lytically towards target cells after 25 h (Figure 36 a).

As before, a control experiment was performed exchanging BiTE-stimulation for anti-CD3 and anti-CD28. Contrary to the measurements after 11 h, there was almost no effect of TGF-β on the high E:T ratios although there was a difference in cytolytic behaviour between TGF-β-treated and -untreated T cells at the lowest E:T ratio tested (0.1:1). When 20 pg/ml MT110 were administered, the difference was around 10 % (Figure 36 b) at this E:T ratio, while for the higher MT110 concentration of 25 ng/ml it was 16 % (Figure 36 d).

Prolonged reaction times of cytotoxicity assays allowed TGF-β-treated stimulated T cells to catch up with T cells that had been stimulated in the absence of TGF-β.

Effector cells of the previously described experiments had been removed from exposure to TGF-β before being subjected to cytotoxicity assays. To investigate the effects of a permanent exposition to TGF-β on T cells, 100 pM TGF-β were added to sample wells at the beginning of a further cytotoxicity reaction.





Figure[CHa34] 37 Effects of permanent exposition to TGF-β on cytolytic activity of T cells
PBMC were (a) left untreated, (b) co-cultured with SW-480 cells (E:T 5:1) and 10 ng/ml MT110 or (c) incubated with platebound anti-CD3 and anti-CD28 for 2.5 days in the presence or absence of 100 pM TGF-β. After pre-treatments, $CD3^+$ T cells were purified from the suspensions and tested for cytolytic activity in a cytotoxicity assay with Kato III cells (E:T 1:1) as target cells and MT110 in various concentrations. 100 pM TGF-β were added to sample wells that contained effector cells whose pre-treatment included TGF-β. After 25 h, redirected lysis of Kato III cells was determined by propidium iodide uptake into cell nuclei using flow cytometric analysis.**[CHa35]**

Cytolytic activity of unstimulated T cells was not influenced by permanent incubation with TGF-β at all (Figure 37 a). The lytic pattern of TGF-β-treated and -untreated stimulated T cells were similar to before but it seemed that the permanent presence of TGF-β in the culture medium intensified and prolonged the effects previously observed for TGF-β-treated T cells. Lytic performance of TGF-β-treated towards -untreated BiTE-pre-stimulated T cells was reduced by 17 % (re-stimulation with Kato III cells and 1 - 25 ng/ml MT110 for 25 h) when TGF-β was permanently present during pre-incubation and cytotoxicity assay (Figure 37 b) whereas it was only reduced by 5 % with TGF-β being present only during pre-incubation (Figure 33 b). T cells stimulated with anti-CD3 and anti-CD28 and under constant exposition to TGF-β were on average 35 % less effective in eliminating tumor cells than untreated T cells (re-stimulation with Kato III cells and 390 pg/ml - 100 ng/ml MT110 for 25 h) (Figure 37 c). When TGF-β treatment was limited to the pre-incubation phase, T cells were at most 14 % less effective (only for the highest MT110 concentration of 100 ng/ml) compared to T cells naïve to TGF-β (Figure 33 c).

Looking at cytolytic performance of TGF-β-treated stimulated T cells over the time course of 25 h, E:T ratio, concentration of MT110 and duration of TGF-β treatment play a decisive role in discerning the intensity and duration of the TGF-β influence.

4.4 Determination of the MABEL of MT110 *in vitro*

For first-in-man clinical studies, a safe clinical starting dose (SSD) has to be found. Generally, the "no observed effect level" (NOEL) or, more usually, the "no observed adverse effect level" (NOAEL) value (expressed in dose per kilogram) determined in preclinical studies in one or more relevant animal species are used to estimate this SSD with reference to allometric scaling and by building in an appropriate safety factor. In light of the incidents concerning the superagonistic monoclonal antibody TGN1412, a wider approach to dose calculation in man is recommended for those investigational medicinal products deemed to be high risk. All relevant preclinical informantion available is to be considered, resulting in the minimal anticipated biological effect level (MABEL). The starting dose for first-in-man clinical trials is to be set below the MABEL value.

Experiments examining MT110-induced up-regulation of T cell activation marker CD25 and redirected lysis of tumor cells were repeated testing 13 different donor PBMC as effector cells. Additionally, the impact of the glucocorticoid methylprednisolone (MPDS) on T cell activation and tumor cell lysis was evaluated. MPDS is administered as pre-medication in clinical trials in order to prevent a harmful over-production of cytokines. The concentration to be used was determined in preliminary experiments. Administration of 10^{-7} M MPDS yielded a remarkable reduction in cytokine secretion while causing only minor impact on the activation status of T cells and BiTE-mediated lysis of tumor cells.

The same target cell line involved in previous experiments was also used for MABEL experiments. Kato III cells have ideal features as target cells as they express the highest known numbers of EpCAM molecules on the cell surface (~ 900,000) (Prang et al. 2005), are most sensitive to BiTE-induced redirected lysis, are most efficacious in T cell activation assays and give robust signals upon flow cytometry analysis. A high effector to target ratio of 10:1 and a long incubation period of 24 h was used to allow for the most sensitive analysis.

4.4.1 MABEL calculated from T cell activation

Up-regulation of T cell activation marker CD25 on PBMC proved to be one of the most sensitive markers to determine MT110-induced biological activity.

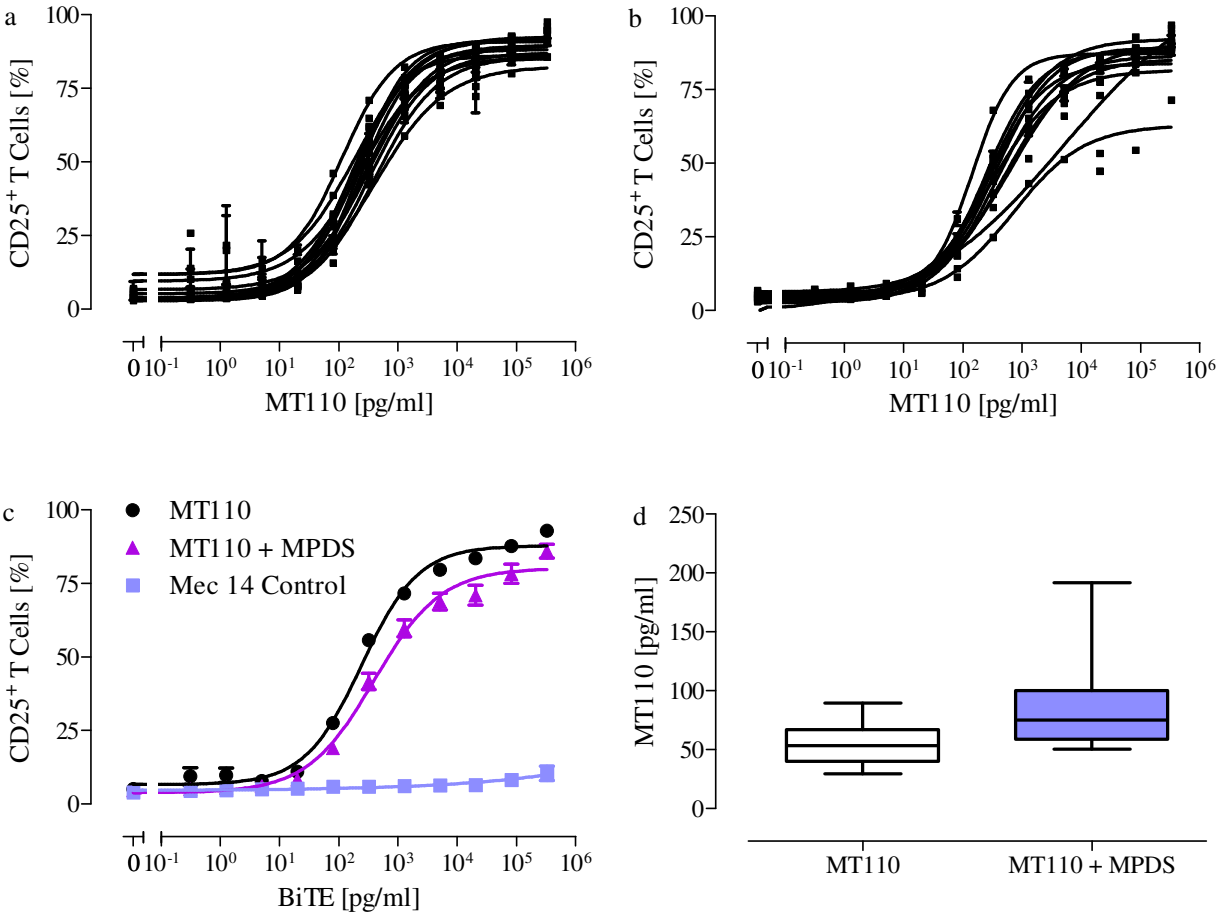


Figure 38 MABEL determination for MT110-induced up-regulation of T cell activation marker CD25 and the effect of glucocorticoid
PBMC from 13 different donors were incubated (a) in the absence or (b) presence of 1 x 10^{-7} M MPDS for 1 h. After pre-treatment, PBMC were incubated with Kato III cells as target cells (E:T 10:1) and with increasing doses of MT110 or a control BiTE, Mec 14. MPDS was added to MPDS pre-treated PBMC to a final concentration of 1 x 10^{-7} M. After 24 h, cells were stained with anti-CD4, anti-CD8 and anti-CD25 and analyzed by flow cytometry. (c) Means of dose-response curves of 13 different donors (d) Box-and-whiskers graph of EC_{20} values. Boxes show the interquartile range between the 25th and the 75th percentile with line drawn at the median. Whiskers indicate minimal and maximal values.

With all 13 different donor PBMC preparations, MT110-induced strictly dose-dependent up-regulation of CD25 on $CD3^+$ T cells. Within 24 h, close to 100 % of all T cells became activated (Figure 38 a and c). Co-incubation with methylprednisolone had a slight inhibitory effect on T cell activation (Figure 38 b and c). Incubation with the negative control BiTE Mec 14, that exclusively binds to CD3 i.e., can not to EpCAM, had no effect on T cell activation (Figure 38 c).

The calculated EC_{20} values for MT110-induced T cell activation, as derived from the individual dose response curves, varied to a certain extent, which can be attributed to inter-donor variations of the PBMC. The mean MT110 EC_{20} value derived from the 13 different donors was calculated as 55 pg/ml with an interquartile range from 45 to 64 pg/ml. In the presence of MPDS, the mean EC20 value was calculated to be 86 pg/ml with an interquartile range from 63 to 109 pg/ml, and thus values appeared to be slightly higher than in the absence of the glucocorticoid (Figure 38 d).

4.4.2 MABEL calculated from cytotoxicity assays

Redirected lysis of tumor cells was also used for determination of the MABEL value of MT110. EC_{20} values served as a measure for MABEL.

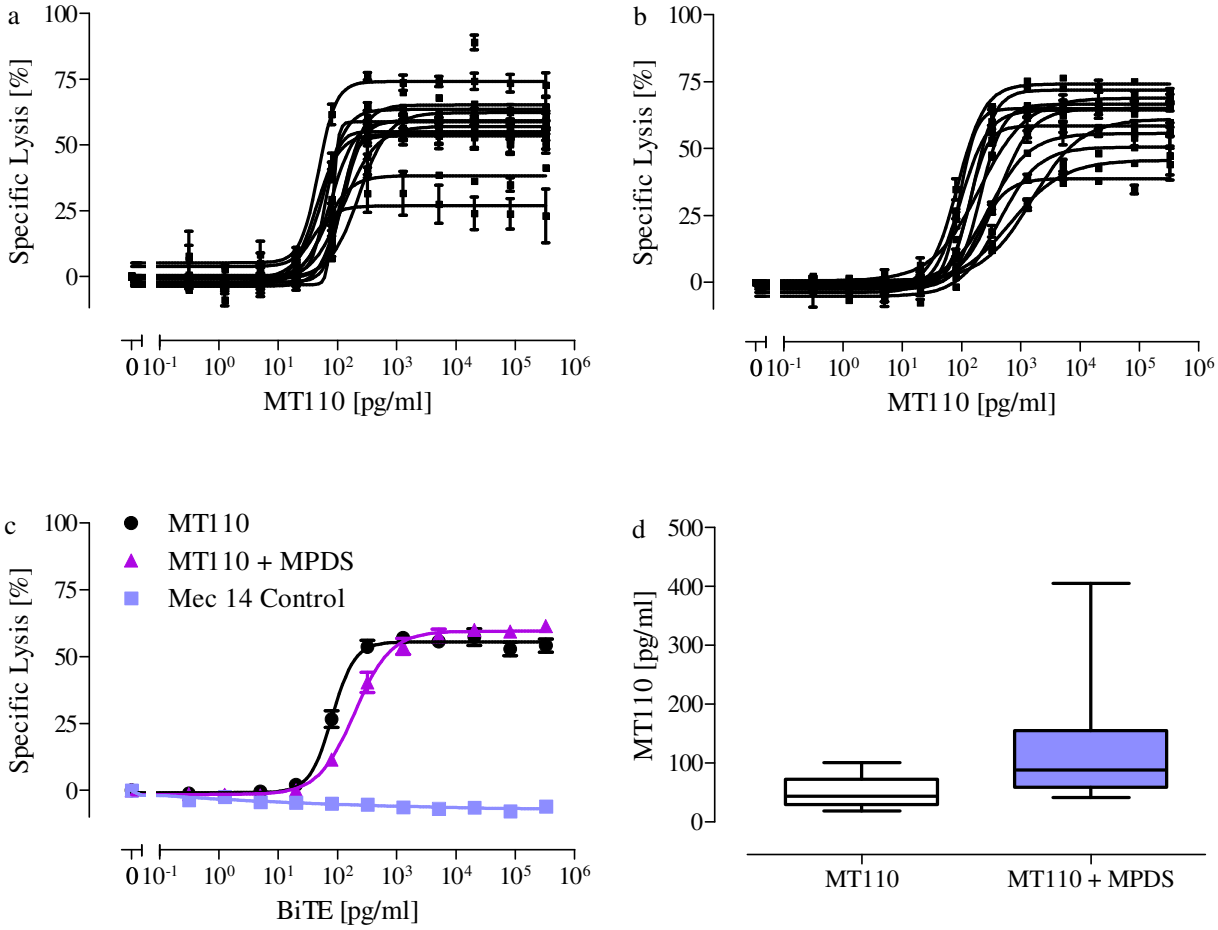


Figure 39 MABEL determination for MT110-induced specific lysis and the effect of glucocorticoid
PBMC from 13 different donors were incubated (a) in the absence or (b) presence of 1 x 10^{-7} M MPDS for 1 h. After pre-treatment, PBMC were incubated with Kato III cells as target cells (E:T 10:1) and with increasing doses of MT110 or Mec 14. MPDS was added to MPDS pre-treated PBMC to a final concentration of 1 x 10^{-7} M. After 24 h, cells were analyzed for vitality by flow cytometry. (c) Means of dose-response curves of 13 different donors. (d) Box-and-whiskers graph of EC_{20} values. Boxes show the interquartile range between the 25th and the 75th percentile with line drawn at the median. Whiskers indicate minimal and maximal values.

Lysis of Kato III cells occurred in a strictly dose-dependent manner for all 13 donor PBMC. Between 30 and 75 % of tumor cells were eliminated after 24 h (Figure 39 a and c). Lysis of tumor cells by PBMC that had been treated with methylprednisolone seemed to be marginally impaired (Figure 39 b and c). Control BiTE molecule Mec 14 did not mediate tumor cell death (Figure 39 c).

Donor variability again led to variation in EC_{20} values. For redirected lysis, the mean EC_{20} value of MT110 was calculated as 51 pg/ml with an interquartile range from 36 to 66 pg/ml. Under the influence of methylprednisolone, the mean EC_{20} value was increased to 119 pg/ml with an interquartile range from 62 to 177 pg/ml (Figure 39 d).

Based on the most sensitive test model, assay conditions and readout systems, the minimal anticipated biological effect level for MT110 was determined to be 50 pg/ml.

5 Discussion

5.1 Mode of action of BiTE molecules

The data presented describe part of a BiTE molecule's developmental path from early preclinical testing via functional analyses to assays required for admission to clinical trials.

BiTE molecules are members of the class of bispecific antibodies. As the name implies, bispecific antibodies do not recognize the same epitope with each of their two binding arms but rather target two different epitopes. The BiTE platform represents a new design for bispecific therapeutical antibodies. BiTE molecules consist of two distinct antibody recognition domains connected by a short peptide linker. They lack an Fc receptor. Without the constant fragments of the heavy and the light chains of an antibody, its molecular weight only amounts to approx. 55 kDa. Its small size may help it to reach the tumor site faster and penetrate into a solid tumor.

In contrast to many conventional anti-cancer antibodies (e.g., trastuzumab, cetuximab, rituximab) that mediate the death of cancer cells via antibody-dependent cellular cytotoxicity, bispecific antibodies of the BiTE class use the potential of T cells as effector cells. BiTE molecules bind with one arm to cancer cells and with the other arm to T cells, bringing the two cell populations into close proximity. In binding to both cell types at the same time, BiTE molecules stimulate T cells to enter an activated state, to establish an immunological synapse with the target cell, and to secrete cytolytic effector molecules towards the target cell.

BiTE molecules do not recognize a tumor-specific antigen on cancer cells but rather a helper epitope that is either almost exclusive to the cancer cells or strongly up-regulated on tumor tissue. This feature allows BiTE molecules to target tumor cells even when variant subpopulations occur in the tumor. After 1 to 2 weeks of progression, the pattern of surface molecules on cells within a tumor often begins to change and the immunodominant epitope of the parental tumor may no longer be expressed (Beck et al. 2001). Neither tumor-specific T cells nor tumor-specific antibodies would be capable of attacking newly formed tumor cells. Due to their independence from tumor antigens, BiTE molecules can fight even those cancer cells that have lost the immunodominant epitope.

BiTE molecules bind to the invariant CD3ε chain of the TCR on T cells. T cells activated by BiTE molecules work independently of a specific T cell receptor, of co-stimulatory signals and of the antigen presenting machinery in tumor cells.

The experiments presented show that the biological activity of MT110 is highly specific. Activation of T cells and lysis of tumor cells are only detectable when MT110, $CD3^+$ T cells and $EpCAM^+$ target cells are simultaneously present. No biological effect is apparent in the

absence of target cells (Figure 22 c). Similarly control BiTE molecules exclusively binding to either the CD3 or EpCAM target alone do not show biological activity (Figure 17 c and d). The same specificity in biological activity has been proven for MT103 (Loffler et al. 2000; Dreier et al. 2002).

Both BiTE molecules tested - MT103 and MT110 - started to effectively eliminate target cells in the respective *in vitro* cytotoxicity assays from a concentration of 625 pg/ml BiTE and greater. When 10 ng/ml MT110 were administered, lysis of target cells reached more than 75 % and seemed to remain around this value as a final plateau (Figure 17 b). With 10 ng/ml MT103, lysis of target cells had not peaked and seemed to rise even higher than 85 % (Figure 6 b). One could conclude that MT103 is more potent than MT110 as it yields higher lysis rates. Yet, one has to take into account that the respective target cells (Mec-1 and Kato III) differ in terms of density, size and availability of antigen. Additionally, it has to be considered that effector cells stem from different donors and might be armed to different degrees. Many experiments have shown that potency and level of activity upon stimulation of PBMC vary widely between individual donors. Inter-donor variation causes differences in EC_{50} values, which themselves can only be compared within one experiment, as in Figure 18 and Figure 28 a, and never between separate assays. The T cell line MC15 was tested in cytotoxicity reactions but found to be unsuitable for long-term assays: all target cells were killed by the T cells after 6 h.

One goal of this study was to investigate the mechanism by which an MT110-induced kill of target cells is achieved. Effector cells for the experiments stemmed from peripheral blood from human donors. These peripheral blood mononuclear cells represented physiological mixtures of effector cells containing $CD8^+$ T cells as well as $CD4^+$ T cells. Both T cell populations contribute to elimination of target cells (Brischwein et al. 2006).

Distinct effects of a BiTE-induced activation were found on the surface of T cells: A co-culture of effector cells and target cells with a relevant BiTE molecule in a sufficient concentration lead to *de novo* expression of activation markers CD69 and CD25 (Figure 11, Figure 22), a characteristic signal of activation that is also observed with T cells activated by cognate antigen plus MHC on an antigen presenting cell (Uchiyama et al. 1981; Yokoyama et al. 1989). BiTE stimulation further caused an increase in expression of adhesion molecules CD2 and LFA-1 on T cells (Figure 13, Figure 23). Both molecules play important roles in the establishment of the immunological synapse between effector and target cell.

Target cells in a cytotoxicity assay undergo cell death as is determined by uptake of the dye propidium iodide into cell nuclei (Figure 6, Figure 17), activation of caspases (Figure 8,

Figure 25 a), cleavage of poly (ADP-ribose) polymerase (Figure 26 b) and fragmentation of chromosomal DNA (Figure 26 c). These events clearly characterize cell death induced by BiTE molecules as apoptosis.

Effector cells that were pre-stimulated lyse target cells more effectively in a subsequent cytotoxicity reaction (Figure 18). Pre-stimulation allows T cells to adopt a highly potent state possibly involving production of large amounts of effector granules. Elevated to this state of high potency, T cells can develop a serial lysis mode, i.e., one T cell can eliminate several cancer cells in series. Serial T cell killers can lyse tumor cells effectively even in mixtures of very low E:T ratios (Figure 18 f). Highest lytic rates were obtained when effector cells were pre-stimulated with anti-CD3 and anti-CD28 antibodies. A pre-stimulation with target cells plus MT110 brought effector cells into a less aggressive state. However with a high BiTE concentration (10 ng/ml and more) and an E:T ratio not smaller than 1:1, BiTE-pre-stimulated effector cells performed almost equally efficiently as PBMC pre-stimulated with anti-CD3 and anti-CD28. Additional co-stimulation with anti-CD28 might account for the more effective pre-stimulation of anti-CD3/anti-CD28 and it is likely that a combination of BiTE and anti-CD28 stimulation would enhance the lytic performance of effector cells compared to BiTE-only stimulation. Incubation with BiTE molecules activates effector memory T (T_{EM}) cells as effector cells, whereas co-stimulation with anti-CD28 also recruits naïve T cells in addition to T_{EM} cells. Yet, there is reason to believe that an anti-CD28 treatment of effector cells is not desirable. As was seen in clinical trials, the potency and efficacy of BiTE-activated PBMC was already very high (Bargou et al. 2008) and an activation of naïve T cells can be dangerous as became evident with the TeGenero case (Suntharalingam et al. 2006).

As mentioned before, a reason for the stimulatory phase enhancing the ability of PBMC to eliminate tumor cells might be an increased production of cytolytic effector molecules like granzyme B and perforin during stimulation. Both BiTE molecules tested - MT103 and MT110 - were shown to up-regulate expression of these two proteins in T cells during the course of a cytotoxicity reaction.

$CD8^+$ T cells responded faster and stronger to BiTE stimulation. Granzyme B^{high} cells were increased 6-fold in the $CD8^+$ T cell subset compared to unstimulated T cells (Figure 7 b, Figure 19 b). The increase in perforin expression was not as high but more persistent, as was seen in long-term assays over 6 days (Figure 9 b). The perforin content in $CD8^+$ T cells seemed to be temporarily exhausted after 3 days but recovered and was even higher after 6 days than on days 2 and 3 (Figure 20 c). Interestingly, this kinetics was not mirrored by the lytic behaviour of T cells.

$CD4^+$ T cells were less responsive. The rise in the granzyme B^{high} $CD4^+$ cell population ranged between minor and 3-fold (Figure 7 a, Figure 19 a). Perforin production in this T cell subset remained on a basal level and could not be increased by any BiTE concentration (Figure 9 a, Figure 20 a).

Exocytosis of granzyme B and perforin by T cells represents one major pathway for elimination of malignant cells. Another pathway involves binding of CD95, TRAIL or TNF-α to their receptors (death receptors) on the surface of target cells. Upon ligand binding, adaptor molecules inside the cell are recruited to the cytoplasmic domain of the death receptors. These adaptors cause activation of caspase 8 and 10 starting the caspase cascade. Induction of apoptosis via CD95, TRAIL and TNF-α is not dependent on the presence of Ca^{2+}-molecules in contrast to perforin, which needs Ca^{2+}. Since depleting Ca^{2+} from the extracellular environment of a cytotoxicity reaction with the chelator molecule EGTA (2 mM) abolishes both nuclear uptake of propidium iodide (Figure 24) and activation of caspases (Figure 25) in target cells, the granzyme B/perforin pathway seems to be the only relevant one for BiTE-induced lysis of tumor cells. Ca^{2+} ions are indispensable for oligomerization of perforin in the target cell membrane. Without functional perforin neither granzyme B nor other granzymes can enter the target cells and cleave their regarding substrates. The influence of EGTA on activation of caspases strongly suggests that presence of functional perforin is necessary for BiTE-mediated cell lysis. However, when supernatants of cytotoxicity reactions with MT110 were transferred to fresh cancer cells no cell lysis was observed (Figure 28 b). These data support the theory that effector molecules granzyme B and perforin are only secreted into the enclosed compartment of the immunological synapse. Furthermore, no other soluble factors that are present in the supernatants (e.g., TNF-α) are capable of mediating target cell death in the chosen cellular system. Target antigen negative cells in the vicinity of target antigen positive tumor cells will be unaffected by BiTE-activated T cells.

When a pan-caspase inhibitor (20 µM) was present during the whole incubation phase of a cytotoxicity reaction, activation of caspases came to a complete halt (Figure 26 a). However, BiTE-mediated tumor cell death was unimpaired (Figure 26 d) although other characteristic signals of apoptosis were reduced but not fully inhibited. Frequencies of PARP cleavage (Figure 26 b) and DNA fragmentation (Figure 26 c) were seemingly decreased by the lack of mature caspases. Yet, as they are not inhibited completely, there must exist additional effector molecules that take over the caspases' functions. Granzyme B is reported to not only cleave pro-caspases but also caspase substrates, e.g., PARP (Harris et al. 1998) and the complex CAD/ICAD (Thomas et al. 2000). Released from its inhibitor ICAD, the endonuclease CAD

cleaves internucleosomal DNA. Direct cleavage of PARP and processing of endonuclease CAD by granzyme B might provide an explanation for cleavage of PARP and fragmentation of DNA even in the presence of a caspase inhibitor. Additionally, granzyme B is described as cleaving the pro-apoptotic Bcl-2 family member Bid (Sutton et al. 2000). Truncated Bid interacts with Bak and Bax resulting in the release of cytochrome *c* from mitochondria (Marsden and Strasser 2003). It is suggested that release of cytochrome *c* is accompanied by liberation of endonuclease G and apoptosis-inducing factor (AIF) from mitochondria that would result in DNA fragmentation independent of caspases (Figure 2) (Barry and Bleackley 2002). Support for the model of caspase-independent apoptosis comes from experiments with the MCF7 cell line. Despite their genetic deficiency for caspase 3, Bax-induced PARP cleavage and cell death were not impaired in MCF7 cells (Kagawa et al. 2001).

Presence of a granzyme B inhibitor (10 µM) in the extracellular environment of a cytotoxicity assay reduced the activation rate of caspases but did not completely inhibit it (Figure 27 a). BiTE-induced cleavage of PARP (Figure 27 b) and tumor cell elimination (Figure 27 c) were found at levels equal to control samples. There is reason to believe that other granzymes are able to - at least partly - replace granzyme B. Granzyme A was shown to induce cytolysis and single-strand DNA breaks in target cells (Beresford et al. 1999); granzyme K has been reported to cause cell death in the presence of perforin (MacDonald et al. 1999); granzymes H (Fellows et al. 2007) and M (Kelly et al. 2004) also have the ability to induce cell death. Granzyme A induces caspase-independent cell death, while PARP does not seem to be a substrate for any granzyme other than granzyme B. However, activation of caspases mediated by granzymes H/K/M would undoubtedly lead to cleavage of PARP. Processing of pro-caspases by several granzymes simultaneously would also provide an explanation for a high level of active caspases in BiTE-stimulated granzyme B^{low}/granzyme B^- $CD4^+$ cells (Figure 7 a, Figure 8).

The serine proteases of the granzyme class hold a central position in BiTE-mediated lysis of tumor cells. However, BiTE-activated T cells can deliver death to tumor cells even in the presence of a granzyme B inhibitor.

BiTE molecules seem to implicate many mechanisms known from MHC-restricted lysis in their mode of action. BiTE-mediated elimination of tumor cells is very potent but at the same time very specific for recognition of proper target cells (Figure 22 d) and is accompanied by T cell proliferation (Figure 32). BiTE stimulation causes production of cytokines (TNF-α, IFN-γ, IL-10 and IL-4) (Figure 16) and up-regulation of expression of the cytotoxic proteins granzyme B and perforin in T cells (Figure 19, Figure 20). The importance of the Ca^{2+}-

dependent protein perforin for BiTE-induced tumor cell lysis is proved by a complete inhibition of lysis in the presence of the calcium chelator EGTA (Figure 24). BiTE-stimulated T cells require the presence of target cells for their activation (Figure 22) and are capable of multiple rounds of target cell lysis as was demonstrated in cytotoxicity assays with low E:T ratios (Figure 18 e and f). Holes are induced into cell membranes during the course of a cytotoxicity reaction including BiTE stimulation allowing large proteins to pass (Figure 21). Caspases play important roles in BiTE-mediated tumor cell lysis (Figure 26 a - c), but even in the absence of active caspase 3, tumor cell death occurs following BiTE stimulation (Figure 26 d).

Yet, the BiTE-induced lysis of tumor cells does not require T cells specific for a tumor antigen or co-stimulation by CD4, CD8 or CD28, and it is not inhibited by the presence of TGF-β which is secreted by many tumor cells to impair T cell development.

5.2 Influence of TGF-β

The mode of action of BiTE-mediated elimination of target cells by T cells omits many of the steps of MHC-restricted kill that can convert into tumor evasion mechanisms when mutated. BiTE molecules abolish the requirement of a tumor antigen in the context of an MHC molecule on cancer cells to establish a connection between tumor and T cell. This way none of the defects concerning the antigen processing and presenting machinery of tumor cells will allow tumor cells to evade the anti-tumor action of the BiTE-supported immune system. Reduction or loss of MHC on the cell surface of tumor cells or deficiencies in the transport of peptides of cytosolic tumor proteins into the endoplasmic reticulum via TAP or defects in loading MHC with tumor antigens will not provide evasion mechanisms for tumor cells facing BiTE molecules. But other immunoediting strategies developed by cancer cells might endanger the success of BiTE treatment. One such strategy is the production of TGF-β by tumor cells. TGF-β directly influences differentiation and proliferation of T cells. A decrease in properly-functioning T cells directly causes a reduction in BiTE-mediated tumor cell death.

The experiments presented show that TGF-β (100 pM) reduced the production of the effector molecules granzyme B (Figure 29) and perforin (Figure 30) and influenced expression of the cell surface molecule foxp3 (Figure 31) by stimulated T cells as described in the literature (Thomas and Massague 2005). Although BiTE molecules do not provide a co-stimulus, stimulation with MT110 yielded similar percentages of granzyme B^+ cells as stimulation via anti-CD3 plus anti-CD28. The rise in production of granzyme B and perforin was reduced (to

various degrees in $CD4^+$ and $CD8^+$ T cells) when TGF-β was present during the 2 day-stimulation phase.

A possible reason for an altered expression profile could be a change in T cell subpopulations due to TGF-β. As a modulator of the immune response, TGF-β has the ability to inhibit differentiation of T cells and keep them in a pluripotent state (Gorelik and Flavell 2002). Out of this state, T cells can quickly develop into any subtype needed as soon as TGF-β is removed from the system (Sad and Mosmann 1994). Some studies point towards TGF-β directing differentiation of naive T cells to central memory T (T_{CM}) cells rather than to effector memory T (T_{EM}) cells upon activation (Swain et al. 1991; Sad and Mosmann 1994). T_{EM} cells are cytolytically active and eliminate target cells upon TCR engagement. However, T_{CM} cells lack effector function proteins and can therefore not contribute to reduction of tumor cells (Sallusto et al. 2004). Although they are more ready to proliferate and replenish the pool of T_{EM} cells that is constantly decreased by frequent cell death of T_{EM} cells (Geginat et al. 2001; Sallusto et al. 2004).

Both mechanisms - inhibition and directing of differentiation - probably occur at the same time in accordance with the current state of each individual T cell. So the reduced level of cytotoxic proteins in TGF-β-treated T cells could be the result of a mix of TGF-β effects: fewer differentiated T cells among which there are fewer cytolytically-active T cells.

An effect of TGF-β on cytolytic performance of BiTE-stimulated T cells was observed under defined circumstances: TGF-β and stimulus had to be present simultaneously for more than 25 h. Exposition to TGF-β for one day, did not diminish cytolytic performance of T cells upon stimulation (Figure 33 a). Neither did a total stimulation time of 3.5 days with TGF-β only present during the last 25 h (Figure 33 b and c). Confirming the results of other groups that found that TGF-β did not affect the cytolytic function of fully activated CTL in most experimental settings (Ranges et al. 1987; Tada et al. 1991). A prolonged incubation phase with TGF-β (2.5 days) followed by a stimulatory phase of 25 h without TGF-β did not change lytic behaviour of T cells (Figure 34). A strong effect was observed when effector cells were stimulated in the presence of TGF-β for 2.5 days. Stimulated TGF-β-treated T cells eliminated less tumor cells than stimulated control T cells (Figure 35). This effect weakened and finally disappeared when TGF-β was removed from the system. Eleven h after stimulated T cells had been transferred into TGF-β-free medium, they lysed up to 30 % less tumor cells than control T cells (Figure 35). Another 14 h later (25 h after removal from TGF-β influence), the curve of TGF-β-treated T cells had come very close to the curve of control T cells (Figure 36). Continuously present TGF-β exerted a persistent influence on effector cells. After 3.5 days of

stimulation in the presence of TGF-β, T cells eliminated only 32 % of target cells compared to 69 % lysed by control T cells (Figure 37 c). The high sensitivity observed with anti-CD3/anti-CD28 pre-stimulated effector cells was consistent with experimental work by Lee and Rich who found TGF-β to reduce the cytolytic responses of enriched anti-CD3 stimulated T cells (Lee and Rich 1993).

An explanation for the observation that simultaneous TGF-β exposition and stimulus is required in order to have an effect on cytotoxicity of T cells might lie with regulatory T cells. TGF-β is known to induce $CD4^+$ regulatory T cells (Chen et al. 2003). Regulatory T cells suppress the response of T cells in the periphery (Curiel et al. 2004). T_{reg} cells need to be activated to suppress the T cell response and they function via cell-cell-contact. In a hypothetic model, T_{reg} cells exert their suppressive function most efficiently in the presence of TGF-β, the cytokine responsible for their induction. As soon as TGF-β-containing medium is replaced for TGF-β free medium, regulatory T cells start to lose their influence. TGF-β-treated T cells recover from suppressive conditions and revert to a state of activation equal to control cells. This model is supported by the experiments of several groups. $CD4^+$ $CD25^+$ T cells were shown to mediate immunosuppression via TGF-β expressed on their surfaces (Nakamura, 2001) and T_{reg} cells inhibited NK cell cytolysis and INF-γ secretion in a TGF-β dependent manner (Ghiringhelli et al. 2005).

Alternatively a change in T cell subset distribution caused by TGF-β could account for weaker cytolytic activity of TGF-β-treated T cells. Under the influence of TGF-β, a higher percentage of T cells remains in a pluripotent state than in control samples. Additionally, differentiation of T cells is shifted towards cytolytically inactive T_{CM} cells instead of more potent T_{EM} cells. After 2.5 days of TGF-β treatment, undifferentiated T cells and T_{CM} cells outnumber T_{EM} cells by far. Accordingly, TGF-β-treated cells show weaker cytolytic potency than control T cells. Upon removal of TGF-β from the reaction system, the mass of undifferentiated pluripotent T cells is ready to differentiate into T_{EM} cells. T_{CM} cells begin to express cytotoxic proteins and turn into T_{EM} cells as well. The number of T_{EM} cells in TGF-β-treated samples increases rapidly and overall cytotoxicity in TGF-β-treated samples reaches the same values as in control reactions.

Both models underline that an effect by TGF-β relies on the presence of TGF-β. Its effects will last as long as the cytokine is present in the cell culture medium but fade soon after removal. Confirmation for this theory was found when T cells were constantly exposed to TGF-β.

When T cells were under the prolonged influence of the cytokine, the effect of TGF-β did not disappear. Stimulated T cells in TGF-β-containing medium continued to perform worse than control T cells. When a co-stimulus was included the drop in cytolytic performance of T cells was most dramatic. However, when T cells were stimulated with MT110, the effectiveness of TGF-β-treated and -untreated T cells differed by a maximum of 17 % (Figure 37).

It seems that the last-described conditions are the most interesting ones as they most ressemble the *in vivo* situation: a tumor containing TGF-β-producing cells under BiTE treatment. As MT110-stimulated T cells lyse 72 % of cancer cells even in the permanent presence of TGF-β (Figure 37 b), this evasion mechanism does not seem to provide an escape for tumor cells from MT110-mediated cytotoxicity.

5.3 MABEL of MT110

According to recommendations by the *Expert Scientific Group on Phase One Clinical Trials* (EMEA/CHMP/SWP/28367/2007), the minimal anticipated biological effect level was determined from a range of *in vitro* experiments in order to have an additional method for estimating the starting dose for first-in-man clinical trials. As a measure for MABEL, the effective MT110 concentrations that induced 20 % of the respective maximal effect (EC_{20} values) were used.

In vitro experiments analyzed MT110-induced effects including T cell activation (Figure 38 a), lysis of tumor cells (Figure 39 a), secretion of cytokines by T cells and release of adenylate kinase by tumor cells. Furthermore, the fractional receptor occupancy was calculated for the envisaged starting dose in man.

As the MT110 treatment in clinical trials was going to be accompanied by a glucocorticoid pre-medication, the effect of methylprednisolone on T cell activation (Figure 38 b) and tumor cell lysis (Figure 39 b) was investigated.

The mean EC_{20} values for T cell activation and tumor cell lysis assays were generally comparable and ranged from 50 to 55 pg/ml MT110. Co-incubation with methylprednisolone (10^{-7} M) resulted in slightly higher mean EC_{20} values that ranged from 85 to 120 pg/ml indicating an approximately 2-fold higher MABEL following glucocorticoid co-administration.

MT110-induced secretion of cytokines and release of adenylate kinase was a less sensitive measure for MABEL. For all cytokine dose response curves, the apparent EC_{20} values were clearly higher as compared to the EC_{20} values derived from the T cell activation and tumor lysis curves. Similarly, EC_{20} values calculated from adenylate kinase release assays with

MDA-MB-453 cells were approximately 14-fold higher confirming that the Kato III system was the most sensitive test system for *in vitro* determination of MABEL of MT110.

The fractional receptor occupancy indicates the fraction of all receptor molecules that are bound to an antibody molecule. Its calculation for MT110 requires K_D values (indicating 50 % receptor occupancy) of the CD3 and the EpCAM binding arm and depends on the serum concentration of the BiTE molecule. Based on the classical NOEL/NOAEL approach a starting dose and treatment duration for first-in-man clinical trials of approximately 1 µg per patient and 24 h was envisaged for MT110. With continuous intravenous infusion intended as the mode of administration, a steady-state serum concentration could be predicted taking into account pharmacokinetic parameters already determined clinically for MT103. The steady-state serum concentration of MT110 was calculated to be 20 pg/ml. Together with the K_D values of the CD3 (K_D = 126 ± 25 nM) and EpCAM binding arms (K_D = 18.4 ± 2.4 nM), fractional receptor occupancies for increasing serum concentrations have been calculated for the CD3 and EpCAM target. The fractional receptor occupancies predicted for the MT110 serum concentration of 20 pg/ml were below 0.002 %. Even a 1000-fold increase in MT110 steady-state serum concentration was considered to result in a receptor occupancy of < 5 %. Therefore, fractional receptor occupancy was not considered a dose-critical component in MABEL calculation.

Based on T cell activation and tumor cell lysis which were shown to be the most sensitive test systems and assay conditions, the MABEL of MT110 was determined to be 50 pg/ml. Co-administration of glucocorticoids increased the MABEL concentration approximately 2-fold.

The glucocorticoid dexamethasone (3×10^{-7} M), tested in the MT103 system, also did not markedly reduce T cell activation (Figure 14), expression of adhesion molecules (Figure 15) or lysis of tumor cells (Brandl et al. 2007) but did inhibit the release of cytokines (Figure 13). During the course of this PhD study, methylprednisolone was shown to be more a favourable co-medication for the clinics than dexamethasone as its serum half life is shorter and its effect therefore better controllable. Furthermore MPDS is lacking dexamethasone's mineral steroid function, which can have a detrimental effect on the kidney.

The starting dose of MT110 for first-in-man clinical trials was assessed to 1 µg per patient and 24 hours.

The starting dose and treatment duration of MT110 for first-in-man clinical trials was proposed to be and accepted as 1 µg per patient and 24 h.

5.4 Outlook

Further experiments should be conducted to find out about the role of perforin in BiTE-mediated cytotoxicity. Does the content of perforin in BiTE-activated T cells correlate with their cytolytic performance? Is perforin capable of or even required for activating caspases? Addition of a specific perforin inhibitor to cytotoxicity assays will provide important information on the latter topic.

Completely blocking BiTE-induced tumor cell lysis by simultaneous administration of granzyme B inhibitor and caspase inhibitor will represent the ultimate experiment confirming that BiTE molecules induce the granzyme B/caspase pathway for elimination of tumor cells.

To confirm the theory that TGF-β influences distribution of T cells into the individual T cell populations, subset analyses should be performed. Is the population of T_{EM} cells decreasing and the population of T_{CM} cells increasing upon exposure to TGF-β? A primary series of experiments has been started to clarify the role of TGF-β in T cell development but identification of individual T cell subsets and which is involved proved difficult. In particular, long-term assays seemed to have an inconsistent influence on the expression of T cell subset markers.

BiTE molecules recruit the most potent effector cells of the human immune system, the T cells, for elimination of cancer cells. Can these small proteins be further engineered to additionally recruit NK cells? An Fc receptor portion connected to a BiTE molecule could potentially activate both T cells and NK cells for tumor cell lysis. Molecular cloning for generation of such a trispecific construct has been performed already. Yet, production and purification are challenging processes and require further research.

6 Summary

Bispecific T cell engager (BiTE) display a novel design among the class of bispecific antibodies and hold great promise to fight diverse cancers. BiTE molecules consist of two different binding entities derived from two human IgG antibodies connected by a short peptide linker. Their binding arms are directed against the CD3ε chain of the T cell receptor on T cells and against an antigen that is specific for (e.g., CD19 for lymphoma in MT103) or over-expressed on (e.g., EpCAM for epithelial cancer in MT110) tumor cells. Without requirement for pre- or co-stimulation, BiTE molecules efficiently redirect $CD3^+$ T cells towards tumor cells expressing the relevant target antigen. Only a BiTE molecule simultaneously bound to both tumor cell and T cell activates the T cell to exert its cytolytic function resulting in tumor cell death.

In T cells stimulated with both BiTE and target cells, elevated levels of caspase activation and increased expression of cytotoxic and signaling proteins are observed. These include cytolytic proteins granzyme B and perforin, activation markers CD69 and CD25 and adhesion molecules CD2 and LFA-1. Activated T cells secrete the usual mix of cytokines, among them pro-inflammatory cytokines IFN-γ and TNF-α. The membrane of tumor cells expressing the relevant target antigen is perforated during the attack of BiTE-stimulated effector cells as can be concluded from adenylate kinase release from the cytosol of tumor cells. Ca^{2+}-chelator EGTA completely blocked BiTE-mediated activation of caspases and tumor cell lysis. As perforin is strictly Ca^{2+}-dependent, a major role for this pore-forming protein is assumed for the elimination of tumor cells via BiTE-stimulated T cells.

Granzyme B and caspases are main players in BiTE-mediated elimination of tumor cells. Inhibitors of granzyme B or caspases reduce or block, respectively the activation of caspases. However, other signals of apoptosis (cleavage of PARP and fragmentation of DNA) were only reduced by granzyme B inhibitor or caspase inhibitor. Most interestingly, the lytic capacity of BiTE molecules was not impaired by granzyme B inhibitor or caspase inhibitor. It seems that there is no requirement for granzyme B and caspases to be present simultaneously. Instead the data presented provide evidence that they can be replaced one at a time by related proteins.

Pre-incubation of effector cells with the glucocorticoids dexamethasone or methylprednisolone resulted in markedly decreased secretion of cytokines by T cells yet only a small reduction in the expression of activation markers and adhesion molecules on T cells and specific lysis of tumor cells upon BiTE stimulation.

Soluble factors secreted in an undirected manner by BiTE-stimulated T cells do not mediate tumor cell death by themselves. Bystander cells negative for the antigen that is recognized by the BiTE molecule will not be compromised by BiTE activity.

The cytokine TGF-β reduced proliferation as well as granzyme B and perforin expression of BiTE-stimulated T cells. Redirected lysis by BiTE-activated T cells was also decreased under the influence of TGF-β, however lysis was still performed at a reasonable rate (72 % of target cells). TGF-β does not exert a deleterious effect on lytic potential of BiTE-stimulated T cells.

The minimal anticipated biological effect level for the BiTE MT110 was determined for the entry of MT110 into phase I clinical studies. Experiments analyzing redirected lysis of tumor cells, expression of activation marker CD25 and cytokine release by T cells revealed a MABEL value of 50 pg/ml for MT110.

7 Zusammenfassung

Bispecific T cell engager stellen mit ihrem neuartigen Design eine eigene Gruppe unter den bispezifischen Antikörpern dar und zeigen sich vielversprechend im Kampf gegen unterschiedliche Krebsarten. BiTE Moleküle bestehen aus zwei unterschiedlichen Bindungsstellen, die von zwei humanen IgG Antikörpern abgeleitet sind und durch einen kurzen Peptidlinker verbunden sind. Die Bindungsstellen sind gerichtet gegen die CD3ε Kette des T-Zell-Rezeptors auf T-Zellen und gegen ein Antigen, das auf den Tumorzellen ausschließlich (CD19 bei Lymphomen in MT103) oder in erhöhtem Maße (EpCAM bei epithelialem Krebs in MT110) exprimiert wird. BiTE Moleküle richten $CD3^+$ T-Zellen gegen Tumorzellen, die das relevante Zielantigen präsentieren. Dabei sind sie nicht auf Vor- oder Kostimulation angewiesen. Nur wenn das BiTE Molekül gleichzeitig an Tumorzelle und T-Zelle gebunden ist, aktiviert es die T-Zelle zytolytisch zu wirken und die Tumorzelle zu töten.

T-Zellen, die mit BiTE und zugleich Targetzellen stimuliert wurden, zeigen erhöhte Raten von Caspaseaktivierung und vermehrte Expression von zytotoxischen und Signalproteinen. Diese beinhalten die zytolytischen Proteine Granzyme B und Perforin, die Aktivierungsmarker CD69 und CD25 und die Adhäsionsmoleküle CD2 und LFA-1. Aktivierte T-Zellen sezernieren die übliche Mischung an Zytokinen, darunter die pro-inflammatorischen Zytokine IFN-γ und TNF-α. Die Freisetzung von Adenylatkinase aus dem Zytosol von Tumorzellen lässt darauf schließen, dass die Membran von Tumorzellen, die das relevante Zielantigen exprimieren, während dem Angriff von BiTE-stimulierten Effektorzellen durchlöchert wird. Der Ca^{2+} Chelator EGTA verhinderte die BiTE-vermittelte Aktivierung von Caspasen und Lyse von Tumorzellen vollständig. Da Perforin in Abhängigkeit von Ca^{2+} wirkt, wird für dieses porenbildende Protein eine entscheidende Rolle in der Beseitigung von Tumorzellen mittels BiTE-stimulierter T-Zellen angenommen.

Granzyme B und Caspasen sind die Hauptakteure in der BiTE-vermittelten Beseitigung von Tumorzellen. Inhibitoren von Granzyme B oder den Caspasen vermindern bzw. hemmen die Aktivierung von Caspasen. Andere Apoptosesignale (PARP-Spaltung und DNA-Fragmentierung) werden von Granzyme B- oder Caspase-Inhibitoren jedoch lediglich reduziert. Bemerkenswerterweise wurde die lytische Kapazität von BiTE Molekülen durch einen Granzyme B- oder Caspase-Inhibitor nicht beeinträchtigt. Es scheint, dass keine Notwendigkeit für die gleichzeitige Anwesenheit von Granzyme B und Caspasen besteht. Stattdessen erbringen die vorgestellten Ergebnisse einen Hinweis dafür, dass diese Proteine jeweils einzeln durch verwandte Proteine ersetzt werden können.

Präinkubation von Effektorzellen mit den Glucocorticoiden Dexamethason oder Methylprednisolon bewirkte eine deutlich verminderte Zytokinsekretion von T-Zellen, jedoch nur eine geringe Abnahme der Expression von Aktivierungsmarkern und Adhäsionsmolekülen auf T-Zellen und der spezifischen Lyse von Tumorzellen in Folge von BiTE-Stimulierung.

Lösliche Faktoren, die von BiTE-stimulierten T-Zellen nicht zielgerichtet abgegeben werden, vermitteln keine Lyse von Tumorzellen. Zellen, die sich in der Nachbarschaft des Tumors befinden, aber das Antigen nicht exprimieren, das vom BiTE Moleküle erkannt wird, werden daher durch BiTE Aktivität nicht in Mitleidenschaft gezogen.

Das Zytokin TGF-β verminderte die Proliferation von BiTE-stimulierten T-Zellen sowie deren Expression von Granzyme B und Perforin. Die gerichtete Lyse von BiTE-aktivierten T-Zellen war unter dem Einflusss von TGF-β ebenfalls vermindert. Trotzdem erreichten die Lysisraten Werte von 72 %. TGF-β übt keinen schädlichen Effekt auf das lytische Potential von BiTE-stimulierten T-Zellen aus.

Die MT110-Konzentration, bei der der geringste biologische Effekt erwartet wird, wurde für den Eintritt von MT110 in klinische Studien der Phase I bestimmt. Auf Grundlage von Experimenten zur gerichteten Lyse von Tumorzellen, zur Expression des Aktivierungsmarker CD25 auf T-Zellen und zu Freisetzung von Zytokinen aus T-Zellen, ergab sich ein MABEL-Wert von 50 pg/ml für MT110.

8 Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
Apaf-1	apoptotic protease activating factor 1
APC	allophycocyanin
APC	antigen-presenting cell
ARG1	arginase-1
ATP	adenosine triphosphate
BiTE	bispecific T cell engager
BSA	bovine serum albumin
C-terminus	carboxyl-terminal end of a protein
Ca^{2+}	calcium (ions)
CAD	caspase-activated DNAse
CAM	cell adhesion molecule
CD	cluster of differentiation
CHO	chinese hamster ovary (cells)
CI-MPR	cation-independent mannose-6-P receptor
COX2	cyclooxygenase-2
CR2	complement receptor 2
CTL	cytotoxic T lymphocytes
Cy	cytochrom
Da	Dalton
DC	dendritic cell
Dex	dexamethasone
DISC	death-inducing signaling complex
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DPPI	dipeptidyl peptidase I
EC_{50}	half-maximal effective concentration
EDTA	ethylenedinitrilotetraacetic acid
E-FABP	epidermal fatty acid-binding protein
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EpCAM	epithelial cell adhesion molecule
Erk	extracellular signal-regulated kinase
E:T	effector to target (ratio)
FACS	fluorescence-activated cell sorter
FADD	Fas-associated protein with death domain
Fc	constant region of an immunoglobulin
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
g	standard gravity, 9.80665 $\frac{m}{s^2}$
HEPES	2-(4-(2-hydroxyethyl)- 1-piperazinyl)-ethansulfonsäure
IC_{50}	half-maximal inhibitory concentration
ICAD	inhibitor of CAD
ICAM-1	intercellular adhesion molecule 1
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-gamma
IgG	immunoglobulin of G subclass
IL	interleukin
kb	kilo base pairs
K_D	equilibrium dissociation constant
L-Arg	L-arginine
LFA-1	lymphocyte function-associated antigen 1
MABEL	minimal anticipated biological effect level
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MPDS	methylprednisolone
N-terminus	amino-terminal end of a protein
NF-κB	nuclear factor-κB
NK	natural killer (cells)
NOAEL	no observed adverse effect level
NOEL	no observed effect level
NOS2	nitric-oxide synthase 2

PARP	poly (ADP-ribose) polymerase
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PE	phycoerythrin
PGE2	prostaglandin E2
PI	propidium iodide
PI-9	proteinase inhibitor 9
RLU	relative light units
SEM	standard error of the mean
SSD	safe clinical starting dose
STAT5	signal transducer and activator of transcription 5
TAP	transporter associated with antigen processing
TCR	T cell receptor
T_{CM}	central memory T (cells)
T_{EM}	effector memory T (cells)
T_{reg}	regulatory T cells
TGF-β	transforming growth factor-beta
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-alpha
TUNEL	terminal deoxynucleotidyltransferase dUTP nick end labeling
TY	thyroglobulin
U	enzyme unit
VEGF	vascular endothelial growth factor
V_L	variable region of the light chain of an immunoglobulin
V_H	variable region of the heavy chain of an immunoglobulin

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10 Vita

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Name	Cornelia Hauff (formerly Haas)
Date of birth	July 12th, 1979
Place of birth	Augsburg
Marital status	married

EDUCATION

Sept. 1985 – Jul. 1989	Elementary school in Augsburg
Sept. 1989 – Jun. 1998	Maria-Ward-Gymnasium in Augsburg
Nov. 1998 – Mar. 2004	Undergraduate and graduate studies in biology at the Julius-Maximilians-Universität in Würzburg
Oct. 2000	Intermediate Master's Examination*
Aug. 2001 – May 2002	Study abroad stipend of the Julius-Maximilians-Universität in Würzburg Work in the laboratory of Dr. Carmen A. Mannella (Wadsworth Center, State University of New York in Albany, NY) in the field of respiratory control in mitochondria
Apr. 2003	Master's Examination*
Jun. 2003 – Mar. 2004	Master's Thesis*: Import of RNA in mitochondria at the Institute for Cell and Developmental Biology at the Julius-Maximilians-Universität in Würzburg Supervisor: Prof. Dr. P. Seibel
Sept. 2004 – Jul.2009	Ph.D. work at Micromet AG in Munich Aspects of the mode of action of bispecific T cell engager (BiTE) antibodies Supervisors: Prof. Dr. P.A. Baeuerle Prof. Dr. G. Krohne

	INTERNSHIPS
Aug. – Sept. 1998	Internship at Clariant GmbH, Gersthofen Key aspects of activity: Handling of metallic and non-metallic materials, chemicals, analytical, measuring and regulating instruments and electronics
July 2001	Professor's aid at the Institute for Behavioral Physiology and Sociobiology at the Julius-Maximilians-Universität in Würzburg: In vitro and in vivo fluorescence microscopy in the nervous system of the honey bee

*The german Diplom is most equivalent to the american Master's degree.

11 List of publications

Brandl, C., Haas, C., d'Argouges, S., Fisch, T., Kufer, P., Brischwein, K., Prang, N., Bargou, R., Suzich, J., Baeuerle, P. A. and Hofmeister, R. (2007). **The effect of dexamethasone on polyclonal T cell activation and redirected target cell lysis as induced by a CD19/CD3-bispecific single-chain antibody construct.** Cancer Immunol Immunother. 56(10)**:** 1551-63.

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Poster:
Haas, C., Brischwein, K., Friedrich, M., Kufer, P., Baeuerle, P. A. and Schlereth, B.
Mode of Action of MT110, a Novel Bispecific Single-chain Antibody of the BiTE® Class for Treatment of Adeno and Squamous Cell Carcinoma Expressing EpCAM (CD326)
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13 Ehrenwörtliche Erklärung

gemäß § 4 Abs. 3 Ziff 3, 5 und 8 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität Würzburg

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Weiterhin erkläre ich, dass diese Dissertation noch in keinem anderen Prüfungsverfahren in gleicher oder ähnlicher Form vorgelegen hat.

Ich versichere, dass ich außer dem Diplom in Biologie keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

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Cornelia Hauff