# Tribodies: Fab-scFv fusion proteins as a platform to create multi-functional pharmaceuticals

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Abstract

Tribodies are multifunctional recombinant antibody derivatives, which utilize the natural in vivo heterodimerization of the heavy chain (Fd fragment) and light chain (L) of a Fab fragment, to form a scaffold, upon which additional functions can be incorporated, such as additional binders - e.g. scFv binding domains.

Each chain can be extended preferably at the C-terminus with an additional scFv binder. The chains are co-produced in mammalian cells, where the host-cell BiP chaperone drives the formation of the heavy chain-light chain heterodimer (Fd:L) - this reaction does not appear to be inhibited by the chain extensions, and leads to a very specific heterodimerization, using molecules abundantly present in serum (non-immunogenic)

These heterodimers are stable, with each of the binders retaining their specific affinities, with the bivalent tribody having higher affinity, and higher activatation of T-cell proliferation and cytotoxicity *in vivo.*

This design allows easy engineering of multispecificity in a single molecule, e.g. bispecific antibodies bivalent for the target and monovalent for effector activation (e.g. for T-cell activation), or trispecific antibodies *pur sang.*

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# 1 Introduction

Our proper immune system is a powerful but highly complex regulated system. Powerful since it is capable of specifically repel bulky cell masses (think of transplantation) and defend us quiet successfully against a pleitropy of invaders , and this with minimal damage to healthy tissue. This is due to a high specificity and self regulation build into a complex system, i.e. a system with a multitude of redundancies and feed-back loops. Antibodies are one way in which the system incorporates specificity. The main function of antibodies is to opsonize bacteria and deliver them to neutrophils and macrophages. They also have a natural function of clearing toxic compounds or circulating viruses out of the blood and target them for clearance. Cellular malignancies such as viral infected cells are more effectively halted by the cellular arm of the system, mainly by the action of T-cells and NK-cells. Only the latter carry a receptor for opsonizing antibodies.

Due to the high specificity and high affinity we can find in antibodies and the ease of developing recombinant proteins and adapt them to our needs antibodies became a first means for specific immunotherapy, with a special interest in trying to specifically target cancer cells. Through their Fc part antibodies are able to induce antibody-induced cell death (ADCC) and complement depended cytotoxicity (CDC). Target-cell bound antibodies are recognized through Fc-receptors on effector cells, such as the high affinity Fc-receptor Fc$\gamma$RIII on macrophages and activated neutrophils, but also to inhibiting receptors such as Fc$\gamma$RIIb, and on Fc$\gamma$RIIa complexes on non-cytotoxic cells such as platelets and B-cells. However, experience with anti-tumor antibodies has shown that effective antibodies also have an influence on the target cell by the nature of the receptor (or even epitope) they target. Ways this can be done is by growth inhibition (e.g. by down-regulation of growth factor receptors), rendering the cancer cells more susceptible to apoptosis, or even induce cell lysis.

Receptors without such a function have been targeted with antibodies with much less clinical anti-tumor effectiveness. This created a possibility to develop molecules with a comparable affinity and specificity for these receptors as antibodies can develop, but with a different effector mechanism. Effective routes under investigation are to couple toxic payloads or functions to the targeting molecules (such as the antibodies), that either need to be internalized or stay at the cell surface. The toxins can be of variable nature, such as protein toxins with enzymatic activity, chemical drugs or radionucleotides. Although such fusions are effective one problem is to target enough into the tumor. The percentage of injected dose (%ID) that actually reaches the tumor is often very low in human patients (can be in the order of less than 1%) (Steffens, Boerman et al. 1997). As long as these "armed" antibodies or antibody-derivatives are not bound to the tumor or excreted they remain circulating and can cause toxic effects elsewhere. Since monoclonal antibodies have a special recue mechanism by the FcRn binding in liver cell endosomes, they can have half lives of up to 3 weeks (Borvak, Richardson et al. 1998). This eventually allows good tumor targeting but results in a bad ratio of targeted versus non-targeted (circulating or deposited) antibody.

So there is a clear need to develop targeting reagents which have a good tumor binding but are cleared more rapidly from the rest of the body. Smaller fragments still containing the antigen binding properties of the antibody have been used as carrying vehicles of toxic functions.

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However, when these proteins are below 60-80 kDa in size they will be cleared rapidly by the kidneys. Kidneys work as an ultrafiltration device with a 6-10 nm cut-off value. As a comparison: a Fab molecules is about 8 x 5 nm, so it can pass readily through the pores. With a glomerular filtration rate in humans of about 7 liters/hour, a short half life can be predicted for these small fragments. This is indeed experimentally noticed using a variety of small antibody fragments. In most cases, half lives were determined to be less than 1 hour, with disappointing tumor accumulation as a result (Smith, Popplewell et al. 2001).

An intermediate sized molecule should avoid kidney clearance and ideally provide a half life sufficient for improved tumor accumulation while also avoiding a body detainment which would hamper targeting toxic function to the tumor.

One way of combating tumor cells different from the normal monoclonal antibody functions is by using antibody functions to cross-link cytotoxic T-cells with tumor cells. This is done using bispecific antibodies (BsAb) targeting both the tumor and the T-cell Receptor (TCR). Such a strategy involves T-cells in the induced immunotherapy. T-cells are naturally involved in combating cellular targets but are left inactive when invoking a cure with monoclonal antibodies (moAb). T-cell engaging bispecific antibodies are thus a complementary approach to moAb therapy. In this strategy it is important to have a monovalent binding towards the T-cell since bivalent binding will lead to initial T-cell activation but then induces T-cells anergy. This is why Orthoclone (OKT3 moAb anti-TCR) or its (Fab)$_2$ fragments is used with the aim to tolerize tissue transplants (Todd and Brogden 1989). On the other hand a bivalent binding towards the tumor cell can be more advantageous since the avidity will increase the functional binding and targeting towards the tumor.

We have created a manifold that combines two scFv fragments with a Fab fragment. The Fab fragment serves as a specific heterodimerization signal, and the two scFv fragments are each fused to a different Fab chain. In this way we obtain a molecule of intermediate molecular weight (100 kDa) which allows incorporating three different antibody fragments (Schoonjans, Willems et al. 2000). This manifold, which we baptized "tribody", can be used to create trivalent molecules as well as bispecific molecules with bivalent binding to only one target, as trispecific molecules.

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# 2 Tribody Structure, Production and Stability

## 2.1 What is a Tribody?

Tribodies are multifunctional recombinant antibody derivatives, which utilise the natural *in vivo* heterodimerization of the heavy chain (Fd fragment) and light chain (L) of a Fab fragment, to form a scaffold, upon which additional functions can be incorporated, such as additional binders - e.g. scFv binding domains.

Each chain can be extended preferably at the C-terminus with an additional scFv binder. This leads to a very specific heterodimerization, using molecules abundantly present in serum (and hence non-immunogenic and non-antigenic). The only non-natural sequences in the molecule are the peptide linkers connecting the VH and VL domains in the scFv moiety and the linkers connecting the Fab chains and the scFv (Schoonjans, Willems et al. 2001) (Schoonjans, Willems et al. 2000). These sequences can however be chosen to resemble natural human linker sequences or be composed of glycin-polymers which are considered to be neither antigenic (recognized by antibodies) nor immunogenic (recognized by T-cells when presented by antigen presenting cells), probably due to the lack of amino acid side chains.

This design allows easy engineering of multispecificity in a single molecule. The Fab chains (Fd and L chain) can be extended at both the N- and C-terminal side. However N-terminal extension at the Fab chains often leads to a hampered binding function of the Fab fragment (unpublished observations), so our preferred model will have the extensions made at the C-terminal side of the Fd and L-chain (Figure 1).
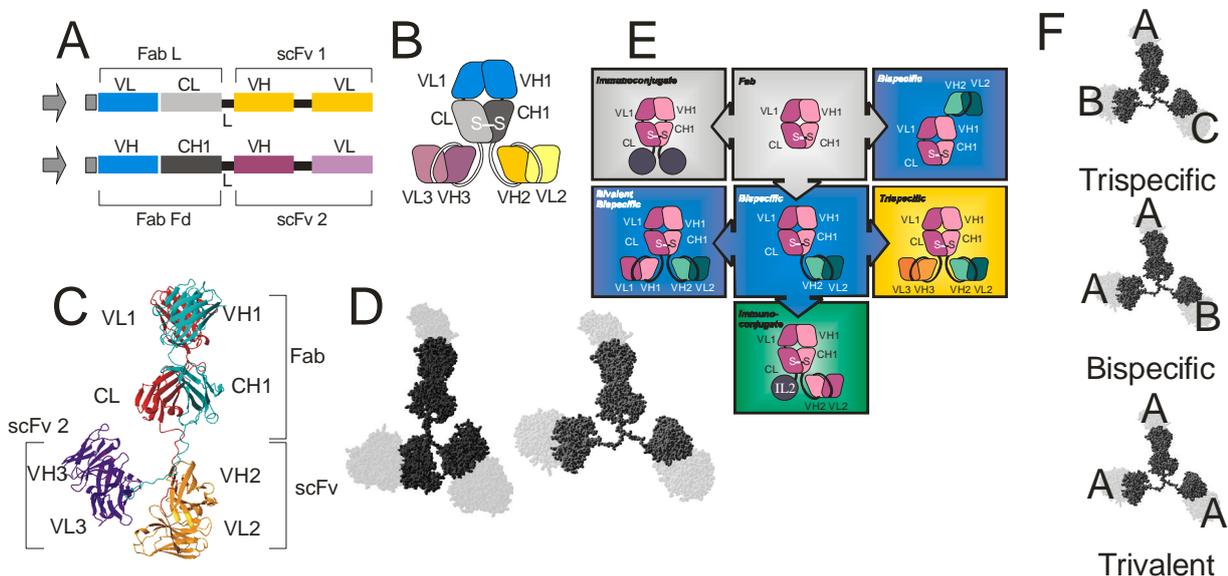


Figure 1: A) Tribodies are genetically constructed by fusing entities (i.e. to the Fd and L chain of a Fab molecule. As a Fab is a natural disulphide stabilized heterodimer, the position of each function is exactly determined and a single homogeneous product is produced. C) Models of the molecule predict no sterical hindrance between the subunits. D) Even as the scFv are N-terminally linked, their binding site is facing outside. E) Tribodies can lead to 3x1 (trispecific), 2+1 (bispecific) or trivalent molecules, as well as immunotoxins, immunocytokines, enzyme fusions,...F) The model easily allows trispecific, bispecific and trivalent antibody derivatives to be produced.
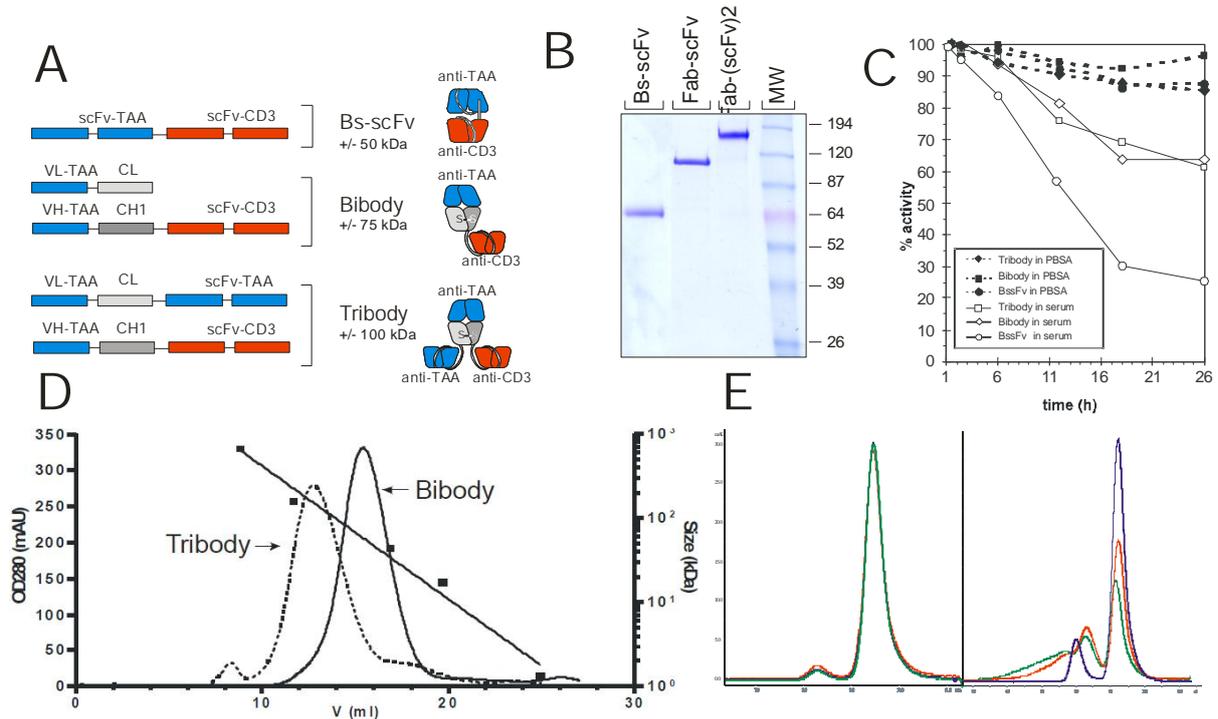
Figure 2: Production and stability. A) Gene and protein structure of a sc(Fv)$_2$ (BiTE), Fab-scFv (Bibody) and Fab-(scFv)$_2$ (Tribody) format. B) Coomassie brilliant blue stained gel of 10 µg of purified fractions. C) Tribody (squares) and Bibody (diamonds) residual activity in T-cell activation assay after 24h incubation at 37°C in PBS (dotted lines) and freshly prepared mouse serum (solid lines) D) Preparative gel filtration of Bibody and tribody structures and column calibration. E) 2 different Tribody structure analyzed on analytical size exclusion before (solid line) and after one (dashed line) or two (dotted line) freeze-thaw cycles (-80°C to 37°C).

The genetic structure of these fusion protein encoding genes thus starts with the coding sequence of either the Fd or the L chain, extended with a linker sequence separating the Fab from the scFv and the scFv molecule. Of course the scFv molecule can be substituted by other binding structures. New developments are producing single domain binders (human, camel, or shark derived) that can substitute for the two-domain scFv molecule. Furthermore, non-antibody domains often selected from more stable proteins allow the insertion of randomized loops and thus can be used for selecting an antibody-like type of binding to any desired target (Table 1). Also other domains or proteins can be included into the scaffold, depending whether the total fusion product can be produced. Examples here are natural protein ligands, cytokines, receptor domains, tags or enzymes.

A molecular model of a Tribody molecule is shown in Figure 1. Noteworthy is the orientation of the binding planes as present in the scFv molecules, which are oriented towards the outside (as opposed to orientation towards the Fab fragment), predicting the possibility to cross-link cells and large molecules. Through the choice of flexible linkers of a reasonable size (e.g 15 amino acids), a large span of the molecule can be predicted, comparable to the span range of a

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Table 1: Examples of alternative binding domains in development.

| Domain for targeting | Derived from | Platform Trade Name | Company |
|---|---|---|---|
| **antibody derived:** | | | |
| scFv | Human antibody VH + VL domains | SCA | Micromet / Enzon |
| Human $V_H$ or $V_L$ | Stable human V domains | dAbs | Domantis GSK |
| Camel/Llama VHH | Camel or Llama heavy chain mAbs | Nanobodies | Ablynx |
| **non-antibody derived:** | | | |
| Ankyrin repeats | Synthetic human ankyrin repeats | DARPins | Molecular Partners |
| Fibronectin | Trimeric human plasminogen binding protein | Tetranectins | Borean Pharma |
| Fibronectin | 10th fibronectin type III domain (human) | AdNectins | Adnexus Therapeutics |
| Transferrin | Non-glycosylated form of human transferin | Trans-bodies | BioRexis (Pfizer) |
| Lipocalins | Derived from *P. brassicae* butterfly | Anticallins | Pieris Proteolab |
| γ-Crystallin | Stable human protein from eye lens | Affilins | Scil Proteins |
| Cystein knots | *E. elaterium* trypsin inhibitor II – cyclotide EETI-II | Microbodies | NascaCell Technologies |
| Synthetic coiled coil | De novo design | Alphabodies | Complix |
| Protein A domains | *S. aureus* protein A domain | Affibodies | Affibody |

monoclonal antibody. We observed however no difference in expression or functionality when using shorter 5 amino acid linkers (Schoonjans, Willems et al. 2000).

## 2.2 Tribody Expression

The two fusion genes Fd-scFv and L-scFv can be produced in a variety of host cells. Production in *Escherichia coli* however resulted in a large fraction of precipitated protein as inclusion bodies. Also, a significant fraction of the produced material was degraded. In one attempt, a final purified batch of *E. coli* produced Tribody yielded only 0.1 mg/L and had a lower specific activity as compared to the same protein produced in mammalian cell culture.

Mammalian cell culture is becoming more and more advanced for high level production of antibodies (especially IgG formats). We found that tribodies are easily expressed in a range of mammalian cells, including HEK293, NS0, SP2/0 and CHO cells. Mammial cells all posses the BiP protein, a HSP70 class of chaperone present in the endoplamatic reticulum. This chaperone is involved in guiding the correct assembly of the antibody Fab fragment. It mainly binds to the CH1 domain of the Fd chain and is only displaced after displacement by correct Fd:L chain pairing (Lee, Brewer et al. 1999). As a consequence, no free Fd chains are found in the culture medium. This quality control mechanism does not appear to be inhibited by the C-terminal extensions to the Fab chains. L:L dimers can be secreted by normal and malignent B-cells and also L-scFv:L:scFv dimers are found in the supernatant of transfected cells. We never found them to interfere with overall production since they are easily removed by simple ion exchange chromatography. It is however a good strategy to select a final cell line which produces less fee light chaint and base a purification strategy on the Fd-chain properties. For easy of evaluation, we often incorporate a hexa-histidine tag at the C-terminus of Fd-containing fusion protein (e.g. Fd-scFv-H6).

Typical transient expression levels in either adherent or suspension HEK239 cells growing in T-flasks or shake flasks are in the tens of mg/L range. These values are obtained using a vector

with an actin promoter, a Kozak-optimized mRNA and a standard IgG kappa chain signal sequence. Expression yields using stable transformed cells are of course highly dependent on the cell selection procedure and thereafter from the bioreactor conditions. Without putting effort in selection of high-level producing cells and using laboratory shake flask conditions, we have examples where yields higher than 100 mg/L were obtained. This leads us to assume that high-level yielding bioreactor runs can be obtained with a > 1 g/L yield as is the case for most IgG productions, but this needs to be proven.

Tribodies can also be expressed in yeast cells (Schoonooghe, Kaigorodov et al. 2009). We have demonstrated more or less similar overall yields using the yeast *Pichia pastoris* with an AOX promoter and the α-mating type prepro sequence. We constructed bicistronic expression plasmids to be used for integration since double integration events occur with very low yield. Also in *Pichia*, no free Fd chains where observed in the medium. This indicates that the yeast chaperone system can substitute for the quality control mechanisms as seen in mammalian cells. Even when using defined medium for growing both host cells, the resulting cleared medium from a *Pichia* fermentation contained considerably more contaminants than cleared medium from serum-free growing mammalian cells. This resulted in a lower overall recovery yield after purification (20% in Pichia pastoris as compared with 70% from mammalian culture supernatans). The tribodies produced in *Pichia* showed the same functionality as those derived from mammalian cells. The α-mating prepro sequence is highly efficient in guiding recombinant proteins to the medium (as
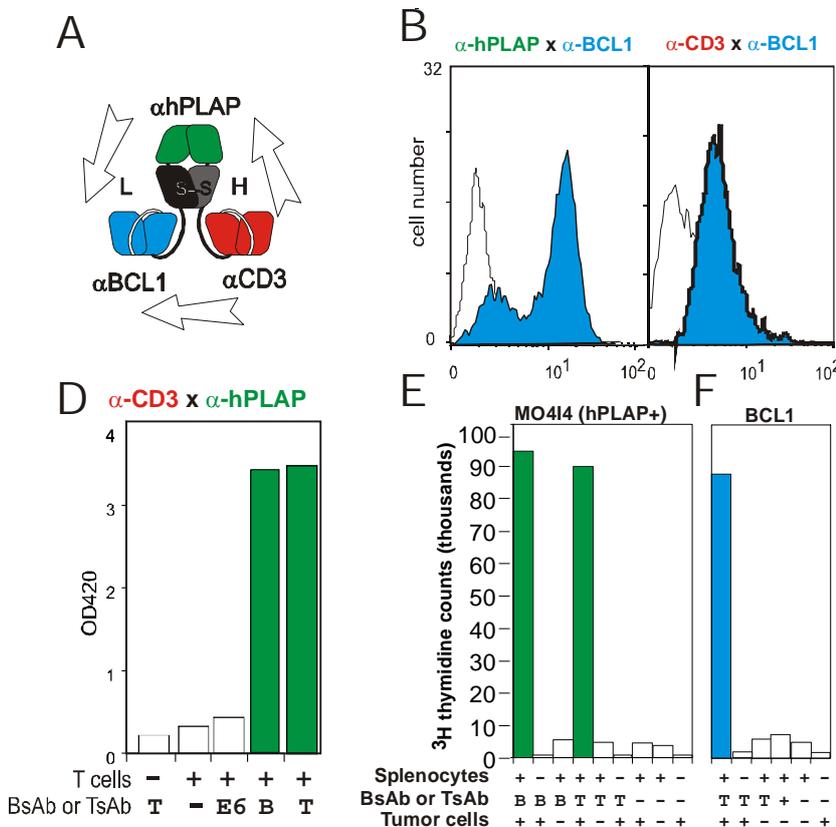


Figure 3: Binding and T-cell activation. A) A Tribody was constructed having specificity for two different TAA: mouse BCL1 and human placental alkaline phosphatase (hPLAP). The functionality of each of the indicated axes is demonstrated by binding to one antigen and detecting with the second. B) binding of the hPLAPxCD3xBCL1 Tribody to mouse T cells and detecting with hPLAP (which has alkaline phosphatase activity). C) Binding of the tribody to MO4I4 hPLAP transfected cells and detected with fluorescent BBCL1 antigen, and the same detection after binding the tribody to mouse T-cells. D) The hPLAPxCD3xBCL1 trispecific antibody acts as a bispecific antibody in a mouse T-cell proliferation assay primed both with hPLAP-positive cells as well as with BCL1 cells.

opposed to a major route to the vacuoles, which is the natural deposit for most yeast secreted proteins). This prepro-sequence is removed by KEX-2 protease.

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This protease prefers a Gln-Ala-Gln-Ala repeat after the cleavage site. These 4 amino acids can be removed by the di-aminopeptidase STE-13. We found the N-terminus of antibody fragments produced with this strategy to be heterogeneous as a result of partial or no removal of the extra amino acids. Also, when producing glycosylated Tribodies in yeast cells, it must be taken into account that yeast glycosylation differs from mammalian type of glycosylation which can lead to severe effects on half live *in vivo* and antigenicity.

## 2.3 Purification and Stability

Since most tribodies have a pI value of 7-8, we found a cation exchange fast flow column at pH 5.5 resolved with discrete step elutions an ideal and practical capture step. Further purification was dependent on the antibody. As mentioned, the inclusion of a hexahistidine tag simplifies this by using a common IMAC column.  In this way a simple purification step based on immobilized metal chromatography can already lead to a highly enriched protein and produces only Fd-scFv:L-scFv heterodimers. Other purification strategies however could also be designed, e.g. based on protein-L, MEP-hypercel or a combination of ion exchange steps (Willems, Leoen et al. 2003). As a polishing step, gel filtration has the advantage to be able to separate dimers and possible aggregates that might have been co-purifying. Typical, a purified and concentrated sample of tribody shows a small fraction of dimer. The amount of dimerized or even multimerized protein is highly dependent on the nature of the scFv proteins used. This is a behavior known to be associated with scFv molecules (Arndt, Muller et al. 1998). It is possibly a result of the inherent domain swapping property of the scFv molecules, which leads to a diabody type of dimerization. We have noticed this behavior to be even more pronounced when
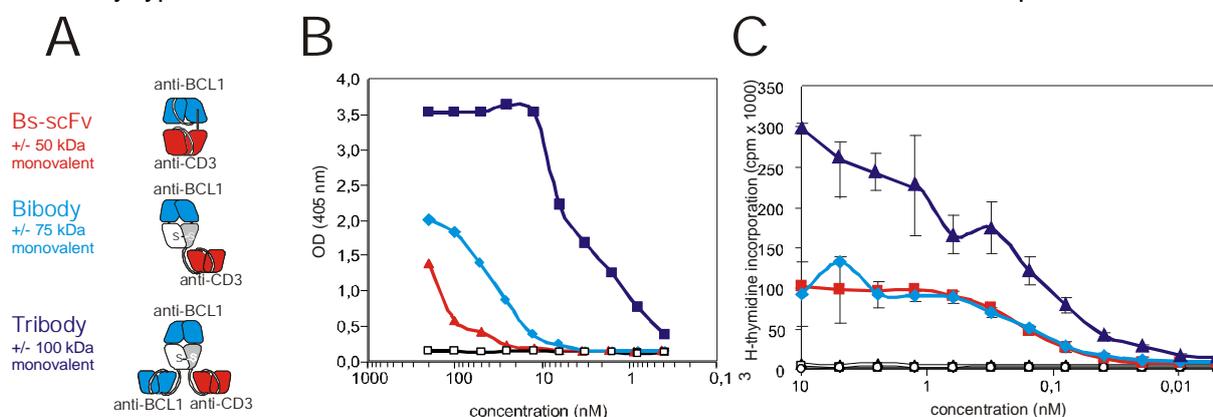


Figure 4. A) In vitro comparison of bispecific antibodies crosslinking BCL1-cells to T-cells in a sc(Fv)$_2$ (BiTE) format, a Fab-scFv (bibody) and Fab-(scFv)$_2$ (tribody) format. B) Apparent affinity of the BsscFv (BCL1xCD3), bibody (BCL1xCD3) and tribody (BCL1xBCL1xCD3). An ELISA plate was coated with the BCL1 IgM tumour antigen and subsequently incubated with respectively the BsscFv (-▲- ), the bibody (-●- ), the tribody (-■- ) and an irrelevant bibody (-□- ). The bound BsAbs were detected via their His-tag, with an anti-Histag Ab and a secondary alkaline phosphatase conjugated anti-mouse-IgG1. Data are representative for 3 independent experiments. C) *In vitro* T cell activating potential of the BsscFv, bibody and tribody. Mitomycin C treated BCL1 cells were co-incubated with syngeneic Balb/c spleen cells in the presence of decreasing concentrations of the indicated BsAb. Each condition was tested in triplicate.

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making sc(Fv)$_2$ constructs. Using the same scFv sequences in a tribody structure (i.e. fusing them to the Fab) reduces the dimers and multimers already considerably. The dependency on the nature of the scFv used thus stresses the importance of proper selection of lead candidate scFv molecules, which should not only be characterized for binding and epitope selection but hence also for its lack of tendency to multimerize when incorporated in a more complex manifold (such as even a sc(Fv)$_2$). Remarkably, for many tribodies we could purify the monomeric fraction and this fraction remained monomeric upon concentration and storage.

Tribody preparations were stored at 4°C in PBS at 1 mg/ml concentration and as such stable for at least 3 months. However, an even higher stability can be achieved in a storage buffer ith a pH of 5-6, well away of the pI of 7-8 of most molecules. Freeze-thawing stress testing again revealed large differences in behavior depending on the antibody V-domain sequences used and even on the position of these domains (Figure 2), again indicating the need for extensive characterization and monitoring starting with building block selection, but even extending to selecting the optimal configuration within the manifold. Some tribodies could be repeatedly frozen and thawed in PBS buffer lacking any cryoprotectant or stabilizing additive without any sign of aggregation, while other were very prone to aggregate upon such treatment.


# 3 Tribody Activity

## 3.1 *in vitro* binding and T-cell activation

In order to study the use of these Tribodies in a natural immune inveronment we constructed BsAbs to target BCL1 (an IgM/λ idiotypic determinant expressed on the murine myeloma cell line BCL1) and murine T-cell receptor determinant CD3ε. The BCL1 system has been well characterized and is a model for NHL in a syngeneic immunocompetent Balb/c mouse model (Brissinck, Demanet et al. 1991). As a comparison, we used the "gold standard" for small bispecific antibody formats: the bispecific scFv (BsscFv or BiTE) format. This is the smallest BsAb (50 kDa) used and was composed of two scFv linked together by a peptide linker. This BsscFv(BCL1xCD3) was previously reported to be successful in treating BCL1 lymphoma bearing mice. We engineered BsAbs of intermediate size (75-100 kDa) in the tribody format by fusing single-chain variable fragments (scFv) to the C-terminus of one or both of the Fd and L chains of a Fab fragment. Starting from the anti-BCL1 scFv, a chimeric anti-BCL1 Fab-fragment was constructed by grafting the variable domains onto murine CL and CH1 constant domains of a Fab fragment, We then fused an anti-mouse CD3 2C11 scFv to the C-terminus of the heavy chain of the chimeric Fab-fragment and an anti-BCL1 scFv to the C-terminus of the light chain of the chimeric Fab-fragment. Co-expression of the heavy chain Fab-scFv fusion gene with respectively the light chain Fab gene or the light chain Fab-scFv fusion gene, lead to the production of an (BCL1xCD3) Fab-scFv bibody, or a (BCL1xBCL1xCD3) Fab-(scFv)$_2$ tribody (Figure 4). The tumor binding mode (anti-BCL1) is different in all three BsAbs: In the BsscFv the tumor is recognized via a scFv, in the bibody via a Fab-fragment and in the tribody via both a Fab-fragment and a scFv. An ELISA experiment determined whether these differences resulted in a different functional affinity for the tumor antigen BCL1. To this end, the BCL1 tumor antigen was coated and subsequently incubated with a serial dilution of the respective BsAbs, using equimolar amounts. The bound Bs-Abs were then detected via the C-terminal His-tag, with a

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His-tag specific mAb. The $B_{50}$ value of the bivalent binding Fab-(scFv)$_2$ tribody was 3 nM, while the monovalent binding Fab-fragment in the bibody had a 10 times higher $B_{50}$ (30 nM). Binding through the scFv alone in the BsscFv lead to a further decrease in apparent binding affinity.

In a T-cell proliferation assay it was determined whether these differences in tumor binding affinity had an effect on the T-cell activating potential of the different BsAbs *in vitro*. A serial dilution of the different BsAbs, starting from equimolar amounts, was incubated with mitomycine inactivated BCL1 tumor cells and syngeneic spleen cells. All three BsAbs were found to be capable of activating T-cells in the presence of tumor cells. Despite the difference in affinity seen with ELISA, no difference in T-cell activating potential could be observed using both the monovalent binding bibody and the BsscFv. However, the bivalent binding tribody exerted a clearly improved T-cell activating potential. The maximum T-cell proliferation reached with the bivalent tribody was 2 -fold higher than with the monovalent bibody or BsscFv (Figure 4). Moreover, the bivalent tribody remained capable of inducing T-cell proliferation at 4 -fold lower concentration compared to the monovalent bibody and the BsscFv. Also in targeting other tumor markers, the increase in valency lead to better tumor binding (Schoonooghe, Burvenich et al. 2010).

### 3.2 *In vivo* model: mouse BCL1 lymphoma

In a next step it was investigated whether the differences in molecular weigh and tumor avidity would also lead to a different therapeutic potential of the BsAbs. Groups of mice (n=13) were inoculated i.p. with 5000 BCL1 cells on day 0 and i.v. (in the tail vein) treated with 4 daily injections on days 2 to 5. Treatment was performed with different equimolar amounts (200, 100 or 50 pmol/inj) of the BsscFv, the bibody or the tribody. In addition, a group of 6 mice were injected with PBS or 200 pmol/inj of anti-Id mAb that targets bivalently to the BCL1 tumor surface. All the animals in the control group receiving PBS or anti-Id mAb treatment developed terminal illness and were euthanized by day 60 or 140, respectively (Figure 5). After dissection the spleen was found to be enormously enlarged by the massive presence of tumor cells. In contrast, the mice treated with 200 pmol/inj of each of the BsAbs demonstrated significant (P<0.0001) protection against tumor formation in the spleen: after treatment with the BsscFv or
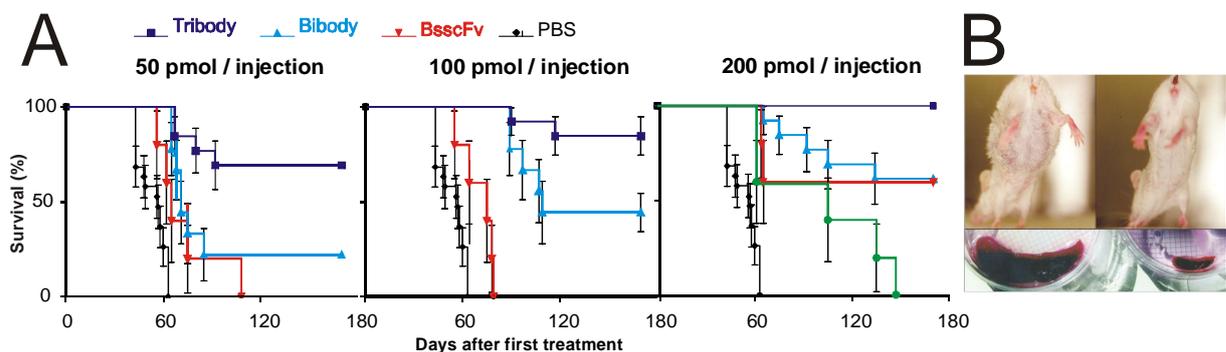


Figure 5. Balb/C mice were mice were inoculated with of 5 000 BCL1 lymphoma cells i.p. in 100µl sterile and endotoxin free PBS on day 1 of each experiment. During treatment, the BsAbs (aggregate and endotoxin free) were injected i.v. in the tail vain on days 2-5 in a volume of 200 µl sterile PBS. Animals were followed until a swollen abdomen could be observed, and then euthanized by cervical dislocation. Results are presented as a Kaplan-Meyer plot.

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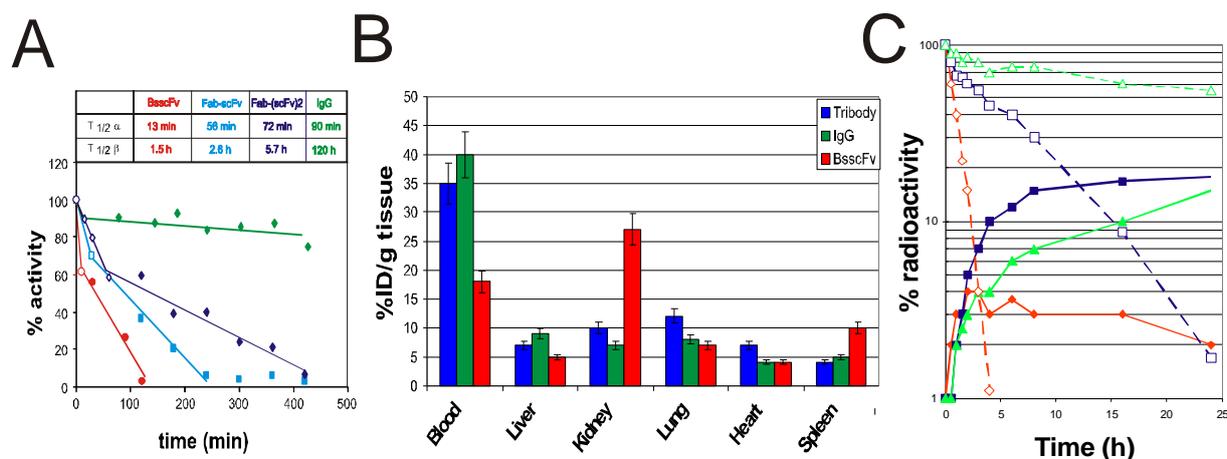*In "Bispecific Antibodies" (ed. R. Kontermann), in publication*

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Figure 6. A) Healthy Balb/c mice were i.v. injected with 800 pmol of the Ab-fragments into the tail vein. At various time points, mice were bled and serum was frozen. The remaining biological activity of the Bs-Abs in the serum was analyzed by a T cell proliferation assay. The activity present 5 min after injection (i.e. time required for anesthetic to take effect) was used as zero time point activity. B) [123]I labeled tribody, IgG1 and BsscFv were injected in the tail vein and a group of 6 mice was sacrificed after 1 h. Selected tissues were counted and compared. C) Comparison of ^{123}I labeled IgG1 (triangles), tribody (squares) and BsscFv (diamonds) serum clearance (dotted lines) and accumulation into a limb tumor nodule (solid lines) over a 24h period, plotted on a logarithmic scale.

the Fab-scFv bibody, 60% survival was observed, while treatment with the Fab-(scFv)$_2$ tribody even resulted in 100% protection of the mice. Lowering the dose of BsscFv treatment to 100 pmol/inj and 50 pmol/inj resulted in loss of protection. The Fab-scFv bibody treatment, however, remained active at 100 pmol/inj resulting in 45% survival, which was found to be statistically significant (P<0.0001). At 50 pmol/inj, the Fab-scFv bibody protected only 20% of the mice.

However, the bivalent Fab-(scFv)$_2$ tribody manifold was able to protect mice at concentrations were other manifolds were ineffective (70%, 85% and 100 % survival at 50, 100 or 200 pmoles injected 4 times, respectively) (P=0.0190, P=0.0371 and P=0.0059). Mice surviving after tribody treatment at the 200 pmole/dose treatment were 100% cured and did not develop tumors during a follow-up of 200 days. This argues for a effective tumor elimination and against the antibody induced dormancy of lymphoma cells.

## 3.3 PK measurements

We also determined whether the size of the different BsAbs significantly influences the *in vivo* blood clearance rate. Mice were i.v. injected in the tail vein with 800 pmoles of either the BsscFv, the bibody or the tribody. At different time points after injection, mice were bled and the remaining biological activity in the serum was determined by a T-cell proliferation assay. A typical biphasic blood clearance was observed for all three antibody derivatives (fig. 5). The initial blood distribution phase half-life ($T_{1/2}\alpha$) was found to be comparable for both the bibody and tribody i.e. 56 min and 72 min, respectively, but was considerably lower for the BsscFv (13 min). The terminal blood elimination phase half-life ($T_{1/2}\beta$) was found to correlate directly with

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the molecular weight of the antibody derivatives. The BsscFv was cleared fast ($T_{1/2}\beta$=1.5h), while the bibody and tribody remained longer in circulation ($T_{1/2}\beta$=2.9h and 5.7h, respectively).

As a test for biodistribution we radiolabeled the anti-BCL1 IgG1, BsscFv and tribody with ^{123}I and analysed 6 mice 1 hour after injection. The distribution in selected tissues is shown in Figure 6B. It can be concluded that the BsscFv format is rapidly accumulating in the kidneys while the IgG1 as well as the tribody is not.

We also compared serum clearance and tumor accumulation of these radiolabeled proteins (Figure 6C). As expected the IgG1 clears very slowly. The BsscFv however is quicly eliminated while the tribody has an intermediate clearing time. The accumulation in a limb injected tumor nodule was followed over a period of 24 h and shows low accumulation of the BsscFv in contrast to an increased accumulation of the IgG1 and the tribody.

These data indicate that introducing bivalency and increasing half life can increase the potency of a reagent *in vivo*. The tribody manifold already does this by incorporating a third binding function.

## 4 Tribody Use and Potential

As mentioned there are a lot of molecules that can be recombinantly fused to be heterodimerized by the Fab chains. I will discuss one example where we engineered a cross-interacting pretargeting system using this scaffold. Bispecific antibodies binding when crosslinking a T-cell via CD3 to the tumor cell do activate the T-cell both for proliferation and cytolytic response. This has been shown as well *in vitro* as in clinical trails (Baeuerle, Kufer et al. 2009). However, T-cells are more readily activated when a co-stimulatory signal is present (Gimmi, Freeman et al. 1993). The CD28 coreceptor is one of the first co-stimulatory signals identified and is probably the most potent one. Most antibodies targeting CD28 alone do not activate T-cells and are as such not toxic, although superagonistic CD28 binders have been described and found to be dangerous. In general the activation trigger for a T-cell comes from CD3 engagement. Without a proper environment (co-stimulus) T-cells can go into anergy or cell death. These co-stimulatory signals are given by activated antigen presenting cells, another control loop to avoid accidental activation of the immune system. A single bispecific or trispecific antibody crosslinking both the CD3 T-cell receptor and the CD28 costimulatory receptor does activates T-cells even without crosslinking to a tumor cell. This might induce systemic T-cell activation with associated cytokines storms and be potentially life-threatening (Stebbings, Findlay et al. 2007).

We have created a couple of Tribodies that had a build-in cross reactivity (cross-reactive bispecific antibodies (CriBs). This was done by including a small peptide in the first and an anti-peptide scFv in the second. One of both targeted tumor and CD28 and was completely harmless, unable to activate T-cells on its own. The second could bind the tumor and CD3 and also the P-peptide includes in the first. This allowed us to pretarget the anti-CD28 activity on the tumor cell, and then give the TAAxCD3xP tribody to activate the T-cell. We found that using this CriBs system, efficient T-cell activation could be achieved at 30 fold lower concentrations of the

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anti-CD3 containing bispecific as compared with a pair of non-cross interacting bispecific antibodies (Willems, Schoonooghe et al. 2005). Also, an optimized stochiometry of co-stimulus versus TCR engagement could be achieved with a large window between effective concentrations and start of marginal non-tumor cell induced T-cell activation (Figure 7). This might illustrate the potential and flexibility of Tribody molecules.

## 5 Conclusion

Clearly, above mentioned data illustrate that the tribody manifold has the potential to generate more active and potent molecules, by posiblilities to create better binding (through multivalency) as wel as though more binding (by targeting more antigens). The molecule also has more favorable properties for toxic payload delivery. It can be argued that delivering a T-cell activating activity can be regarded as a toxic function. Antibodies with such a function remaining in the body for a longer time have the potency to accumulate aspecifically or form deposits in healthy tissue.

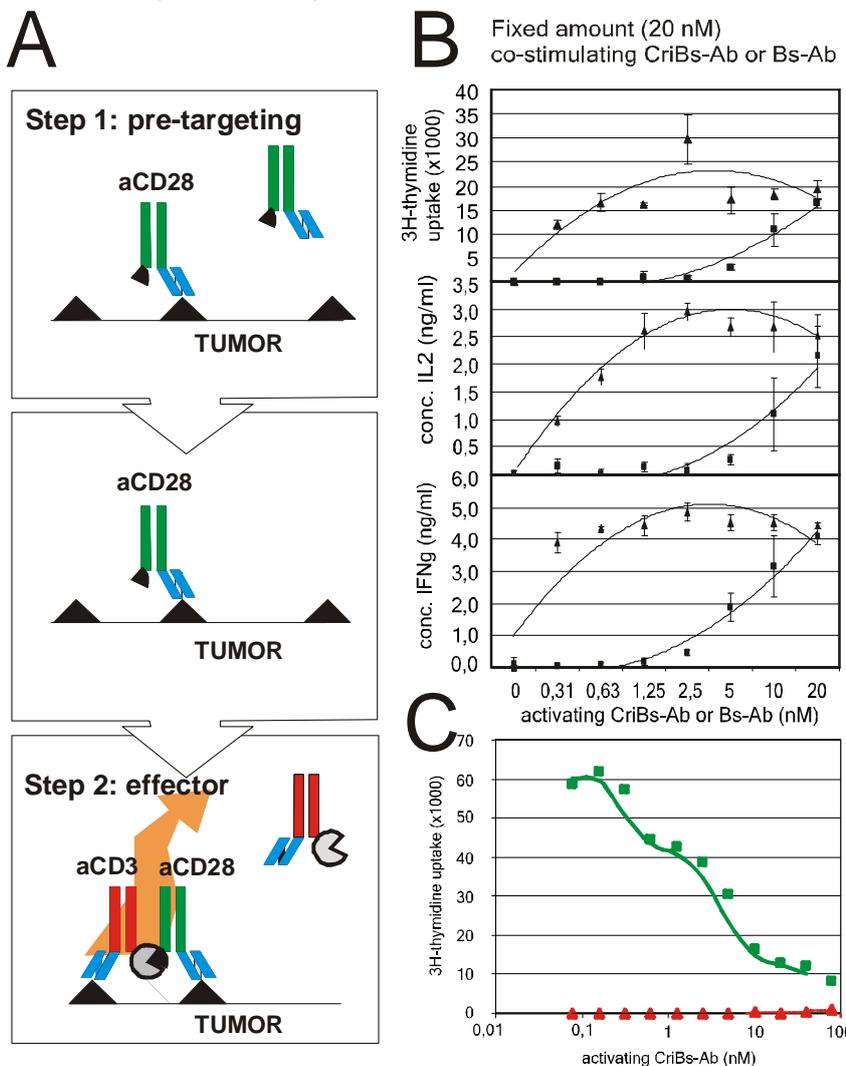To be eligible for drug development, the tribody needs to be able to be produced to high titers



Figure 7. A) A couple of cross-interacting bispecific antibodies (CRIBs) was constructed where an non-activating tribody is pretargeted to crosslink the tumor cell with the T-cell CD28 co-stimulus. This tribody contains a unique peptide P. A second tribody bind tumor, CD3 on the T-cell and the P-peptide and can crosslink with the first upon binding by the P-αP interaction. B) Mitomycine treated tumor cells were pre-incubated for 1h with a fixed amount (20 nM) of anti-(TAAxCD28) BsAb (-□-) or anti-(TAAxCD28)P CriBs-Ab (-Δ-) followed by a 1h incubation with a serial dilution (0-20 nM) of anti-(TAAxCD3) BsAb (-□-) or anti-(TAAxCD3xP) CriBs-Ab (-Δ-). Pre-incubated tumor cells were thern co-incubated with T cells. T cell activation was measured by proliferation, IL-2 production and IFNγ production. Each condition was tested in triplicate. C) Dose-dependent activation of T-cells in the presence (squares) and absence (triangles) of tumor cells in a range from 0.1 to 100 nM.

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(g/L) and remain stable under standard manufacturing conditions. The tendency to form dimers or multimers can be a problem both in manufacturing and hmper several applications, tumor cell dependent T-cell activation being one example.

Surprising differences can be seen with distinct building blocks (scFv as well as Fab moieties). This can be situated on the expression level, binding performance as well as on the level of tendency to form multimers. Moreover, permutations within the scaffold of the same moieties can induce the same differences. In our experience, a well integrated and professionally run technology platform which closely monitors these factors can produce drug development ready tribody lead candidates in relative short time frames.

## References

Arndt, K. M., K. M. Muller and A. Pluckthun (1998). "Factors influencing the dimer to monomer transition of an antibody single-chain Fv fragment." Biochemistry **37**(37): 12918-12926.

Baeuerle, P. A., P. Kufer and R. Bargou (2009). "BiTE: Teaching antibodies to engage T-cells for cancer therapy." Curr Opin Mol Ther **11**(1): 22-30.

Borvak, J., J. Richardson, C. Medesan, F. Antohe, C. Radu, M. Simionescu, V. Ghetie and E. S. Ward (1998). "Functional expression of the MHC class I-related receptor, FcRn, in endothelial cells of mice." Int Immunol **10**(9): 1289-1298.

Brissinck, J., C. Demanet, M. Moser, O. Leo and K. Thielemans (1991). "Treatment of mice bearing BCL1 lymphoma with bispecific antibodies." J Immunol **147**(11): 4019-4026.

Gimmi, C. D., G. J. Freeman, J. G. Gribben, G. Gray and L. M. Nadler (1993). "Human T-cell clonal anergy is induced by antigen presentation in the absence of B7 costimulation." Proc Natl Acad Sci U S A **90**(14): 6586-6590.

Lee, Y. K., J. W. Brewer, R. Hellman and L. M. Hendershot (1999). "BiP and immunoglobulin light chain cooperate to control the folding of heavy chain and ensure the fidelity of immunoglobulin assembly." Mol Biol Cell **10**(7): 2209-2219.

Schoonjans, R., A. Willems, J. Grooten and N. Mertens (2000). "Efficient heterodimerization of recombinant bi- and trispecific antibodies." Bioseparation **9**(3): 179-183.

Schoonjans, R., A. Willems, S. Schoonooghe, W. Fiers, J. Grooten and N. Mertens (2000). "Fab chains as an efficient heterodimerization scaffold for the production of recombinant bispecific and trispecific antibody derivatives." J Immunol **165**(12): 7050-7057.

Schoonjans, R., A. Willems, S. Schoonooghe, J. Leoen, J. Grooten and N. Mertens (2001). "A new model for intermediate molecular weight recombinant bispecific and trispecific antibodies by efficient heterodimerization of single chain variable domains through fusion to a Fab-chain." Biomolecular Engineering **17**(6): 193-202.

Schoonooghe, S., I. Burvenich, L. Vervoort, F. De Vos, N. Mertens and J. Grooten (2010). "PH1-derived bivalent bibodies and trivalent tribodies bind differentially to shed and tumour cell-associated MUC1." Protein Eng Des Sel.

Schoonooghe, S., V. Kaigorodov, M. Zawisza, C. Dumolyn, J. Haustrate, J. Grooten and N. Mertens (2009). "Efficient production of human bivalent and trivalent anti-MUC1 Fab-scFv antibodies in Pichia pastoris." BMC Biotechnol **9**(1): 70.

Smith, B. J., A. Popplewell, D. Athwal, A. P. Chapman, S. Heywood, S. M. West, B. Carrington, A. Nesbitt, A. D. Lawson, P. Antoniw, A. Eddelston and A. Suitters (2001). "Prolonged in vivo residence times of antibody fragments associated with albumin." Bioconjug Chem **12**(5): 750-756.

Stebbings, R., L. Findlay, C. Edwards, D. Eastwood, C. Bird, D. North, Y. Mistry, P. Dilger, E. Liefooghe, I. Cludts, B. Fox, G. Tarrant, J. Robinson, T. Meager, C. Dolman, S. J. Thorpe, A. Bristow, M. Wadhwa, R. Thorpe and S. Poole (2007). ""Cytokine storm" in the phase I trial of monoclonal antibody TGN1412: better understanding the causes to improve preclinical testing of immunotherapeutics." J Immunol **179**(5): 3325-3331.

Steffens, M. G., O. C. Boerman, J. C. Oosterwijk-Wakka, G. O. Oosterhof, J. A. Witjes, E. B. Koenders, W. J. Oyen, W. C. Buijs, F. M. Debruyne, F. H. Corstens and E. Oosterwijk (1997). "Targeting of renal cell carcinoma with iodine-131-labeled chimeric monoclonal antibody G250." J Clin Oncol **15**(4): 1529-1537.

Todd, P. A. and R. N. Brogden (1989). "Muromonab CD3. A review of its pharmacology and therapeutic potential." Drugs **37**(6): 871-899.

Willems, A., J. Leoen, S. Schoonooghe, J. Grooten and N. Mertens (2003). "Optimizing expression and purification from cell culture medium of trispecific recombinant antibody derivatives." J Chromatography B **786**(1-2): 161-176.

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Willems, A., S. Schoonooghe, D. Eeckhout, G. De Jaegher, J. Grooten and N. Mertens (2005). "CD3 x CD28 Cross-Interacting Bispecific Antibodies Improve Tumor Cell Dependent T-cell Activation." <u>Cancer Immunol Immunother</u> **54**: 1059-1071.

# Bispecific Antibodies (to be published)

**edited by Roland Kontermann**
**published by Springer Heidelberg**
The concept of using bispecific antibodies for cancer therapy by retargeting immune effector cells has been developed more than 25 years ago. However, initial clinical studies were rather disappointing mainly due to low efficacy, severe side effects and immunogenicity of the bispecific antibodies. A deeper understanding of effector cell biology and especially developments in the field of antibody engineering has led to the generation of new classes of bispecific antibodies capable of circumventing many of these obstacles. Furthermore, new applications were established for bispecific antibodies, such as pre-targeting strategies in radioimmunotherapy and dual targeting approaches in order to improve binding, selectivity and efficacy. In this book, the different ways to generate bispecific antibodies are described, with an emphasis on recombinant formats, and information on the various applications of bispecific antibodies, e.g. in cellular cancer immunotherapy, radioimmunotherapy and pretargeting strategies, but also emerging applications such as dual targeting strategies, i.e. simultaneous inhibition of two targets (cytokines, receptors, etc.) are provided. This book is intended for a broad readership in the field of antibody engineering, mainly from the pharmaceutical and biotechnology sector, but also academic researchers working in this field.

## Content (provisional titles)

**1 Bispecific antibodies - a historical perspective**
Roland Kontermann (University of Stuttgart, Germany)
**2 Bispecific antibodies from hybrid hybridoma**
Gerd Moldenhauer (DKFZ, Germany)
**3 Generation of bispecific antibodies by chemical conjugation**
Justin Scheer (Genentech, USA)
**4 Bispecific tandem scFv molecules and triple bodies**
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