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# The role of stimulating bystander cells in bispecific antibody - mediated T cell activation

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# List of abbreviations

| | |
|---|---|
| ° C | Degree Celsius |
| 7-AAD | 7-aminoactinomycin |
| APC | Antigen-presenting cell |
| ADCC | Antibody-dependent cellular cytotoxicity |
| ADCP | Antibody-dependent cellular phagocytosis |
| ALL | Acute lymphoblastic leukemia |
| BCR | B-cell receptor |
| BiTE® | Bispecific T cell engager, see: bssc |
| BSA | Bovine serum albumin |
| BsMAb | Bispecific monoclonal Antibody |
| bssc | Bispecific single-chain format |
| C-Terminus | Carboxy-terminus |
| CAR | Chimeric antigen receptor |
| CD | Cluster of differentiation |
| CDC | Complement-dependent cytotoxicity |
| CDCC | Complement-dependent cellular cytotoxicity |
| CDR | Complementarity-determining regions |
| Ci | Curie |
| Conc. | Concentration |
| CSPG-4 | Chondroitin sulfate proteoglycan 4 |
| CTL | Cytotoxic T lymphocyte |
| CTLA-4 | Cytotoxic T lymphocyte-associated Protein 4 |
| CXCL8 | Chemokine ligand 8 (former Interleukin 8) |
| Da | Dalton |
| DMSO | Dimethylsulfoxide |
| DN | Double negative (T cells) |
| DNA | Desoxyribonucleic acid |
| DP | Double positive (T cells) |
| DPBS | Dulbecco’s phosphate buffered saline |

| | |
|---|---|
| E:T | Effector to target ratio |
| ECGM | Endothelial cell growth medium |
| EDTA | Ethylendiaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGP2 | Anti-epithelial glykoprotein 2 |
| ELISA | Enzyme-linked immunosorbent assay |
| Fab | Fragment antigen-binding |
| Fabsc | Fragment antigen-binding single chain format |
| FACS | Flourescence-activated cell sorting |
| Fc | Fragment crystalizable |
| FCS | Fetal calf serum |
| FDA | Food and drug administration |
| g | Gram |
| Gy | Gray |
| h | Hour |
| HC | Heavy chain |
| Her-2 | Human epidermal growth factor receptor 2 |
| HLA | Human leukocyte antigen |
| HUVEC | Human umbilical vein endothelial cell |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IMDM | Iscove's modified Dulbecco's medium |
| ITAM | Immune-receptor tyrosine-based activation motifs |
| IU | International Units |
| K | Kelvin |
| l | Liter |
| LC | Light chain |
| LFA | Leukocyte function-associated antigen |
| LPS | Lipopolysaccharides |

| | |
|---|---|
| M | Molar |
| mAb | Monoclonal antibody |
| MAC | Membrane attack complex |
| Mac-1 | Macrophage 1-antigen |
| MFI | Mean flourescence intensity |
| MHC | Major histocompatibility complex |
| min | Minute |
| MTD | Maximal tolerated dose |
| N-Terminus | Amino-terminal |
| NK cell | Natural killer cell |
| $OD_{280}$ | Optical density at $\lambda$=280 nm |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| PD | Programmed death |
| PHA | Phytohemagglutinine |
| PRR | Pattern recognition receptor |
| PSGL-1 | P-selectin glykoprotein ligand 1 |
| PSMA | Prostate specific membrane antigen |
| R | Receptor |
| Ref | Reference |
| RER | Rough endoplasmic reticulum |
| RPMI | Roswell Park Memorial Institute Medium |
| s | Second |
| SBC | Stimulating bystander cells |
| scDiabody | Single-chain diabody format |
| scFv | Single-chain fragment variable |
| SDPD | Succinimidyl 3-(2-pyridyldithio)propionate |
| TAA | Tumor-associated antigen |
| TAP | Transporter associated with antigen processing |
| TCR | T cell receptor |
| TGF | Transforming growth factor |

| | |
|---|---|
| TH | T helper cell |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| U | Unit |
| v/v | Volume/Volume |
| VEGF | Vascular endothelial growth factor |
| $\times$ g | Multiple gravitational acceleration |

# 1 Introduction

## 1.1 The Immune System

The immune system consists of cells and molecules in order to provide protection (immunity) against malignant and infectious diseases.

The immune system is made up of two distinct parts; the innate and the adaptive immune system, with the former being comprised of neutrophils, macrophages, monocytes, dendritic cells, NK cells, the complement system and cytokines. The latter, adaptive immune system consists of T and B cells, also known as lymphocytes, and antibodies.

Cells of the innate system are capable of detecting pathogens, using only a limited number of germline-encoded invariant receptors called toll-like receptors (TLRs) [Beutler 2004]. These receptors recognize pathogen-associated molecular patterns (PAMPs), which are found in a vast array of molecules, for example, lipopolysaccharides (LPS) from gram-negative bacteria, bacterial flagellin and ssRNA derived from viruses [Akira and Hemmi 2003]. Proteins produced after activation of innate immune cells include, complement components, defensins, lysozyme, C-reactive protein and cytokines. Cytokines are small proteins (approximately 25 kDa) that play an important role in cell signaling and regulation of immune responses. Multiple cell types are capable of releasing and binding cytokines, which are not only released by a vast range of immune cells such as NK cells, neutrophils or macrophages, but also by endothelial cells, fibroblasts and other stromal cells. Maturation, proliferation, activation, apoptosis, and the downregulation of immune cells are all regulated by different cytokines including Interleukins (IL), Interferons (IFN) and Tumor-necrosis factor (TNF). In response to antigenic stimulation, macrophages and dendritic cells secrete IL-1$\beta$, IL-6, CXCL8, IL-12 and TNF$\alpha$. Whereas CXCL8 redirects immune effector cells to the site of infection, IL-1$\beta$, IL-6 and TNF$\alpha$ further induce the acute-phase response in the liver, and mediate fever.

In fact, the innate immune system reacts similarly to every case of infection, so is therefore known as non-adaptive. Due to this limited specificity, virus-infected cells and

malignant cells can easily surpass these defense mechanisms [Drake, Jaffee, and Pardoll 2006].

The acquired immune system is characterized by the ability to recognize different antigens by specific receptors. During lymphocyte development, gene rearrangement in V(D)J regions, which encode the antigen-binding sites of lymphocyte receptors, takes place. As a result, human lymphocyte receptors have a huge variability and can recognize millions of different antigens. Thus, an acquired immune response is highly specific and refined over time. Furthermore, repeated contact with a specific pathogen leads to a faster and stronger immune response due to the development of memory cells that are specific for the pathogen. The establishment of immunological memory, and the continuous adaptation, means that the acquired immune system is also known as the adaptive immune system [Janeway et al. 2005].

A key feature of the immune system is antigen presentation on the surface of major histocompatibility complex (MHC) molecules, also known as human leukocyte antigens (HLA) in humans. All nucleated cells express MHC class I, which presents endogenously, synthesized and processed peptides on the cell surface. Presented peptides are derived from antigens such as cytosolic proteins or, as in the case of infection, viral proteins. Before presentation, endogenous antigens have to undergo processing in the proteasome. After delivery via transporter associated with antigen processing (TAP), the peptides are loaded onto the rough endoplasmic reticulum (RER), and eventually peptides comprised of 8-10 amino acids are presented on the cell surface. [Rammensee, Falk, and Rötzschke 1993]

Dendritic cells, macrophages, monocytes and B lymphocytes, all bear MHC class II molecules, which are involved in presenting exogenous peptides. After phagocytosis of bacteria, and their subsequent processing in endosomes, short peptides comprised of 12-25 amino acids are associated with MHC II molecules, thus forming a MHC II:peptide complex. The MHC II:peptide complex is then presented on the surface of these specialized antigen-presenting cells (APCs).The exogenous peptides presented by MHC II molecules are derived primarily from bacterial proteins, that are taken up and fragmented into peptides [Watts 1997].

Every lymphocyte has a genotypic surface receptor that binds specifically to its corre-

sponding antigen. After binding to an antigen, IL-2 stimulated lymphocytes proliferate and thus a large group of monoclonal cells is formed. So-called clonal selection takes place in lymphoid organs, such as lymph nodes and the spleen [Burnet et al. 1957]. The T cell arm of the adaptive immune system is known as cell-mediated immunity, while the B cell arm is known as humoral immunity.

### 1.1.1 T Lymphocytes

T cells are a central part of the adaptive immune system, and are involved in cell-mediated immunity. During T cell development, lymphoid progenitors leave the bone marrow and migrate to the thymus. Early committed T cells do not express the T cell receptor (TCR), or either of the costimulatory receptors CD4 or CD8 and are therefore termed double-negatives (DNs). In the later stages of their maturation, double-positive (DP) T cell precursors in the cortex exhibit the TCR, and both CD4 and CD8 molecules on their surface. They then undergo positive selection, which means they are tested for recognition of MHC molecules. In the medulla, negative selection takes place, which is when the T cells are tested for recognition of self-antigens. Any T cells with high affinity for self-antigens undergo apoptosis to prevent them from attacking the host's own cells. Double-positive precursors then differentiate further into either $CD8^+CD4^-$ cytotoxic T cells (CTLs), or $CD4^+CD8^-$ T helper cells, before leaving the thymus. As a result, CD4 and CD8 are mutually exclusive in their expression on T cells in the peripheral blood. Mature T cells reside in the lymph nodes where $CD4^+$ T cells undergo another step of differentiation. Stimulation of $CD4^+$ T cells with interleukin 12 (IL-12), secreted by macrophages, induces differentiation into $TH_1$ cells, whereas stimulation with IL-4 induces differentiation into $TH_2$ cells, and both TGF-$\beta$ and IL-6 mediate the differentiation of $CD4^+$ T cells into $TH_{17}$ cells [Germain 2002]. $TH_1$ cells secrete IFN-$\gamma$ and thereby activate themselves in a positive feedback manner, as well as macrophages and $CD8^+$T cells. IL-4 and IL-10, secreted by $TH_2$ cells, inhibit the activation of $TH_1$ cells. In contrast, $TH_2$ cells secrete IL-4, IL-5, IL-10 and IL-13 and thus recruit eosinophils and induce activation, proliferation and differentiation of B cells. IFN-$\gamma$ secreted by $TH_1$ cells inhibits $TH_2$ cells [Murphy and Reiner 2002].

The CD4 and CD8 costimulatory molecules on T cells play an important role in T cell activation. The TCR on $CD4^+$ T cells recognizes antigen presented by APCs via MHC II. After antigen recognition, $CD4^+$ T cells are activated and support proliferation and differentiation of other leukocytes, either by cytokine release or by direct cell-cell contact. The direct destruction of infected cells by $CD4^+$ T cells is possible, but plays a subordinate role.

In contrast to $CD4^+$ T cells, $CD8^+$T cells work specifically by the direct killing of infected and malignant cells. Endogenous peptides, presented by nucleated cells via MHC class I, are recognized by the TCR on CTLs. After antigen recognition, naive $CD8^+$T cells are activated to become CTLs, which then release perforin and granzyme in order to kill infected or malignant cells. Perforin punches a hole in the membrane of the target cell, allowing the serine protease, granzyme to enter the target cell and induce apoptosis. Another important defense mechanism involves the expression of Fas ligang (CD95L) by T cells, and the subsequent induction of apoptosis in target cells. It is evident from research that the downregulation of MHC I is one of the major mechanisms, that tumor cells use to avoid destruction by the immune system [Garcia-Lora, Algarra, and Garrido 2003].

Another functionally distinct T cell population is the regulatory T cells ($T_{regs}$). $T_{regs}$ are of great importance in controlling autoreactive T cells by the release of anti-inflammatory cytokines, such as IL-10 and TGF-$\beta$, and through direct cell-cell contact. It is believed that $T_{regs}$ could promote tumor growth as they are preferentially found in the tumor environment [H. Y. Wang and R.-F. Wang 2007] [Aggarwal, Das, and Sharma 2016].

All T cells express an antigen receptor, known as the T cell receptor (TCR), with the vast majority having a TCR consisting of an $\alpha$ chain and a $\beta$ chain. A small group of T cells express a TCR that is comprised of a $\gamma$ and a $\delta$ chain, which function as immunoregulatory cells, and can be found particularly in children and patients that have received a bone marrow transplant [W. Haas, Pereira, and Tonegawa 1993].

The TCR is associated with some transmembrane proteins that are essential for its surface expression and signal transduction. The most important of these structures is the CD3 complex, which is comprised of CD3$\gamma$,CD3$\delta$, two chains of CD3$\epsilon$ and two $\zeta$ chains. As the TCR has no intrinsic signalling activity, the CD3 complex is necessary for sig-

nal transduction. In the case of TCR binding, phosphorylation of the immune-receptor tyrosine-based activation motifs (ITAMs) within the CD3 complex eventually leads to delivery of phospholipase C-$\gamma$ (PLC-$\gamma$). Activation of the PLC-$\gamma$, however, requires costimulation of the T cell [Lin and Weiss 2001] [Exley, Terhorst, and Wileman 1991]. Naive T cell activation requires two signals; the first signal is provided by APCs, which present antigens via MHC molecules, and the second signal, also known as costimulatory signal, is given by the interaction of CD28 on naive T cells and B7 (CD80/CD86) on APCs. During antigen presentation, a spatially close contact between T cell and APC is established, which is referred to as the immunological synapse. Certain activating cytokines that are present in high doses are also capable of providing a costimulatory signal [Curtsinger et al. 1999].

The stages of T cell activation can be identified based on the expression of specific surface molecules on these cells. Only a few hours after receiving the stimulatory and costimulatory signals, T cells start to express the early activation marker, CD69. Expression of the C-Lectin, CD69, is sustained for approximately 48 hours, probably providing a signal that keeps the T cells from leaving the lymph node [Cotner et al. 1983] [Shiow et al. 2006]. 24 hours after the primary activation of the T lymphocyte, the late activation marker, CD25, begins to be upregulated. CD25, the alpha chain of the IL-2 receptor, associates with the constitutively expressed $\beta$ (CD122) and $\gamma$ (CD132) chains, thus forming an IL-2 receptor with high affinity. T cells can now leave the lymph nodes and relocate to the site of infection, where they eliminate pathogens. Once the antigen is eliminated, a vast majority of the clonal T cells undergo apoptosis due to lack of stimulatory signals. However, a small subpopulation of the T cells form memory cells that can easily be reactivated in the case of recurring contact with their specific antigen. The resulting memory T cell response is much more potent and faster than the primary adaptive immune response [Sprent and Surh 2002].

Muronomab (OKT3), a monoclonal antibody that is directed against the $\epsilon$ subunit of CD3, was the first antibody to be approved as a therapeutic agent. Binding of muronomab to CD3 leads to the transient activation of T cells, followed by inactivation and apoptosis. Its mode of action made muronomab a candidate for the prevention of transplant rejection. The activation of T cells is a cause of side effects mediated by muronomab,

such as cytokine release and systemic inflammatory reactions, therefore, the application of muronomab must also be accompanied by the application of steroids [Sgro 1995].

### 1.1.2 B Lymphocytes

After antigen recognition by a B cell, it undergoes somatic to refine antigen binding, followed by monoclonal proliferation to produce a large number of identical B cells. The majority of these B cells become short-lived antibody-secreting plasma cells, whereas a small amount of B cells become memory B cells. Memory cells have a long half-life and are capable of secreting monoclonal antibodies for a long period of time after the first antigen-lymphocyte contact took place. Reactivation of existing memory cells is much more rapid than the initial B cell activation, differentiation and proliferation, which creates relative immunity after the first contact or after a vaccination. B cells express surface markers such as CD19, CD20 and CD21, which are crucial for B cell activation and proliferation [Poe, Hasegawa, and Tedder 2001].

### 1.1.3 Antibodies

Antibodies, also known as Immunoglobulins (Ig), are proteins, synthesized by plasma cells, consisting of two light chains ($\kappa$ and $\lambda$) and two heavy chains linked by disulfide bonds. These bonds allow the antibody to form the characteristic Y-shape, which is crucial for their function. In humans, the five different isotypes of immunoglobulins (Ig$\alpha$, Ig$\delta$, Ig$\epsilon$, Ig$\gamma$ ,Ig$\mu$), can be distinguished by their different constant domains and specific properties.

In case of infection, B cells recognize foreign antigens via the B cell receptor (BCR) and are activated. Subsequent BCR-mediated endocytosis leads to processing of the foreign antigen and presentation via MHC class II molecules. A second signal that exerts B cell activation is provided by the interaction of CD40 on B cells with CD40L on $TH_2$ cells. As a result, the B cell undergoes activation, affinity maturation, and antibody production. The first antibody isotype to be produced during the initial humoral immune response is Ig$\mu$. IL-10, IL-4 and CD40L provide signals that then induce an isotype switch from Ig$\mu$ to Ig$\gamma$ [Malisan et al. 1996] [Allen et al. 1993].

Ig$\alpha$ provides immunity in the mucous membranes, whereas Ig$\gamma$ and Ig$\mu$ are preferentially found in the blood. In contrast to the pentameric Ig$\mu$, the monomeric Ig$\gamma$ can easily extravasate towards sites of injury or infection. The latter is a desired feature, which makes Ig$\gamma$ the most auspicious isotype for therapeutic antibodies. Ig$\gamma$ is a monomer and has a molecular weight of approximately 150kDa. Furthermore, intact Ig$\gamma$ interacts with FcRn, which prolongs the immunoglobulin's half-life significantly [Roopenian and Akilesh 2007].

The antigen-binding, or Fab, site of an antibody is comprised of the variable domains of both the light and heavy chains. The complementarity-determining regions (CDR), which consist of short amino acid sequences, form a paratope that is able to bind specifically to the epitope of an antigen. The CDR determines the antigen specificity of an antibody, and its high variability allows the formation of a vast range of human antigen-binding sites. Possible epitopes include proteins, glycoproteins, and saccharides. The crystallizable, or Fc, part of an antibody is non-variable and binds to Fc receptors on different immune cells.

Physiologically, antibodies bind to pathogens, such as bacteria and viruses, and are able to neutralize pathogens, or at least mark them as exogenous. In addition, antibodies recruit and activate immune effector cells in a direct or indirect mode of action.

Direct antibody effects are mediated independently of Fc receptor interactions, and are therefore not isotype-specific. This suggests that F(ab) or $F(ab)_2$fragments, or any other format bearing an F(ab) part, can sufficiently induce direct antibody effects, such as inhibition of ligand-receptor interactions, antagonistic, or agonistic properties. Antibodies directed against a distinct growth receptor can impede binding of its ligand, thus preventing a tumor growth signal. Antagonistic antibodies have similar properties when directed against growth receptors. Agonistic antibodies can provide apoptotic signals to tumor cells via the CD95 pathway. Aside from that, activation of immune effector cells through binding of agonistic antibodies (e.g. to CD3) is also feasible.

Indirect properties of antibodies are Fc receptor dependent, and therefore isotype-specific. Fc dependent mechanisms include complement activation via the classical pathway, as well as the recruitment and activation of immune effector cells. Complement activation is restricted to target sites where immobilized Ig$\gamma$ or Ig$\mu$ molecules are present.

This prerequisite is needed to avoid complement activation by soluble antibodies. Complement-dependent cytotoxicity (CDC) is induced by binding of the soluble C1q protein to the Fc region ($C_{H2}$ domain) of at least one Ig$\mu$ or two Ig$\gamma$ molecules. As a result, the complement cascade is initiated, leading to the opsonization of the antigen by C3b/C4b, chemotaxis, and formation of the membrane attack complex (MAC). The immune effects that are induced by binding of complement proteins to antibodies, and culminate in death of the target cell, are termed complement-dependent cellular cytotoxicity (CDCC).

Recruitment and activation of immune effector cells are dependent on the interaction of the Fc$\gamma$ regions with specific Fc$\gamma$ receptors. Five different types of Fc$\gamma$ receptors have been characterized, four of which (Fc$\gamma$RI, Fc$\gamma$RIIA, Fc$\gamma$RIIIA, Fc$\gamma$RIIIB) have activating properties, whereas binding of Ig$\gamma$ to the fifth, Fc$\gamma$RIIB, leads to the inhibition of cellular activity. To ensure a well-balanced immune response, most immune cells bear both activating and inhibiting Fc$\gamma$ receptors. $\alpha\beta$T cells do not express Fc$\gamma$ receptors, whereas B cells only express the inhibitory Fc$\gamma$RIIB. In contrast, NK cells solely express activating Fc$\gamma$RIII, which makes them the most important effector cells in antibody-dependent cellular cytotoxicity (ADCC). After binding of immobilized Ig$\gamma$ to Fc$\gamma$RIII on NK cells the latter undergoes multimerization which leads to the activation of the NK cell. As a result, NK cells release perforine and granzyme, and eventually lyse the target cell. It has been reported that other FcR-bearing cells are capable of inducing ADCC, too. For example, $\gamma\delta$T cells exhibit Fc$\gamma$RIIIA on their surface and are thus able to induce ADCC *in vitro* [Lafont et al. 2001] [Fisher et al. 2015], and although the exact mechanism remains unclear, neutrophils have been shown to exert ADCC, too [Hernandez-Ilizaliturri et al. 2003] [Egmond and Bakema 2013].

In addition, phagocytosis of Ig$\gamma$1-coated target cells by macrophages has been observed *in vivo*, and Fc$\gamma$RIIA seems to play a major role in antibody-dependent cellular phagocytosis (ADCP) [Oflazoglu et al. 2009].

### 1.1.4 Leukocyte Adhesion

Chemoattractant cytokines (Chemokines) are small proteins that mediate chemotaxis, which is the directed migration of immune effector cells. Chemokines mainly act in a

paracrine manner and allure monocytes, neutrophils and lymphocytes towards the site of infection [Zlotnik and Yoshie 2000].

Leukocyte migration is a multistep process that always works in a similar pattern, composed of three stages; chemoattraction, adhesion, and diapedesis [Springer et al. 1994]. At first, innate immune cells, such as dendritic cells and macrophages, release TNF-$\alpha$, IL-1$\beta$, and other cytokines in response to antigen contact. Endothelial cells in close proximity to the source of the chemokines then express selectins. First, preformed P-selectin (CD62P) from Weibel-Palade bodies is externalized and expressed on the surface of endothelial cells. Shortly after, endothelial cells translate E-selectin (CD62E), and present it on the surface, thus replacing P-selectin. Selectins are adhesion molecules expressed by activated endothelium that bind to saccharide derivatives on leukocytes, such as Sialyl-Lewis$^x$ and P-selectin glycoprotein ligand 1 (PSGL-1, CD162). Both P-selectin and E-selectin are crucial for leukocyte adhesion. Triggered by weak selectin-carbohydrate interactions, passing leukocytes adhere to endothelial cells. However, these bonds are constantly interrupted by the shearing forces of blood flow. Ultimately, leukocytes end up rolling along the endothelium.

The following step of leukocyte migration depends on strong interactions between heterodimeric integrins on leukocytes, and monomeric intercellular adhesion molecules (ICAMs) on endothelial cells. An integrin molecule consists of an $\alpha$ chain and a $\beta$ chain, with multiple types of both $\alpha$ and $\beta$ chains being described. A distinct subfamily of integrins shares a $\beta$ chain, which is associated with different $\alpha$ chains. Among the most important integrins necessary for leukocyte migration are leukocyte function antigen 1 (LFA-1, CD11a:CD18 or $\alpha_L\beta_2$) and macrophage 1-antigen (Mac-1, CR3, CD11b:CD18 or $\alpha_M\beta_2$). LFA-1 and Mac-1 are expressed on all innate immune cells, while LFA-1 is additionally expressed on T cells.

ICAMs are membrane-bound molecules that belong to the immunoglobulin superfamily. Both ICAM-1 (CD54) and ICAM-2 (CD102) bind to LFA-1 and Mac-1, with ICAM-2 being more important for extravasation of immune cells through unactivated endothelium. In addition to endothelial cells, ICAM-1 is expressed on activated leukocytes, and ICAM-2 is expressed on dendritic cells [Fougerolles et al. 1991].

After leukocyte rolling, activating cytokines induce an upregulation of ICAM-1 on en-

dothelial cells and conformational changes within LFA-1 on leukocytes. On non-activated T cells, LFA-1 appears in a flexed state that has only low affinity to ICAM-1 [Kim, Carman, and Springer 2003]. TNF-$\alpha$ and chemokines such as CXCL8 in particular, induce a cytoskeletal transformation of leukocytes that mediates affinity state changes of LFA-1 towards an intermediate-affinity state in an extension shape [Salas et al. 2006]. It has been shown that activation of the T cell receptor and subsequent CD3 signal transduction induces similar affinity changes within the LFA-1 molecule [Dustin and Springer 1989] [Van Kooyk et al. 1989].

This in turn allows ICAM-1 to bind to LFA-1 with a 10,000-fold affinity, compared to the flexed state and thus induces another conformational change towards a high-affinity state [Shimaoka et al. 2003]. The resulting bond between LFA-1 and ICAM-1 is firm and allows the leukocyte to start diapedesis, which involves the migration of a leukocyte between endothelial cells.

## 1.2 Cancer immunotherapy

In 2000, Hanahan and Weinberg proposed six hallmarks that are shared by all malignant cells. These include (1) self-sufficiency in growth signals, (2) insensitivity to anti-growth signals (3) evading apoptosis, (4) limitless replicative potential, (5) sustained angiogenesis, and (6) tissue invasion and metastasis. This publication became one of the most cited cancer research papers ever [Hanahan and Weinberg 2000], and 11 years later, the same authors published an updated version of the hallmarks, adding two new features: (7) abnormal metabolic pathways (8) evading the immune system [Hanahan and Weinberg 2011].

Indeed, immune evasion by tumors and immune recognition by cellular and humoral factors has become a major area of research in recent years. According to the observation of the cancer papers published since then, it can be stated that cancer immunology and immunotherapy is one of the fastest-evolving research fields. There are multiple approaches that all intend to redirect immune responses against tumor cells, most of which involve T cells because of their adaptive potential. Strategies include HLA-presented peptide vaccinations, chimeric antigen receptors (CAR), cytokine therapy, and *in vitro* priming of

autologous immune cells. The most popular category of therapeutic agent is the antibody. In fact, a vast majority of approved immunological cancer treatments involve antibodies.

### 1.2.1 Therapeutic Antibodies for Cancer Therapy

In the early 1880s Emil von Behring discovered that the serum of mice, immunized with diphtheria bacteria can provide a short-term immunity to non-immunized individuals [Von Behring 1890]. At this time, the causative agent to provide humoral immunity was not yet known. Paul Ehrlich, founder of the concept of chemotherapy, had a vision in which magic bullets could bind specifically to malignant or infected cells, and thus destroy them. His dream became a reality less than 100 years later.

The evolution of cancer immunotherapy with antibodies was made possible because of three milestones in antibody research: identification of antibody structure, hybridoma technology, and genetic modification of antibodies. The first step to selective cancer therapy was the elucidation of antibody structure. In 1958, Rodney Porter used papain for the digestion of immunoglobulins, producing three parts of approximately 50 kDa, with two of them being identical Fab parts that bind to the same antigen, and the third being an inert Fc part [Porter 1963] [Porter 1959]. Only three years later, Alfred Nisonoff showed that the digestion of immunglobulins with pepsin creates two identical Fab fragments. Furthermore, he created the first bispecific antibody through chemical linkage of two Fab fragments [Nisonoff and Rivers 1961].

Nevertheless, production of antibodies with predefined specificity remained a formidable challenge that was successfully met in 1975, when Georges Köhler and Cesar Milstein published a paper entitled: continuous cultures of fused cells secreting antibody of predefined specificity [Köhler and Milstein 1975]. This publication marked the birth of modern antibody technology.

In the following years, multiple attempts to produce antibodies for therapeutic application were made. Most of the antibodies generated, however, were mouse antibodies and therefore immunogenic in humans. In fact, these xenogenic antibodies had a short serum half-life and provoked undesired immune responses [Bertram et al. 1986]. Despite these disadvantages, the mouse anti-CD3 antibody Muromonab was the first approved

monoclonal antibody for therapeutic purposes.

To reduce the immunogenicity of mouse antibodies, non-binding regions were later genetically modified. Thus, the first chimeric (-ximab) and humanized(-zumab) antibodies were produced, in which the constant domains of the antibody were exchanged with human immunoglobulin sequences, while the mouse antigen-binding regions were not altered. Murine, chimeric, humanized, and human antibodies are depicted in Fig. 1.1.

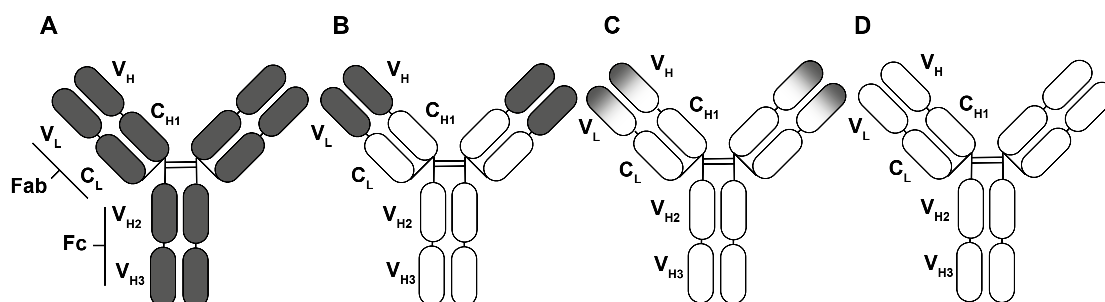


**Figure 1.1:** ***Murine, chimeric, humanized, and human Ig$\gamma$ antibodies A*** *Fully murine Ig$\gamma$ antibody.* ***B*** *Chimeric antibody comprised of murine variable domains and human constant domains.* ***C*** *Humanized antibody with murine CDRs.* ***D*** *Fully human Ig$\gamma$ antibody.*

Besides their reduced immunogenicity, humanized antibodies also interact more efficiently with human Fc$\gamma$ receptors, which is another desired feature. Several preclinical and clinical trials were conducted in the early 1990s to identify promising target antigens on tumor cells, but most of them did not meet the expectations.

Therapeutic antibodies exert their activity by different modes of action, but by far the most important mode of action is ADCC, which is induced by interaction of Ig$\gamma$ with the Fc$\gamma$RIIIA on NK cells. Some antibodies work by blocking the growth receptors on tumor cells, while others work by the inactivation of soluble molecules that amplify tumor growth or angiogenesis. A fourth mode of action is the blockade of check-point molecules on T cells in order to support the anti-tumor activity of those cells.

**ADCC.** The first anti-tumor antibody ever approved was Rituximab (MabThera®), in 1998, a chimeric mouse/human anti-CD20 antibody for the treatment of B-cell lymphomas [Maloney et al. 1997]. Rituximab combines multiple modes of action and thus creates a potent anti-tumor response *in vivo*. The most important mode of action

provided by Rituximab is antibody-dependent cell cytotoxicity (ADCC). Furthermore, complement-dependent cytotoxicity is also induced [Maloney et al. 1997]. Nowadays, Rituximab is part of major drug regimens for the treatment of lymphomas.

**Blocking of growth receptors.** In 2000, trastuzumab (Herceptin®) was approved for the treatment of Her2-positive breast cancer. Trastuzumab, a fully humanized antibody, binds to Her2 (Erb2), which is a growth factor receptor expressed on breast cancer cells. Binding of the Her2 receptor provides a growth-stimulating signal to the tumor cells, so the binding of an anti-Her2 antibody, means that the tumor cells lack growth signals and are thus more susceptible to chemotherapy. As with any therapeutic antibody with an intact Fc binding region, trastuzumab is capable of inducing ADCC [Slikowski et al. 1999].

**Antiangiogenic therapy.** Many kinds of tumor cells can provide vasculo-proliferative signals to endothelial cells. This is to ensure a decent oxygen supply to all tumor cells. On the one hand, the ability to induce neo-vasculaturization is one of the main malignancy criteria, whereas on the other hand, researchers have found it to be another target for anti-tumor therapy. In the late 1990s, vascular endothelial growth factor (VEGF) was characterized as a soluble factor secreted by tumor cells that is capable of inducing proliferation of vessels in the tumor environment. In 2005, Bevacizumab (Avastin®), a humanized anti-VEGF antibody was introduced and approved for the treatment of metastasized colon cancer [Willett et al. 2004].

**Checkpoint blockade.** A more recently developed approach for antibody-mediated immunotherapy is checkpoint blockade, which does not involve the interaction of antibodies with Fc receptors. As mentioned earlier, binding of CD28 on T cells to B7 on APCs provides a second activation signal to T cells, which leads to proliferation. In contrast, binding of CTLA-4 on T cells to B7 inhibits T cell proliferation. Therefore, blocking antibodies directed against CTLA-4 are able to prolong and reinforce T cell activation. Since the interaction of CTLA-4 and CD28 marks a checkpoint in the T cell immune cascade, anti-CTLA-4 antibodies are called checkpoint inhibitors. Ipilimumab(Yervoy®), was the

first checkpoint inhibitor, and was launched in 2014 as a first line therapy for metastasized melanoma.

Another immune checkpoint includes the interaction of programmed death-receptor 1 (PD-1) with its ligand. Physiologically, interaction of PD-1 on APCs with PDL-1 on T cells prevents overwhelming immune reactions. However, tumor cells with a high concentration of PDL-1 on the cell surface can create an immunosuppressive tumor environment, which promotes tumor growth. Therefore, antibody-mediated blocking of PD-1, enhances anti-tumor activity of T cells. In 2015, Pembrolizumab (Keytruda®), a humanized anti-PD-1 antibody, was approved for the treatment of metastasized melanoma.

It was the success of checkpoint inhibitors, among other developments, that prompted Science to call cancer immunotherapy the most important breakthrough of the year 2013. This happened because of the therapeutic goals reached by checkpoint inhibitors, such as anti-CTLA4 or PD-1 antibodies for the treatment of melanoma. Today, antibody therapy is still a promising field, with antibodies and their related products being the fastest growing class of therapeutic agents [Beck et al. 2010].

**Table 1.1:** *Selected monoclonal anti-tumor antibodies*

| Antibody | Approval | Target | Cancer type | Mode of action |
|---|---|---|---|---|
| Alemtuzumab | 2002 | CD52 | CLL | ADCC, CDC, apoptosis |
| Bevacizumab | 2005 | VEGF | Colorectal | Antivascular |
| Cetuximab | 2004 | EGFR | Colorectal, Head & neck | Anti-growth factor, ADCC |
| Daratumumab | pending | CD38 | Multiple Myeloma | ADCC, CDC |
| Ipilimumab | 2011 | CTLA-4 | Melanoma | Checkpoint inhibitor |
| Pembrolizumab | 2015 | PD-1 | Melanoma and SCC | Checkpoint inhibitor |
| Rituximab | 1998 | CD20 | NHL | ADCC,CDC, apoptosis |
| Trastuzumab | 2000 | HER2 | Breast | ADCC,Anti-growth factor |

### 1.2.2 Bispecific Antibodies in Cancer Therapy

Monospecific antibodies are capable of inducing ADCC and thus promote the killing of tumor cells by NK cells. However, T cells lack Fcy receptors that allow cells to bind to the Fc portion of Immunoglobulins. Therefore, monospecific antibodies cannot activate T cells, which are known to be the most potent immune cells.

In the 1980s, scientists developed bispecific monoclonal antibodies (BsMAb) [1], that were able to promote tumor cell killing by T cells, however, there were three prerequisites that must be met. First, the antibody has to have two binding sites directed against a tumor-associated antigen (TAA) and a T cell antigen, thus mediating a spatially close contact between the T cell and the target cell. Second, the T cell binding site must provide a mitogenic signal to ensure on-target T cell activation. For example, binding of specific antibodies to the TCR/CD3 complex provides a strong and persistent activation signal to T cells. Finally, T cell activation must be limited to the tumor site. There should be no off-target activation, this prerequisite is referred to as target cell restriction.

In 1985, Staerz et al. showed that monoclonal antibodies directed against the T cell receptor have a mitogenic effect on CTL clones when coupled to different cell types. Furthermore, they coupled two monoclonal antibodies with SDPD, a heterobifunctional crosslinker. One of the two monoclonal antibodies was directed against the Thy-1.1 alloantigen on leukemic cells and another one was directed against the TCR (T1). The resulting full-size antibody heteroconjugates - as they called them - induced a highly efficient CTL response *in vitro*. Specific lysis of $TAA^+$ tumor cells by mouse CTLs was observed [Staerz, Kanagawa, and Bevan 1985].

In the same year, Perez et al. created heteroconjugates composed of an anti-human CD3 binding site (OKT3), and an anti-murine $K^k$ alloantigen binding site. Four different anti-HLA-DPw2 CTL clones were used as effector cells against murine $K^{k+}$ tumor cells. The formation of multicellular tumor-CTL conjugates and subsequent target cell lysis was observed. As human CTL clones showed efficient lysis of xenogenic murine tumor cells, it became clear that CD3 targeting with bispecific antibodies is independent of MHC presentation. Perez et al. suggested the use of different conjugates containing Fc regions to activate lymphocytes as well as NK cells and macrophages [Perez et al. 1985].

Only two years later, Staerz et al. showed the effective lysis of influenza virus-infected cells *in vivo* after the application of a bispecific antibody, composed of anti-TCR and anti-hemagglutinine binding sites [Staerz, Yewdell, and Bevan 1987].

[1] In the context of this thesis, the abbreviation BsMAb(Bispecific monoclonal antibody) - if not otherwise specified - refers to bispecific antibodies with a CD3/TCR stimulating binding site and another binding site directed against a tumor associated antigen.

In 1986, Jung et al. showed that not only mouse CTL clones, but also human peripheral blood mononuclear cells (PBMCs) can be activated by hybrid antibodies. As a result, human Raji and M21 tumor cells coated with antibodies were killed effectively by PBMCs preincubated with OKT3. Lysis could be stopped by the excess addition of the monoclonal antibody, OKT3. The bispecific antibodies that Jung et al. used were composed of the monoclonal anti-CD3 antibody OKT3 and different antibodies directed against TAA. Chemical linkage with SPDP was used to generate the hybrid antibodies [Jung, Honsik, et al. 1986].

In 1988, Jung et al. suggested that it may be preferable to use F(ab')$_2$ fragments rather than intact antibodies to rule out any effects mediated by binding of conjugates to Fc$\gamma$ receptors *in vivo* [Jung and Müller Eberhard 1988] [Jung, Freimann, et al. 1991].

As an alternative to chemical linkage, generation of bispecific antibodies can be realized by the fusion of two different hybridoma cell lines. The resulting cell line secretes both pristine monospecific antibodies, as well as bispecific antibodies [Staerz and Bevan 1986].

In the years that followed, advances in recombinant DNA technology allowed the generation and improved production of bispecific antibody molecules. On the one hand, humanization and miniaturization of bispecific antibodies reduced the immunogenicity markedly. However, on the other hand, recombinant DNA technology has allowed the generation of engineered antibodies in new formats.

An important building block for the generation of recombinant BsMAbs, is the so-called single chain fragment (scF$_v$). A scF$_v$ is composed of the V$_H$ and the V$_H$ domain of an antibody, with both of them being included in a single polypeptide chain and joined with a peptide linker. Combination of two or more scF$_v$, allows the generation of bi-, tri- and multispecific antibodies. Linkage of two scF$_v$ in one polypeptide chain results in formation of the bispecific single chain scF$_v$ format (bssc, also termed bispecific T-cell engager (BiTE)), whereas heterodimerization of two scF$_v$ peptide chains creates a BsMAb in the single chain diabody (scDiabody) format [Holliger, Prospero, and Winter 1993] [Mack, Riethmüller, and Kufer 1995]. Different bispecific antibody formats are depicted in Fig. 1.2.

BsMAb in both formats are small (approximately 55 kDa) and have a very short serum

half-life due to impaired interaction with Fc$\gamma$Rn [Müller and Kontermann 2010]. BsMAb in formats that resemble the structure of Ig$\gamma$ more closely could exhibit a longer serum half-life. Durben et al. established a FLT3$\times$CD3 BsMAb in a new Fabsc format [Durben et al. 2015].

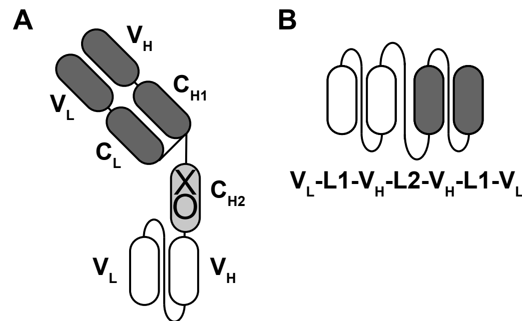


**Figure 1.2:** ***Bispecific antibody formats*** ***A*** *Fabsc format. The antibody is comprised of a Fab part derived from an antibody directed against a TAA and a single chain fragment derived from an antibody directed against CD3. X indicates replacement of the amino acid cystein in order to avoid dimerization. Replacement of several amino acid residues in the CH2 domain in order to prevent binding to Fc receptors is depicted as O.* ***B*** *Bssc/BiTe format. The single chain antibody is comprised of two $V_L$ and two $V_H$ parts derived from two antibodies, with one of them being directed against a TAA and the other against CD3. L1 indicates a glycine-serine linker, and L2 indicates a amino acid sequence derived from the elbow fragment of human Ig$\gamma$1.*

### 1.2.3 Characteristics of Bispecific PSMA x CD3 Antibody NP-CU

All experiments included in this thesis were conducted using a bispecific PSMA$\times$CD3 antibody (NP-CU) in a new recombinant Fabsc format. NP-CU was generated and characterized by Dipl.-biol. Fabian Vogt at the Department of Immunology in Tübingen. The structure of Fabsc constructs is more similar to the mutual Ig$\gamma$ structure than the established bssc format. It consists of a Fab region, a linker CH2 domain with genetic modifications to impair FcR-binding, and a single chain antibody with a second specificity. In contrast to the Fab-derived format described by Schoonjans et al. the Fabsc-format described here contains a CH2-domain separating the two specificities in order to reduce undesirable interactions between them [Durben et al. 2015] [Schoonjans et al. 2000].

The PSMAxCD3 Fabsc antibody was composed of the PSMA-antibody J591 (Fab) and the CD3-antibody UCHT1 (single chain) [H. Liu et al. 1997] [Beverley and Callard 1981].

### 1.2.4 Current Issues in Bispecific Antibody Therapy

#### Dose Limitation

Recently, the bispecific CD19xCD3 antibody Blinatumomab was approved for the treatment of $CD19^+$ acute lymphoblastic lymphoma (ALL) after showing efficacy in killing tumor cells [R. Bargou et al. 2008]. Blinatumomab is applied as a continuous infusion due to its short serum half-life of approximately 2.11 hours. The suggested daily dose for patients who weigh at least 45kg is 9$\mu$g/day on days 1-7 and 28$\mu$g/day on days 8-28. At these clinical doses for the treatment of relapsed/refractory ALL, the mean steady-state serum concentration was 211 pg/mL and 621 pg/mL, respectively [AMGEN 2015].

Notably, this concentration is 10,000 fold lower than those achieved with monospecific antibodies [Adams and Weiner 2005].

One should recognize that this exceptionally low serum concentration does not provide saturation of the target antigen at the tumor cell surface.Dense coating of tumor cells by bispecific antibodies can effectively induce tumor cell killing, therefore, it is believed that higher concentrations would greatly enhance the anti-tumor effect of bispecific antibodies. In fact, it is widely held that serum concentrations of the BsMAb blinatumomab are suboptimal.

However, severe dose-dependent side effects seen in clinical studies do not allow the application of higher doses [Topp et al. 2011] [Klinger et al. 2012]. Two major problems have been observed repeatedly in clinical trials with BsMAb: Lymphocyte drop, and cytokine release syndrome, with the latter being more severe and thus life-threatening.

#### Cytokine Release Syndrome after treatment with BsMAbs

Severe systemic side effects upon the clinical application of bispecific antibodies have been observed during previous clinical trials.

For example, in 1994, Kroesen et al. published a phase 1 study of intravenously applied bispecific antibody in renal cell cancer patients (n=14). The antibody used in this study was a (Fab')$_2$ bispecific antibody that was generated by hybrid-hybridoma technology. The resulting quadroma, produced a fusion protein composed of an anti-CD3 antibody (Ig$\gamma$3) and an anti-epithelial glycoprotein 2 (EGP2) antibody (Ig$\gamma$1). All patients had received prior subcutaneous IL-2 treatment that was maintained during the clinical trial. During the clinical trial, patients received BIS-1 (Fab')$_2$ at doses of 1, 3, and 5 $\mu$g per kg body weight. Acute toxicity was observed in 2 out of 4, and 5 out of 5 patients that received 3 and 5 $\mu$g per kg, respectively. The observed symptoms included chills, peripheral vasoconstriction with increased diastolic tension, dyspnea, and fever. The maximal tolerated dose (MTD) was 5 $\mu$g per kg. Patients who received BIS-1 at 5 $\mu$g per kg were tested for elevated TNF-$\alpha$ and IFN-$\gamma$ levels. In fact, TNF-$\alpha$ production increased up to 180 pg/ml and IFN-$\gamma$ levels reached 12 U/ml. Peak levels of TNF-$\alpha$ and IFN-$\gamma$ were observed 2 and 3 hours after the start of the application. After 24 hours, cytokine levels had returned to normal [Kroesen et al. 1994].

In 1993, Tibben et al. conducted a study of intravenously applied BsMAb in ovarian carcinoma patients (n=5). The tested antibody was a (Fab')$_2$ BsMAb named OC/TR. OC/TR consists of two Ig$\gamma$1 fragments, with binding sites to CD3 and a tumor-associated folate-binding protein. The BsMAb was obtained after fusion of the MOv18 hybridoma with spleen cells from a BALB/c mouse, immunized with a human T cell clone. During the clinical trial, the first patient received an intravenous infusion of 1mg BsMAb. Unfortunately, the patient observed grade 3-4 adverse events including headache, fever with hypotension, fatigue, chills, nausea, vomiting, and diarrhea [J. Tibben et al. 1993]. Due to this toxicity, patients 2-4 were treated at doses of 0.1 and 0.2 mg, however, similar adverse events were observed, and symptoms had to be treated with paracetamol and metoclopramide, resulting in the termination of the study.

Cytokine analysis showed increased levels of IL-2, TNF-$\alpha$ and IFN-$\gamma$ in the patient's serum. Whereas elevated serum levels of IL-2 were only observed in patients that received higher doses (1mg or 0.2mg), all patients showed high levels of TNF-$\alpha$ and IFN-$\gamma$. Peak levels of all cytokines were observed 2 hours after infusion, but they normalized at 24h after infusion. Serum levels of IL-2 and TNF-$\alpha$ seemed to correlate well with clinical

symptoms [J. G. Tibben et al. 1996].

In 2011, an open-label, multicenter, single-arm, phase 2 clinical study with the bssc CD3×CD19 BsMAb blinatumomab(MT103) was conducted, including 21 patients suffering from minimal residual (MRD) B-lineage ALL. Blinatumomab was applied as a continuous intravenous infusion for four weeks at a dose of $15g/m^2/24$ hours. The resulting serum concentration was 0.6 ng/ml. This exceptionally low dose makes the therapeutic effect all the more remarkable. During this trial, transient peaks of cytokines could be observed in the majority of patients. Cytokine release started 2 hours after the first infusion, and was limited to 48 hours. Out of 21 patients, six patients showed an increase in IL-6 plasma levels only, four patients in IL-6 and IFN levels, two patients in IL-6, IL-2 and IFN levels, and one patient in IL-6, IFN and TNF-$\alpha$ levels. Despite the very low antibody serum levels, patients experienced fever, chills, decrease in blood immunoglobulin, and hypokalemia [Topp et al. 2011].

The *in-vivo* administration of T cell recruiting bispecific antibodies led to cytokine release in past experiments. It is believed that off-target effects play a mayor role in the development of cytokine release syndromes.

Off-target activation can be induced via two different mechanisms: (1) The tumor-associated antigen (TAA) is not entirely tumor specific. Thus, the BsMAb binds to healthy cells expressing TAA. Subsequent T cell binding and activation leads to cytokine release and killing of TAA$^+$cells. This, however, is not really off-target activation in a strict sense, as it is still induced by TAA$^+$cells. However, the depleted cells are not tumor cells and therefore 'off-target' means 'off-tumor' here. Blinatumomab obviously suffers from this kind of off-target T cell activation, since the target antigen, CD19 recognized by this molecule is not only expressed on human cells of the B-ALL lineage, but also on normal B cells.

(2) Binding of BsMAb via its CD3 binding site leads to T cell activation, independent of target cells. This means, BsMAb-induced T cell activation is not - as it should be - target cell restricted. This type of off-target activation takes place in the absence of TAA$^+$cells.

Off-target T cell activation, upon application of BsMAb may promote cytokine release that significantly limits safely applicable doses. It is therefore of utmost importance to

understand and prevent this side effect.

Another phenomenon that was repeatedly observed upon application of BsMAb was a drop in lymphocyte counts within the peripheral blood.

### Lymphocyte drop

Conductors of a phase 1 study of intravenously applied bispecific antibody in renal cell cancer patients, observed lymphopenia in various patients. They described a rapid reduction in the number of PBMCs in the peripheral blood during the infusion of 5 $\mu$g per kg BsMAb. Whereas the number of granulocytes showed no consistent changes, monocyte and lymphocyte counts were significantly reduced, two hours after the start of the infusion.

Lymphocyte subset analysis showed a preferential reduction in the percentage of LFA-la-bright and HLA-DR$^+$ cells within the CD8$^+$ lymphocyte population [Kroesen et al. 1994].

Another clinical study with ovarian cancer patients showed similar effects. Approximately 30 minutes after infusion, lymphocyte counts in almost all patients dropped. However, they showed a full recovery, when they were analyzed 48 hours later [J. G. Tibben et al. 1996].

During a phase 2 clinical trial in 2011, blinatumomab was applied to 21 ALL patients as a continuous intravenous infusion for four weeks at a dose of $15g/m_2/24$ hours. The resulting serum concentration was 0.6 ng/ml.

Eleven out of the 21 patients experienced grade 3 or 4 adverse events, with lymphopenia being the most frequent event (7 out of 21 patients). However, as with the previous study, every case of lymphopenia resolved within 48 hours. Granulocytopenia and thrombocytopenia were also observed, but only in one patient [Topp et al. 2011].

Clinical studies performed since the 1990s have shown a transient lymphocyte drop after administration of a T cell-activating bispecific antibody. However, the lymphocyte count was restored after a few hours, suggesting a transient adhesion of T cells to the endothelium and possible migration.

Transient lymphopenia by itself does not seem to be a severe side effect, however, lymphopenia could play an important role in the early development of cytokine release,

since T cell adhesion promotes T cell activation [Hynes 1992]. It is possible that blocking T cell adhesion could inhibit overwhelming cytokine release, thus allowing application of BsMAb at higher dose levels.

### The Link Between Cytokine Release and Lymphocyte Drop

Molema et al. observed both cytokine release and lymphocyte drop during their clinical study with bispecific antibodies [Kroesen et al. 1994]. *In-vitro* studies showed that the application of bispecific antibodies leads to the upregulation of ICAM-1 and other adhesion molecules on endothelial cells, which results in an increased adhesion of T cells to endothelial cells [Molema et al. 2000]. As mentioned previously, binding of LFA-1 (CD18:CD11a) to ICAM-1 (CD54) induces mitogenic signals in T cells. This suggests that increased adhesion of T cells, as observed in patients after application of bispecific antibodies, contributes to off-target T cell activation.

### Prevention of Cytokine Release Syndrome

Prophylactic administration of steroids before the application of bispecific antibodies is performed to prevent cytokine release [Brandl et al. 2007] [Mori et al. 2015] [AMGEN 2015]. However, corticosteroids are one of the most potent immunoinhibitory drugs in humans, and work through a variety of different mechanisms [Claman 1972]. It is possible that steroids diminish the therapeutic potential of bispecific antibodies.

## 1.3 Aim and Objective of this Thesis

Severe systemic side effects after administration of T cell-activating bispecific antibodies are well-known phenomena. Maximal tolerated doses of recently approved bispecific antibodies remain sub-optimal in terms of saturation of the tumor-associated antigen. In contrast to the importance of this phenomenon, only a few research projects have been conducted in an attempt to fully understand the involved mechanisms. The work described in this thesis aims at a better understanding of bispecific antibody-mediated off-target T cell activation. To this end, the costimulatory effects of endothelial cells (HUVECs), and some certain lymphoid cells, designated stimulating bystander cells (SBCs), have been further explored. A special focus lies in the characterization of molecules mediating the interaction of T cells and SBCs with the intention to interfere with this interaction, such that undesired off-target T cell activation is diminished.

# 2 Material and Methods

## 2.1 Material

### 2.1.1 Equipment

| | |
|---|---|
| Cell harvester | Inotech Biosystems International, Inc. Rockville, USA |
| Centrifuge 5417C | Eppendorf AG, Hamburg, GER |
| Centrifuge Heraeus Biofuge fresco | Thermo Electron, Waltham, USA |
| Centrifuge Heraeus Megafuge 1.0 | Thermo Electron, Waltham, USA |
| Centrifuge Sorvall RC5C Plus | Thermo Fisher Scientific GmbH, Schwerte, GER |
| Clean bench 3F120-II GS | Integra Biosciences GmbH, Fernwald, GER |
| Clean bench 3F150-II GS | Integra Biosciences GmbH, Fernwald, GER |
| ELISA reader Spectra Max 340 | Molecular devices, Ismaning, GER |
| FACS Calibur | BD Biosciences, Heidelberg, GER |
| FACS Canto II | BD Biosciences, Heidelberg, GER |
| Freezer Colora E80 | Colora Messtechnik GmbH, Lorch, GER |
| Gammacell 1000 Elite | MDS Nordion, Ottawa, CAN |
| Incubator APT.line CD(E6) | Binder GmbH, Tuttlingen |
| Incubator Heraeus function line | Thermo Electron, Waltham, USA |
| MicroBeta$^2$ Counter | Perkin Elmer, Rodgau, GER |
| Microscope Zeiss IM 35 | Carl Zeiss AG, Oberkochen, GER |
| Microscope Axiovert 25 | Carl Zeiss AG, Oberkochen, GER |
| Multichannel pipette | Abimed GmbH, Langenfeld, GER |
| Nanodrop™1000 | Thermo Fisher Scientific GmbH, Schwerte, GER |
| pH-meter Seven Multi | Mettler-Toledo GmbH, Giessen, GER |
| Pipetboy acu | Integra Biosciences GmbH, Fernwald, GER |
| Pipettes DV1000, DV200, DV100, DV20, DV10, DV2 | Abimed GmbH, Langenfeld, GER |

| | |
|---|---|
| Refrigerator Liebherr Premium | Liebherr Hausgeräte GmbH, Ochsenhausen, GER |
| Scale PT600 | Sartorius AG , Göttingen, GER |
| Vortex VF2 | IKA-Werke GmbH& Co. KG, Staufen, GER |
| Water bath Julao 19 | Julabo Labortechnik GmbH, Seelbach, GER |
| xCELLigence RTCA | Roche Diagnostics GmbH, Mannheim, GER |

### 2.1.2 Glass and plastic supplies

| | |
|---|---|
| 15ml Falcon | BD Biosciences, Heidelberg, GER |
| 50ml Falcon | Greiner Bio One, Frickenhausen, GER |
| Cannulas Microlance 3 | BD Biosciences, Heidelberg, GER |
| Cell Culture Flasks 25 $cm^2$, 75 $cm^2$, 175 $cm^2$ | Greiner Bio One, Frickenhausen, GER |
| Cell culture plates 6 well | BD Biosciences, Heidelberg, GER |
| Cell culture plates 96 well | Greiner Bio One, Frickenhausen, GER |
| Cell strainer 40um | BD Biosciences, Heidelberg, GER |
| Centrifuge beaker | Thermo Fischer Scientific GmbH, Schwerte, GER |
| Centrifuge tubes | Thermo Fischer Scientific GmbH, Schwerte, GER |
| Combitips | Eppendorf AG, Hamburg, GER |
| Cryo vials 2ml | Greiner Bio-One, Frickenhausen, GER |
| Dialysis Tube Visking Type 27/32 | Carl Roth GmbH & Co. KG, Karlsruhe, GER |
| ELISA Plates Nunc Maxisorp F-Bottom, 96well | Nunc GmbH & Co. KG, Langenselbold, GER |
| E-Plate | Roche Diagnostics GmbH, Mannheim, GER |
| Eppendorf Cups 1,5ml, 2ml | Eppendorf AG, Hamburg, GER |
| Flow cytometry Tubes | Sarstedt AG & Co., Nürmbrecht, GER |
| Freezing Container Mr. Frosty® | Thermo Fischer Scientific GmbH, Schwerte, GER |
| Glass Beaker | Schott AG, Mainz, GER |
| Glass bottles 1l, 2l | Schott AG, Mainz, GER |
| Gloves Nitrile | Paul Hartmann AG, Heidenheim, GER |
| Neubauer counting chamber | Brand GmbH & Co KG, Weinheim, GER |

| | |
|---|---|
| Parafilm | Pechiney Plastic Packaging, Chicago, USA |
| Pipette tips 10, 20, 200, 1000ul | Starlab GmbH, Ahrensburg, GER |
| Pipette tips 100ul | Biozym Scientific GmbH, Hessisch Oldendorf, GER |
| Plastic Beaker | Vitlab GmbH, Großostheim, GER |
| Printet Filtermats A | Perkin Elmer, Rodgau, GER |
| Reagents Reservoir Costar | Sigma-Aldrich Chemie GmbH, München, GER |
| Safety-Multifly ® Set 21G | Sarstedt AG & Co., Nürmbrecht, GER |
| Serological pipettes 2ml | BD Biosciences, Heidelberg, GER |
| Serological pipettes 5,10,25,50ml | Corning Inc., Corning, USA |
| Single-use syringes 5,10,20,50ml | BD Biosciences, Heidelberg, GER |
| Syringe filter units 0,22um, diameter 4, 13mm | Millipore, Schwalbach, GER |

### 2.1.3 Chemicals and reagents

| | |
|---|---|
| Biocoll separating solution | Biochrom GmbH, Berlin, GER |
| $\beta$-mercaptoethanol | Carl Roth GmbH & Co. KG, Karlsruhe, GER |
| Bovine serum albumin (BSA) | Carl Roth GmbH & Co. KG, Karlsruhe, GER |
| Dimethyl sulfoxid (DMSO) | Merck KGaA, Darmstadt, GER |
| Ethanol | Merck KGaA, Darmstadt, GER |
| Ethylenediaminetetraacetic acid | Sigma-Aldrich Chemie GmbH, München, GER |
| [methyl-$^3$H] Thymidine | Hartmann Analytik GmbH, Braunschweig, GER |
| Phytohemagglutinin (PHA-L) | Sigma-Aldrich Chemie GmbH, München, GER |
| rIFNgamma | Pan-Biotech GmbH, Aidenbach, GER |
| Sodium azide | Merck KGaA, Darmstadt, GER |
| Trypan blue 0,4% | Sigma-Aldrich Chemie GmbH, München, GER |
| Tween® 20 | Merck KGaA, Darmstadt, GER |
| ULTIMA Gold™ liquid scintillation cocktail | PerkinElmer, Rodgau, GER |

### 2.1.4 Buffers and media

#### Supplements for buffers

| | |
|---|---|
| Dulbecco's Phosphate buffered Saline (DPBS) | Life Technologies, Darmstadt, GER |
| RPMI 1650 Medium | Life Technologies, Darmstadt, GER |
| Iscove's Modified Dulbeccos's Medium | Lonza Group Ltd, Basel, CH |
| Sodium Pyruvate | Biochrom GmbH, Berlin, GER |
| L-Glutamine | Lonza Group Ltd, Basel, CH |
| MEN non essential amino acids | Biochrom GmbH, Berlin, GER |
| Penicilline/Streptomycine | Sigma-Aldrich Chemie GmbH, München, GER |
| b-mercapto Ethanol | Carl Roth GmbH & Co. KG, Karlsruhe, GER |
| Fetal Calf Serum | PAA Laboratories GmbH, Pasching, A |
| Endothelial Cell Growth Medium 2 | PromoCell, Heidelberg, GER |
| Accutase | Sigma-Aldrich Chemie GmbH, München, GER |
| Trypsin-EDTA | PAA Laboratories GmbH, Pasching, A |

#### Cell culture media

| | |
|---|---|
| RPMI 1640 Complete Medium | RPMI 1640 Medium<br>10% (v/v) FCS (heat inactivated at 56 °C for one hour)<br>1 mM Sodium Pyruvate<br>1x MEM non-essential amino acids<br>100 U/ml Penicilline<br>100 $\mu$g/ml Streptomycine<br>50 $\mu$M $\beta$-mercaptoethanol |

| | |
|---|---|
| IMDM Complete Medium | IMDM Medium<br>10% (v/v) FCS (heat inactivated at 56 °C for one hour)<br>1mM Sodium Pyruvate<br>1x L-Glutamine<br>1x MEM non-essential amino acids<br>100 U/ml Penicilline<br>100 $\mu$g/ml Streptomycine<br>50 $\mu$M $\beta$-mercaptoethanol |
| Endothelial Cell Growth Complete Medium | Endothelial Cell Growth Medium 2<br>100 U/ml Penicilline<br>100 $\mu$g/ml Streptomycine |
| Freezing Medium | 90% (v/v) Fetal Calf Serum<br>10% (v/v) Dimethyl sulfoxide |

### Cell counting and viability staining

| | |
|---|---|
| Trypan-Blue Solution | 0,05% (v/v) Trypan-Blue |
| Tuerk Solution | Sigma-Aldrich Chemie GmbH, München, GER |

### Magnetic activated cell sorting

| | |
|---|---|
| MACS buffer | DPBS<br>0.5% (v/v) Bovine serum albumin<br>2mM EDTA |

### Flow cytometry

| | |
|---|---|
| 7AAD viability staining buffer | BioLegend GmbH, Fell, GER |
| FACS Flow | DPBS<br>0,02% (w/v) Sodium azide<br>1% (v/v) Fetal Calf Serum |

### ELISA buffer

| | |
|---|---|
| Blocking solution | 10% (v/v) BSA |
| | in $H2O$ bidest |
| Washing buffer | 0,05% Tween-20 |
| | in PBS |
| Dilution buffer | 10% (v/v) BSA |
| | in $H_2O$ bidest |
| Stop solution | 1M $H3PO4$ |

### 2.1.5 Antibodies

#### Conjugated antibodies

| Antigen | Clone | Species | Conjugate | Origin/Purchase |
|---|---|---|---|---|
| Human CD8 | RPA-T8 | Mouse | FITC | Biolegend, Fell, GER |
| Human CD69 | FN50 | Mouse | PE | Biolegend, Fell, GER |
| Human CD54 | HCD54 | Mouse | PE | Biolegend, Fell, GER |
| Human CD45 | 2D1 | Mouse | AmCyan | BD Biosciences, San Jose, USA |
| Human CD4 | OKT4 | Mouse | PacificBlue | Biolegend, Fell, GER |
| Human CD34 | 581 | Mouse | APC/Cy7 | Biolegend, Fell, GER |
| Human CD33 | WM53 | Mouse | APC | Biolegend, Fell, GER |
| Human CD14 | HCD14 | Mouse | PE/Cy7 | Biolegend, Fell, GER |
| Human CD117 | A3C6E2 | Mouse | PE/Cy7 | Biolegend, Fell, GER |
| Human CD105 | 43A3 | Mouse | APC | Biolegend, Fell, GER |
| Human CD8 | OKT8 | Mouse | FITC | homemade |
| Isotype Control | MOPC-21 | Mouse | FITC | Biolegend, Fell, GER |
| Isotype Control | MOPC-21 | Mouse | PE | Biolegend, Fell, GER |
| Isotype Control | MOPC-21 | Mouse | PacificBlue | Biolegend, Fell, GER |
| Isotype Control | MOPC-21 | Mouse | APC/Cy7 | Biolegend, Fell, GER |
| Isotype Control | MOPC-21 | Mouse | APC/Cy7 | Biolegend, Fell, GER |
| Isotype Control | MOPC-21 | Mouse | PE/Cy7 | Biolegend, Fell, GER |
| Human IgG, Fcgamma spec | | Goat | RPE | Jackson ImmunoResearch, West Grove, USA |
| Mouse IgG+IgM | | Goat | RPE | Jackson ImmunoResearch, West Grove, USA |

### Unconjugated antibodies

| Name/Clone | Antigen | Species | Isotype | Origin/Purchase |
|---|---|---|---|---|
| 136726 | human CD275 (LICOS) | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| 11C3.1 | human CD252 (OX40L) | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| 2D10 | human CD80 | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| 3.9 | human CD11c | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| 4G7 | human CD19 | Mouse | Ig$\gamma$1 | M. Hofmann, Tübingen, GER |
| 4G8 | human FLT3 | Mouse | Ig$\gamma$1 | H.J. Bühring, Tübingen, GER |
| 4G8 SDIEM | human FLT3 | Human | Ig$\gamma$1 | M. Hofmann, Tübingen, GER |
| 5C3 | human CD40 | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| Bu15 | human CD11c | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| CBR-1C2/2 | human CD102 (ICAM-2) | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| CBRM1/5 | human CD11b | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| DREG-56 | human CD62L | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| Flebo Gamma | polyclonal | human | | Grifols International S.A. |
| HA58 | human CD54 (ICAM-1) | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| HAE-1f | human CD62E | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| HCD54 | human CD54 (ICAM-1) | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| HI111 | human CD11a | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| ICRF44 | human CD11b | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| Infliximab | human TNF-a | Mouse/Human | Ig$\gamma$1 | Janssen Biotech Inc., Horsham, USA |

| IT2.2 | human CD86 | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
|---|---|---|---|---|
| N19-CU N297Q | human CD19, hum. CD3 | Mouse/Human | bsFab | M.Durben, Tübingen, GER |
| NFAP-CU N297Q | human FAP, hum. CD3 | Mouse/Human | bsFab | F. Vogt, Tübingen, GER |
| NF-CU N297Q | human FLT3, hum. CD3 | Mouse/Human | bsFab | M. Durben, Tübingen, GER |
| NG-CU N297Q | human GD2, hum. CD3 | Mouse/Human | bsFab | D. Schmiedel, Tübingen, GER |
| NM-CU N297Q | human CSPG4, hum. CD3 | Mouse/Human | bsFab | L. Große-Hovest, Tübingen, GER |
| NP-CU N297Q | human PSMA, hum. CD3 | Mouse/Human | bsFab | F. Vogt, Tübingen, GER |
| RPA-2.10 | human CD2 | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| TKS-1 | human CD137L (4-1BBL) | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| TS1/18 | human CD18 (Integrin beta2) | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| TS2/4 | human CD11a | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| TS2/9 | human CD58 | Mouse | Ig$\gamma$1 | Biolegend, Fell, GER |
| UCHT-1 | human CD3 - epsilon | Human | IgG2a | L. Große-Hovest, Tübingen, GER |
| Tocilizumab | human IL-6 | Mouse/Human | Ig$\gamma$1 | Hoffmann-La Roche AG, Basel, CH |

### 2.1.6 Cell lines

| Name | Type | Origin/Purchase | Literature |
|---|---|---|---|
| HUVEC | Human umbilical vein endothelial cells | Promocell, Heidelberg, GER | Jaffe et al. 1973 |
| JY | Human B lymphoblastoid leukemia | European Collection of Cell Cultures | Swaroop and Xu 1993 |
| LN-18 | Human glioblastoma | M. Weller, Tübingen, GER | Diserens et al. 1981 |
| NALM-16 | Human acute lymphoblastic leukemia | DMSZ GmbH, Braunschweig, GER | Kohno, Minowada, and Sandberg 1980 |
| SKMel63 | Human malignant melanoma | B. Gückel, Tübingen, GER | J. Fogh, J. M. Fogh, and Orfeo 1977 |
| SKW 6.4 | Human B cell lymphoma | P.H. Krammer, Heidelberg, GER | Saiki and Ralph 1983 |

## 2.2 Methods

### 2.2.1 Cell Biology Methods

#### Isolation of Peripheral Blood Mononuclear Cells (PBMCs)

Freshly drawn, heparinized blood from healthy donors was diluted 1:1 with PBS. 25 ml of diluted blood was layered over 14 ml of Biocoll Separating Solution in a sterile 50 ml Falcon tube and centrifuged at 360 $\times$ g for 30 min, at 25°C, without brake. Subsequently, the interphase (buffy coat) was collected, which included the peripheral blood mononuclear cells (PBMCs). The cells were washed twice with PBS for 10 minutes at 25 °C (first run at 300$\times$g, then at 200$\times$g) to remove traces of Biocoll solution and the cell pellet was resuspended in RPMI 1640 Complete Medium.

#### Culture of Tumor Cell Lines

All tumor cell lines were cultured at 37°C, 5% $CO_2$, and 90% relative humidity in $75cm^2$ cell culture flasks, containing 12 ml of RPMI 1640 complete medium.

#### Culture of PBMCs

PBMCs were cultured at 37°C, 5% $CO_2$, and 90% relative humidity in $75cm^2$ cell culture flasks, containing 12 ml of RPMI 1640 complete medium.

#### Culture of Human Umbilical Vein Endothelial Cells (HUVECs)

HUVECs were cultured at 37°C, 5% $CO_2$, and 90% relative humidity in $75cm^2$ cell culture flasks, containing 10 ml of Endothelial Cell Growth Complete medium.

#### Culture of Antibody-Producing Transfectants

Antibody-producing transfectants were cultured at 37°C, 5% $CO_2$, and 90% relative humidity in $175cm^2$ cell culture flasks, containing 50ml of IMDM complete medium.

### Harvesting of Adherent Cells

To harvest adherent cells (e.g., 22RV1, HUVECs, LN-18), the cell culture medium was carefully removed from the flask. Then, cells were rinsed once with 10 ml of PBS. Afterwards, 1 ml of Accutase cell detachment solution was added, and the flask was kept in an incubator for 5 minutes Detachment was confirmed by microscopy, and the cells were resuspended in cell culture medium and removed from the flask.

### Harvesting of Non-Adherent Cells

To harvest non-adherent cells, culture flasks were carefully agitated. Detachment was confirmed by microscopy, and the cell suspension was removed from the flask.

### Cryopreservation of Cells

After harvesting, the cell suspension was centrifuged for 10 minutes at 310×g and the supernatant was discarded. The cell pellet was resuspended in 1.5 ml of freezing medium, and placed in a cryo tube. Tubes were immediately put into a Mr. Frosty™ freezing container and stored for at least 12 hours in a -80 °C freezer, in order to secure steady freezing of approximately 1 K per hour.

### Thawing of Cryopreserved Cells

25 ml of cell culture medium was prepared in a 50 ml Falcon tube and warmed to 37 °C. Afterwards, cryo tubes containing preserved cells were thawed in a water bath at 37 °C. The cell suspension was transferred into the Falcon tube containing the prewarmed medium, and immediately centrifuged for 10 min at 310×g. The cell pellet was resuspended in 12 ml of cell culture medium. Viability was assured microscopically, 24 hours later.

### Cell Counting and Viability Staining of Cells

40$\mu$l of cell suspension and 40$\mu$l of 0.05% Trypan blue solution were mixed together and pipetted into a Neubauer chamber. Only unstained, viable cells were counted. The cell

number was determined using the following equation:
cells/ml $=$ counts per quadrant $\times$ chamber constant$(10^4)\times$ dilution factor

### Inactivation of Tumor Cells

Tumor cells were irradiated at a dose of 120 Gy using a GammaCell irradiation chamber, containing $^{137}$Caesium, in order to prevent them from proliferation during T cell proliferation assays.

## 2.2.2 Analytical Methods

### Flow Cytometry

Flow cytometric analysis is based on an antigen-antibody reaction with fluorochrome-conjugated antibodies. Combined with information about granularity and size of microscopically small particles, such as cells, cell types can be easily identified by their individual expression of surface molecules.

Flow cytometry was used to determine the expression of surface molecules on multiple cell lines. Furthermore, the total number of leukemic blasts in a flow cytometry based kill assay was measured. Finally, the expression of activation molecules on T cells after stimulation with a bispecific antibody was determined.

### Flow Cytometric Staining of Surface Molecules

To determine the expression of surface molecules on multiple cell lines, flow cytometric staining was performed.

Cells were harvested as described above, and adjusted to a concentration of $10^6$ per ml. 200 $\mu$l of the cell suspension was plated in a 96-well plate. Subsequently, the cells were centrifuged for three minutes at 561 $\times$ g and the supernatant was discarded. The cell pellet was washed by resuspending in 200 $\mu$l of FACS buffer, and centrifuging for three minutes at 561 $\times$ g. The washing step was repeated one more time. Murine antibodies directed against surface molecules were diluted in FACS buffer and adjusted to a concentration of 5 $\mu$g/ml. 50 $\mu$l of the antibody dilution was added per well. The cell pellet was resuspended

and the plate was kept in the dark at 4 °C for 30 minutes. Afterwards, the plate was centrifuged at 561 $\times$ g for three minutes, and the supernatant was discarded. Next, the cells were washed twice. Goat anti-mouse PE conjugated antibodies were diluted 1:200 in FACS buffer. 50 $\mu$l of the dilution was added to each well. The cell pellets were resuspended and the plate was kept in the dark at 4 °C for 25 minutes. Afterwards, the plate was centrifuged at 561 $\times$ g for three minutes and the supernatant was discarded. Next, the cells were washed twice and the cell pellet was resuspended in 250 $\mu$l of FACS buffer. FACS analysis was performed with a FACSCalibur.

### Flow Cytometry Based Activation Assay

Flow cytometry was used to determine the expression of activation molecules on T cells after stimulation with a BsMAb.

Blood was drawn from healthy donors and PBMCs were isolated, as described previously. PBMCs were adjusted to a concentration of 3 $\times$ $10^6$/ml in RPMI Complete Medium, and plated in a 96-well plate. BsMAb and monospecific blocking antibodies were diluted in RPMI Complete Medium and 50 $\mu$l of the dilution was added per well. The plate was kept in a humidified atmosphere at 37 °C and 5% $CO2$. After two days of incubation, the cells were centrifuged for three minutes at 561 $\times$ g, and the supernatant was discarded. In order to wash the cells, the cell pellet was resuspended in 200 $\mu$l of FACS buffer and centrifuged for three minutes at 561 $\times$ g. The washing step was repeated one more time. Conjugated murine anti-human antibodies directed against CD4, CD8, CD45 and CD69 were adjusted to a concentration of 5 $\mu$g/ml in FACS buffer and added to the cells. The cell pellet was suspended in 50 $\mu$l of the antibody dilution and kept in the dark at 4 °C for 30 minutes. The plate was then centrifuged at 561 $\times$ g for three minutes, and the supernatant was discarded. Next, the washing step was carried out twice. A solution containing 12 $\mu$l 7AAD viability staining solution per ml FACS buffer was added ten minutes prior to the start of the FACS analysis. FACS analysis was performed with a FACSCanto.

### Flow Cytometry Based Kill Assay

Flow cytometry was used to determine the exact number of leukemic blasts in a kill assay using autologous immune cells from patients. Additionally, T cell proliferation, and the expression of activation markers on T cells could be measured.

Blood was drawn from leukemia patients and PBMCs, including leukemic blasts, were isolated as described previously. 500,000 PBMCs, including leukemic blasts, were plated per well in a 96-well plate. BsMAb and monospecific blocking antibodies were diluted in RPMI Complete Medium and 50 $\mu$l of the dilution was added per well. The plate was kept in a humidified atmosphere at 37 °C and 5$\%$ $CO2$. After three days of incubation, the cells were centrifuged for three minutes at 561 $\times$ g, and the supernatant was discarded. To wash the cells, the cell pellet was resuspended in 200 $\mu$l of FACS buffer and centrifuged for three minutes at 561 $\times$ g. The washing step was repeated one more time. Conjugated mouse anti-human antibodies were adjusted to a concentration of 5 $\mu$g/ml in FACS buffer. The cell pellet was suspended in 50 $\mu$l of the antibody dilution, and kept in the dark at 4 °C for 30 minutes. Afterwards, the plate was centrifuged at 561 $\times$ g for three minutes, and the supernatant was discarded. Next, the washing step was performed twice. A solution for viability staining and standardization was prepared, containing 12$\mu$l 7AAD Viability staining solution and two drops of BD negative beads per ml FACS buffer. FACS analysis was performed with a FACSCanto.

### ELISA (Enzyme-Linked Immunosorbent Assay)

Cytokine ELISAs were performed to assess the production of IFN$\gamma$ by activated T cells after stimulation with a bispecific antibody. An ELISA allows the quantification of cytokines in cell culture or assay supernatant. The antigen to be measured can be detected by two non-cross-reacting antibodies. First, the antigen binds to the capture antibody, which is bound to a microtitre plate, the detection antibody then binds to the antigen. The total amount of bound detection antibody correlates with the amount of antigen. In order to determine the amount of antigen, the detection antibody is conjugated with biotin, which forms a stable complex with streptavidin. Streptavidin is added afterwards, and is conjugated with peroxidase, an enzyme that can convert a substrate added later. The con-

version of the substrate is determined by a change of color, which can be measured using a photometer. In fact, the extinction correlates with the total amount of bound antigen. In order to determine the absolute amount of IFN$\gamma$, a standard curve was added to the test samples.

First, 96-well Maxisorp plates were coated with the capture antibody. To this end, the coating antibody was adjusted to a concentration of 1 $\mu$g/ml. 50 $\mu$l of the dilution was plated per well. The plate was covered with a sealer and incubated overnight at 4 °C. The next day, the microtitre plates were washed five times with a washing buffer and thereafter loaded with 200 $\mu$l blocking buffer, and then incubated for one hour at room temperature. After the washing procedure, standards and samples were diluted 1:2 and 50 $\mu$l of each was transferred into the wells in triplicate, and incubated for one hour at room temperature.

A serial dilution of recombinant IFN$\gamma$ served as the standard, whereas supernatants from the proliferation assays were the test samples. After the washing step, a biotinylated antibody was adjusted to a concentration of 1$\mu$g/ml and 50$\mu$l was plated per well. The incubation period of one hour at room temperature was followed by a washing step. Next, streptabidin-HRP conjugate was diluted 1:5000, and 50 $\mu$l of the dilution was transferred to each well. After the incubation for 30 minutes at room temperature, the washing step was performed again. Eventually, 50$\mu$l of substrate solution was added per well. After a short incubation period in the dark, enzymatic color development was confirmed. The enzymatic process was immediately stopped by adding 50$\mu$l stop solution. The specific extinction was measured using a SpectraMax 340 ELISA reader.

### 2.2.3 Functional Immunological Assays

#### XCELLigence Assay

XCELLigence assays were performed to observe real-time killing of adherent tumor cells by PBMCs in the presence of BsMAb, and blocking reagents.

Adherent tumor cells (22RV1) were harvested as described previously, and adjusted to a concentration of $1 \times 10^6$ per ml. 50 $\mu$l of RPMI 1640 Complete Medium per well was plated in an E-Plate 96. The plate was installed in a RTCA XCELLigence System

in an atmosphere of 37 °C, 5% $CO2$ and 90% humidity. Afterwards, 50 $\mu$l of the cell suspension was seeded onto the E-plate to reach a total cell number of 50,000 per well. The plate was installed in the reader and kept for 20 hours in the atmosphere described above. Measurement of cell indices was performed every 15 minutes. Cell indices are an indicator of the total number of viable adherent cells, as killing and subsequent detachment of tumor cells results in lower cell indices. After the first incubation period, PBMCs and different antibodies were plated. The whole assay was incubated for approximately four days, and cell indices were determined every 15 minutes.

### [methyl-$^3$H] Thymidine Activation Assays

Cell proliferation was measured as an indicator of the activation of T cells. To determine the proliferation of T cells, the incorporation of [methyl-$^3$H] thymidine into newly synthesized DNA was measured.

To this end, human PBMCs were isolated as described above, adjusted to a concentration of $2 \times 10^6$ per ml in RPMI 1640 complete medium, and pipetted at a concentration of 100,000 per well in a 96-well flat bottom plate. Then, 100,000 of either irradiated target cells or SBCs were added per well. Finally, BsMab at a concentration of 1 $\mu$g/ml and monoclonal blocking antibodies at a concentration of 1 $\mu$g/ml were added. The assay was incubated for approximately 48 hours at 37 °C, 7.5% $CO2$ and 90% humidity. Afterwards, the cells were pulsed with 0.5 $\mu$Ci per well of [methyl-$^3$H] thymidine. This was followed by a further incubation of approximately 16 hours. The mixture was then harvested with the cell harvester on Printet Filtermat A mats. Only incorporated thymidine binds to the mats, whereas non-incorporated thymidine does not. Next, the filter mats were dried at 50 °C for 30 minutes. The level of radioactive DNA was determined after addition of a scintillation fluid, using the MicroBeta$^2$ scintillation.

### Adhesion Assay

Lymphocyte adhesion assays were performed in 24-well culture plates. HUVECs were seeded at a concentration of 50,000 per square centimeter in Endothelial Cell Growth Medium and cultivated for two days. Formation of a HUVEC monolayer was confirmed

by microscopy. Afterwards, HUVECS were washed twice with 1000 $\mu$l of RPMI Complete Medium. PBMCs were isolated as described above, and adjusted to a concentration of 500,000 per ml. They were then stimulated with a BsMAb at a concentration of 1 $\mu$g/ml for one hour. Then, 2500 $\mu$l of the PBMC suspension was plated into the 24-well plates. Immediately afterwards, blocking antibodies were added at a concentration of 1 $\mu$g/ml. After an incubation of three hours, non-adherent cells were removed by washing with 1000 $\mu$l RPMI Complete Medium three times. next, adherent cells were detached by addition of Accutase detachment solution. HUVECs and adherent PBMCs were collected, and flow cytometry was performed to determine the absolute numbers of adhering $CD45^+$ cells.

# 3 Results

## 3.1 The ability of T cell-activating bispecific antibodies to induce off-target activation

It is widely held, that BsMAb work in a target cell-restricted manner, thus limiting the T cell effector functions to the site of cells that express the tumor-associated antigen (TAA) that the antibody is directed against. Binding of the anti-CD3 arm to T cells is thought not to induce any T cell activation in the absence of target cells. However, off-target T cell activation, especially in the blood, could explain various side effects seen with BsMAb. The activation assays in this work were conducted using PBMCs, isolated from peripheral blood, and a BsMAb termed NP-CU, which is composed of a binding site to PSMA, an antigen that is not present on PBMCs, and the aCD3 antibody UCHT1.

### 3.1.1 [methyl-$^3$H] Thymidine activation assays

To determine the ability of the bispecific PSMA×CD3 antibody NP-CU to induce off-target T cell proliferation, thymidine uptake assays were performed. 100,000 freshly isolated PBMCs per well in a 96-well plate were cocultivated with NP-CU at a concentration of 1 $\mu$g/ml for three days.

The results show that the PSMA×CD3 antibody is capable of inducing moderate T cell proliferation in the absence of target cells. This suggests that a monovalent CD3 stimulus can lead to some T cell activation. This particular effect is dose-dependent and could be reproduced using PBMCs from 12 healthy donors. The required minimum dose to generate this effect is around 300 ng/ml.

An average proliferation of 10% of the maximum was observed, with phytohemagglutinin (PHA) at a concentration of 10 $\mu$g/ml defining 100% activation. PBMCs alone do not show any significant thymidine uptake. (Fig. 3.1)

Off-target induced T cell activation was also induced by other TAA×CD3 BsMAb than

NP-CU. A bispecific chondroitin sulfate proteoglycan 4 (CSPG4) × CD3 antibody was capable of inducing similar T cell activation in an $TAA^-$ environment. (Data not shown)

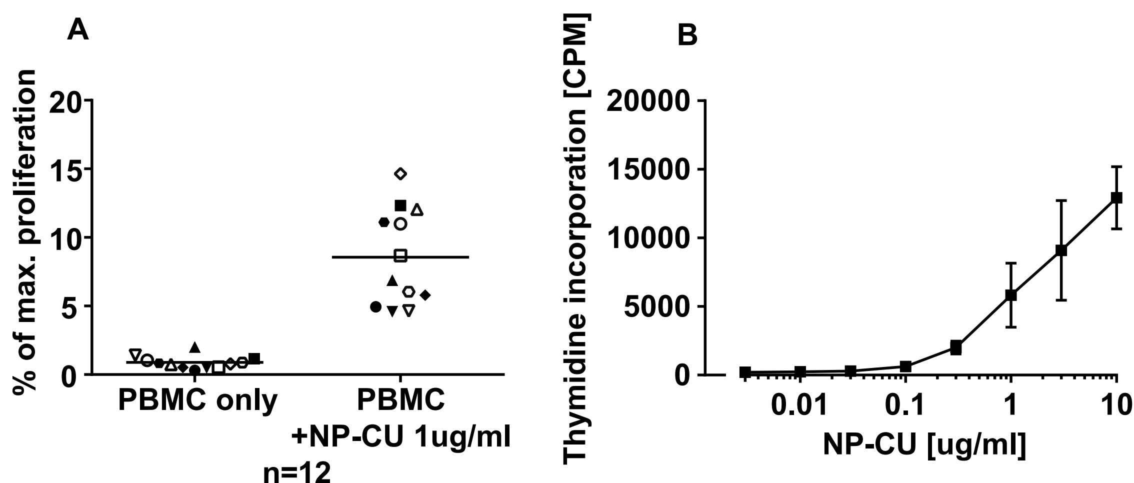


**Figure 3.1:** ***T cell proliferation in response to off-target activation with the BsMAb NP-CU*** *Freshly isolated PBMCs from a healthy donor were cocultivated with a PSMA×CD3 antibody at a concentration of 1μg/ml for two plus one days and T cell proliferation was assessed using a thymidine incorporation assay.* ***A*** *The experiment was repeated 12 times using PBMCs from different healthy donors.* ***B*** *Concentration dependency of this effect. Representative results from one out of three experiments with PBMCs from different healthy donors are shown. Each data point represents the mean value of triplicate samples.*

### 3.1.2 FACS-based activation assays

CD69 is a well-established early activation marker on both natural killer cells and T cells. The following experiments addressed the expression of CD69 on T cells after monovalent CD3 stimulation with NP-CU, independent of target cells. 100,000 freshly harvested PBMCs and BsMAb at a concentration of 1 μg/ml were cultured for 48 hours. Then, the CD69 expression on $CD4^+$ and $CD8^+$ T cells was assessed using flow cytometry.

The results show that a monovalent CD3 stimulus can induce expression of CD69 on both $CD4^+$ and $CD8^+$ T cells. After stimulation with either NP-CU or a CSPG4×CD3 antibody at a concentration of 100 ng/ml, 20% of the $CD4^+$ T cells and 15% of all CD8 $^+$ T cells expressed CD69 (Fig. 3.2). A CD19×CD3 antibody induced CD69 expression in 60% - 70% of all T cells due to on-target activation by $CD19^+$ B cells. A FLT3×CD3

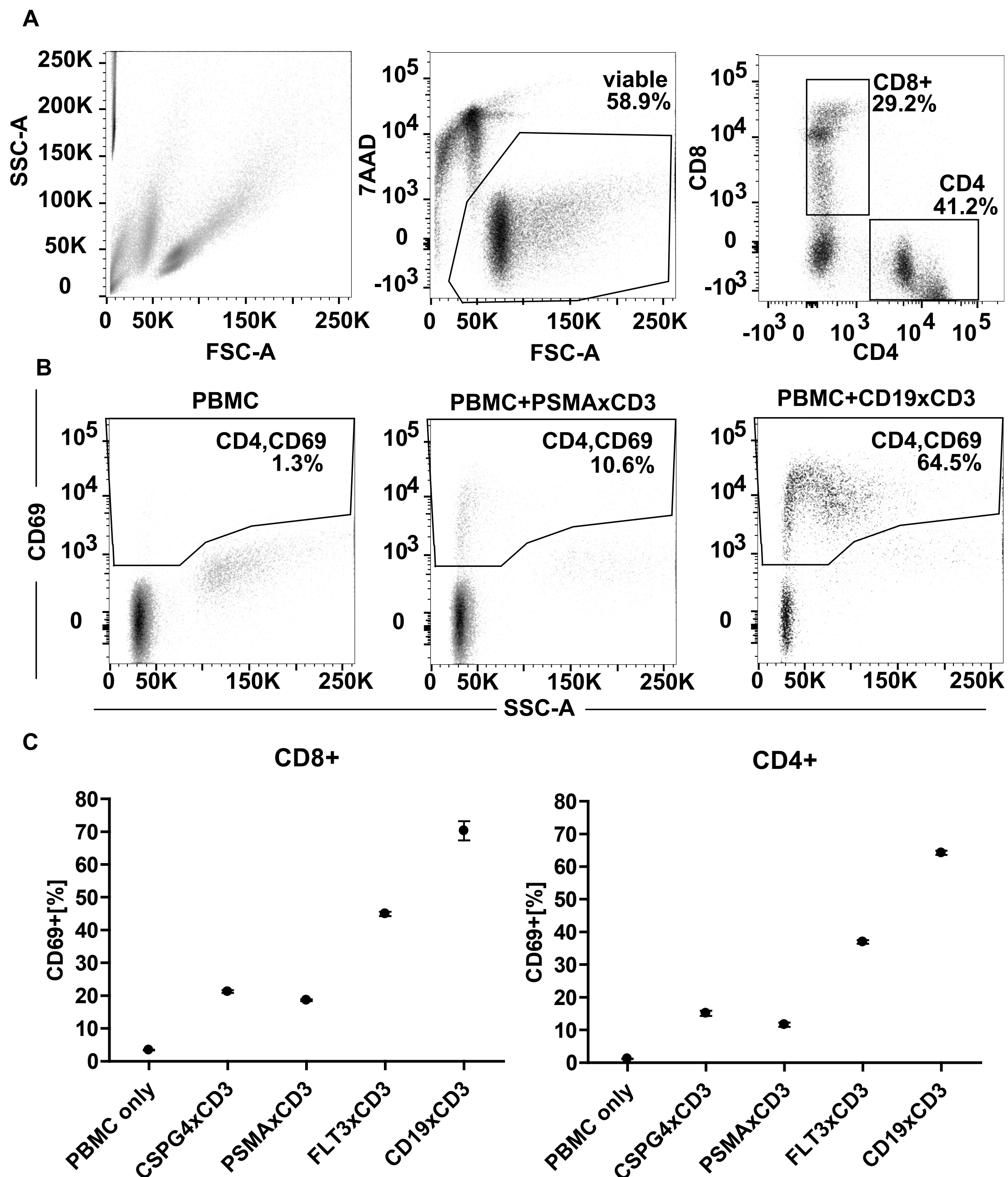


**Figure 3.2:** ***CD69 expression on T cells after off-target stimulation with different BsMAb*** *PBMCs were incubated with PSMA×CD3 or CSPG4×CD3 bispecific antibodies at a concentration of 100 ng/ml for 48 hours. After that, CD69 expression on T cells was assessed via flow cytometry.* ***A*** *T cells were defined as $CD45^+$, $CD8^+$ or $CD4^+$.* ***B*** *CD19xCD3 or FLT3xCD3 antibodies served as positive controls. All tests were performed in triplicates.* ***C*** *Representative results from one out of three experiments with PBMCs from different healthy donors are shown.*

BsMAb induced CD69 expression in 40% of all cells. FLT3 is present on monocytes at a small amount compared with CD19 on B cells. These findings confirm that monovalent CD3 stimulation by BsMAb induces activation of T cells and subsequent expression of CD69/IL-2 receptor. In comparison with the thymidine uptake assays, the flow cytometry-based activation assays seem to be more sensitive, as the BsMAb induced T cell activation at a concentration as low as 100ng/ml.

## 3.2 Identification of stimulating bystander cells

As lymphocyte adhesion to vascular endothelial cells happens rapidly after administration of BsMAb, it is likely that these cells amplify off-target activation by BsMAb. Lymphoid cells within the lymph nodes could induce similar effects. The following experiments addressed the influence of certain lymphoid and endothelial cells on the induction of off-target activation. As a first step, flow cytometric surface staining of various cancer cell lines was performed to rule out expression of PSMA or CSPG4 on these potential bystander cells. To investigate further, whether SKW6.4, JY, and NALM-16 lymphoid cells, and HUVECs amplify off-target T cell activation, thymidine uptake assays were conducted.

It was shown that some lymphoid cell lines, such as SKW6.4 and JY, are capable of enhancing the activation of T cells by BsMAb, while others fail to do so (Fig. 3.3). Thus, the stimulating cells will be referred to as stimulating bystander cells (SBCs).

However, not all lymphoid cell lines tested could be characterized as SBCs. NALM-16 cells, derived from an acute precursor B cell leukemia, showed no enhancement of off-target T cell activation.

To rule out any allogeneic effects, several other tumor cell lines were tested for stimulating properties in an off-target setting. It was shown that neither LN-18 glioblastoma cells, nor SKMel melanoma cells are capable of enhancing off-target activation.

HUVECs are a widely accepted model of endothelial cells. As endothelial cells are most likely involved in BsMAb-induced leukocyte adhesion and activation, they were tested for stimulating properties as described above. The results show that HUVECs are able to enhance off-target activation markedly. However, off-target activation triggered

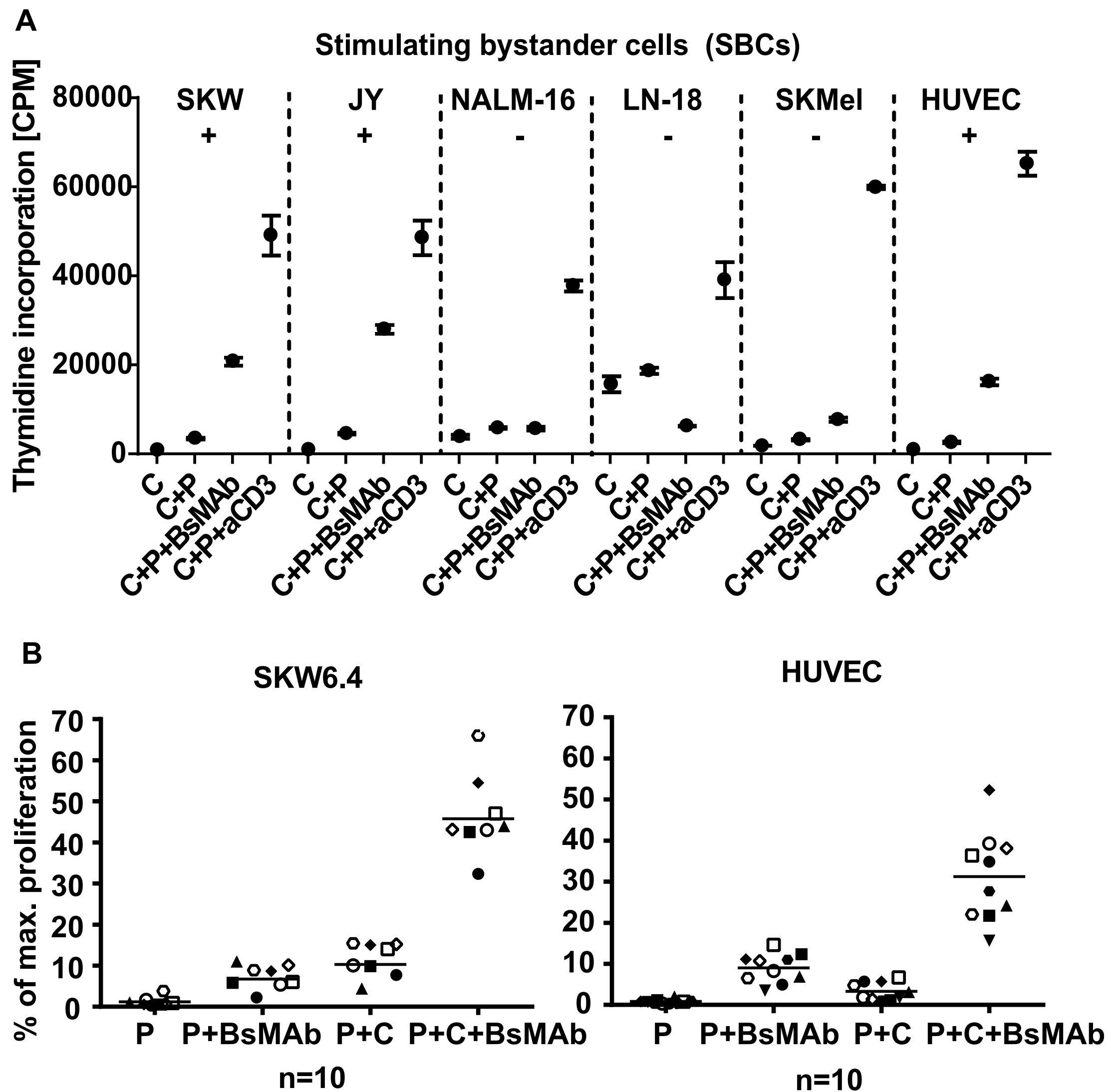


**Figure 3.3:** ***Stimulating properties of different cell lines*** ***A*** *Different lymphoid cells (SKW6.4, JY, NALM-16), glioblastoma cells (LN-18), melanoma cells (SKMel) and human umbilical vein endothelial cells (HUVECs) were either cultivated alone (depicted as C), together with PBMCs (depicted as C+P), or with PBMCs and a PSMA×CD3 BsMAb (NP-CU) at a concentration of 1 μg/ml (depicted as C+P+BsMAb), or with PBMCs and a monoclonal CD3 antibody (UCHT1) at a concentration of 1 μg/ml (depicted as C+P+aCD3) for three days and incorporated thymidine was determined. The effector:target ratio was 1:1 with 100,000 cells per well. Cell lines were evaluated as stimulating (depicted as +) or non-stimulating (depicted as -).* ***B*** *The experiment was repeated ten times using PBMCs from different healthy donors. Each data point represents the mean value of triplicate samples derived from one individual.*

by HUVECs is slightly less pronounced than that obtained by JY or SKW6.4 cells.

## 3.3 The mechanism of off-target activation

**Table 3.1:** ***Expression of costimulatory molecules on cell lines*** *Flow cytometric expression analysis was performed as described in the material&methods section. Strong expression (depicted as +), weak expression (depicted as (+)) and absence of the molecule (depicted as -) was distinguished.*

| Molecule | HUVEC | NALM-16 | SKW6.4 |
|---|---|---|---|
| CD54 (ICAM-1) | (+) | + | + |
| CD102 (ICAM-2) | + | + | + |
| CD80 | - | - | + |
| CD86 | - | - | + |
| CD252 (OX-40L) | (+) | (+) | (+) |
| CD137L (4-1BB) | - | - | + |
| CD275 (LICOS) | + | + | + |
| CD62E (E-Selectin) | - | - | - |
| CD62L (L-Selectin) | - | - | + |
| CD40 | + | - | + |
| CD58(LFA-3) | + | + | + |

The following experiments were conducted to address the influence of costimulatory molecules on SBCs during the induction of off-target activation.

As a first step, expression of costimulatory molecules on stimulating cells was compared with the expression on nonstimulating bystander cells via flow cytometry.

To this end, SKW6.4 cells and HUVECs (stimulating) were compared with NALM-16 cells (non-stimulating).

The results are depicted in Table 1.1.

It could be shown that the stimulating cell line SKW6.4 expresses a greater variety of costimulatory molecules than NALM-16, which is characterized as nonstimulating. It is further remarkable that HUVECs only show a weak expression of CD54.

Nevertheless, CD40 was the only costimulatory molecule tested, which is present on stimulating cell lines, but not on non-stimulating cells.

## 3.4 Inhibition of off-target activation by blocking antibodies and steroids

In the previous experiments, it was shown that SBCs bearing a variety of costimulatory molecules enhance BsMAb-induced off-target T cell activation. The following experiments were conducted to block costimulatory molecules on SBCs with various antibodies. For this purpose, [methyl-$^3$H] thymidine uptake assays with blocking antibodies in comparison with steroids were performed.

### 3.4.1 [methyl-$^3$H] Thymidine activation assays

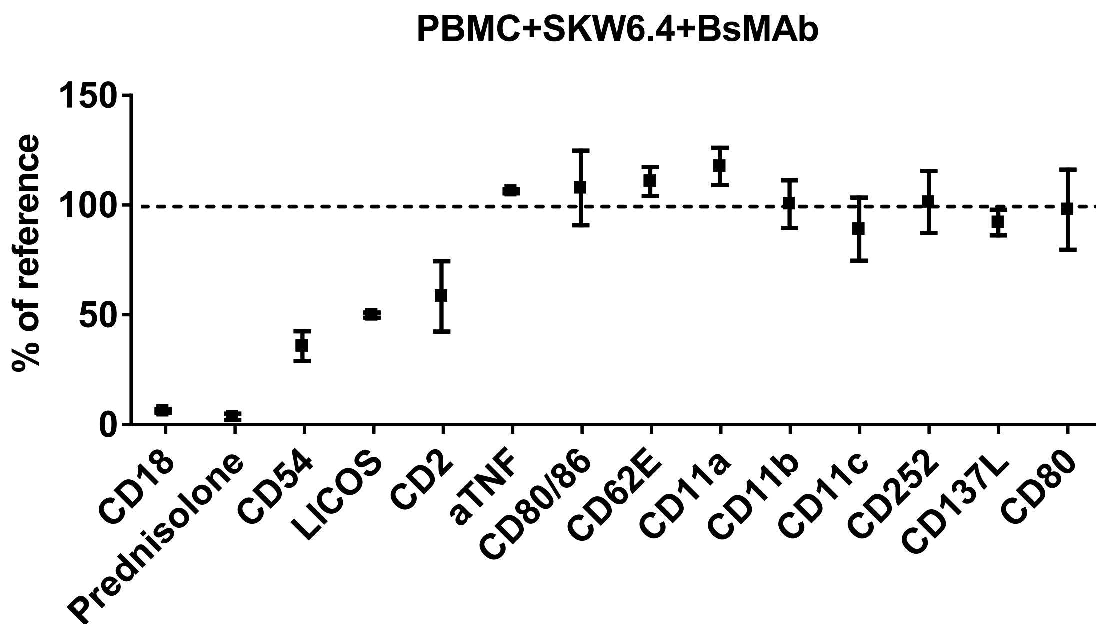


**Figure 3.4:** ***Inhibition of off-target T cell activation in the presence of SKW6.4*** *Freshly isolated PBMCs from healthy donors and SKW6.4 cells were cocultivated with both a PSMA×CD3 antibody and a blocking antibody at a concentration of 1 $\mu$g/ml or a steroid at a concentration of 10 $\mu$g/ml for three days. Then, incorporated thymidine was determined. PBMCs incubated with SKW6.4 and NP-CU at a concentration of 1 $\mu$g/ml were used as reference. Representative data from one out of three experiments are shown. Mean values and standard deviations of triplicate samples are indicated.*

Off-target T cell activation induced by BsMAb in the presence of SBC SKW6.4 could be reduced by different agents. However, one should note that, in all cases, blocking

activity of the respective antibodies has not been confirmed in separate independent experiments.

Although blocking antibodies directed against the established costimulatory molecules CD80 and CD86 had no effect, antibodies directed to LICOS or the adhesion molecules ICAM-1 (CD54), LFA-3 (CD2), or LFA-1 (CD18) exerted a variable blocking effect, the one induced by the anti-CD18 antibody being most pronounced, reaching almost complete inhibition, comparable to that achieved by prednisolone at a concentration of 10$\mu$g/ml.

In a next step, an anti-TNF$\alpha$ antibody was also assessed. It was shown that off-target T cell activation by BsMAb in the presence of SKW6.4 is not affected by this antibody. Fig 3.4 summarizes the results.

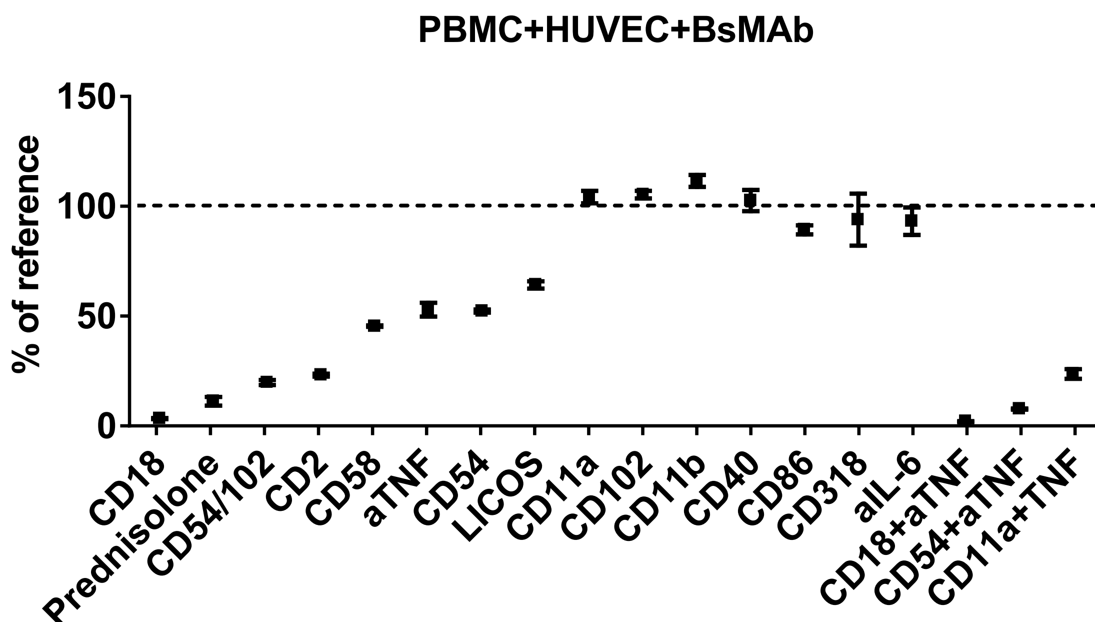


**Figure 3.5:** ***Inhibition of off-target T cell activation in the presence of HUVECs*** *Freshly isolated PBMCs from healthy donors and HUVECs were cocultivated with both a PSMA$\times$CD3 antibody and a blocking antibody at a concentration of 1 $\mu$g/ml or a steroid at a concentration of 10 $\mu$g/ml for three days. Incorporated thymidine was determined afterwards. PBMCs incubated with SKW6.4 and NP-CU at a concentration of 1 $\mu$g/ml were used as reference. Representative data from one out of three experiments are shown. Mean values and standard deviations of triplicate samples are indicated.*

Similar results were obtained, when HUVECs were used as SBCs instead of SKW6.4

cells (Fig. 3.5). Again, CD18 blockade and prednisolone were most effective. In contrast to the experiments conducted with SKW6.4, TNF$\alpha$ blockade seems to reduce off-target T cell activation triggered by HUVECs to some extent.

However,blocking of CD40, which is the only costimulatory molecule that is expressed on stimulating, but not on nonstimulating cells, showed no effect.

The results observed during off-target blocking assay suggest that different costimulatory and adhesion molecules amplify off-target activation.

### 3.4.2 FACS-based activation assays

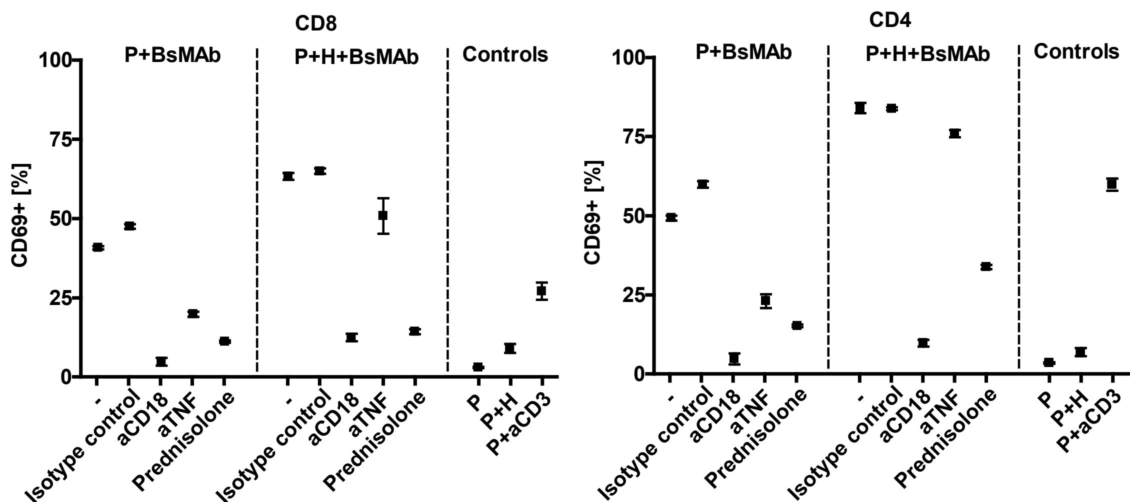


**Figure 3.6:** ***Inhibition of CD69 expression after off-target activation*** *PBMCs from a healthy donor were incubated either with a PSMA$\times$CD3 BsMAb at a concentration of 1$\mu$g/ml (depicted as P+BsMAb), or together with HUVECs and a BsMAb (depicted as P+H+BsMAb) in the presence or absence of blocking reagents at a concentration of 1 $\mu$g/ml. After 48 hours, CD69 expression on T cells was assessed via flow cytometry. Representative results from one out of three experiments with PBMCs from different healthy donors are shown.*

Flow cytometry-based activation assays were performed to investigate whether some of the identified blocking reagents are capable of reducing CD69 expression on T cells after off-target activation in the presence and absence of SBCs. For this purpose, aCD18 antibodies as well as TNF$\alpha$ blockers and prednisolone were assessed.

Addition of a BsMAb to PBMCs induced CD69 expression in approximately 50% of

all T cells. Antibody-mediated blocking of CD18 as well as application of prednisolone brought CD69 expression on both $CD8^+$ and $CD4^+$ T cells down to the baseline level, with the inhibition induced by prednisolone being slightly pronounced regarding $CD4^+$ T cells. Addition of aTNF$\alpha$ antibodies almost completely blocked off-target T cell activation in the absence, but not in the presence of HUVECs. One should note that aTNF$\alpha$ antibodies can effectively block T cell proliferation in a thymidine incorporation assay, but fail to inhibit CD69 expression on T cells (Fig. 3.6).

### 3.4.3 IFN$\gamma$ ELISA

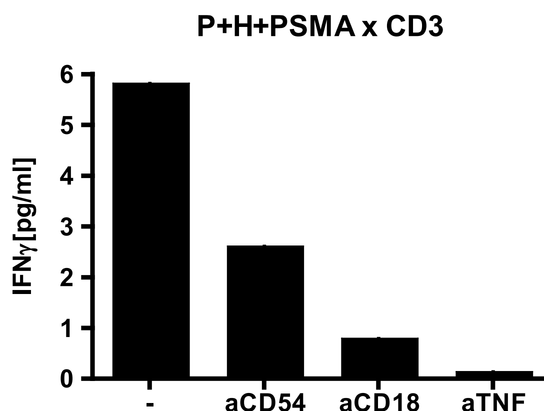


**Figure 3.7:** ***Inhibition of off-target induced IFN$\gamma$ release*** *PBMCs (P) from a healthy donor were incubated with HUVECs (H), a PSMA$\times$CD3 BsMAb at a concentration of 1ug/ml and different blocking antibodies at a concentration of 1 $\mu$g/ml for 48 hours. After that, IFN$\gamma$ release was determined by an ELISA. Mean values of triplicate samples are indicated.*

IFN$\gamma$ is an important pro-inflammatory cytokine. Distribution of IFN$\gamma$ is an indicator of the activation of both innate and adaptive immune cells.

To determine whether off-target T cell activation induces IFN$\gamma$ release, enzyme-linked immunosorbent assays (ELISAs) were performed. Therefore, supernatants from two-day activation assays with PBMCs, HUVECs and BsMAb were analyzed. It was further investigated, whether blocking agents such as aCD18 antibodies, aCD54 antibodies, and aTNF$\alpha$ antibodies reduce IFN$\gamma$ release after off-target stimulation.

The experiments showed that off-target T cell activation triggered by BsMAb in the

presence of HUVECs induces IFN$\gamma$ release of 6 pg/ml. Addition of aCD54 antibodies and a CD18 antibodies could reduce the release by 50% and 80%, respectively. While off-target activation was only reduced by 50% in the presence of antibodies against TNF$\alpha$, they completely blocked IFN$\gamma$ production by immune cells (Fig. 3.7).

In summary, these experiments showed that antibodies against CD18, CD54, CD2, and TNF$\alpha$, as well as prednisolone are capable of reducing off-target T cell activation, thus preventing proliferation, CD69 expression and cytokine release, with an aCD18 antibody being the most effective blocking reagent.

## 3.5 The impact of blockade on on-target activation

A possible blocking reagent for the clinical application should not interfere with the BsMAb-mediated on-target T cell activation, which is necessary for anti-tumor effects. The following experiments were performed to assess, whether blockade of leukocyte antigens or cytokines will impair on-target activation of T cells mediated by BsMAb. To this end, the previously identified antibodies against CD18, CD54, CD2, and TNF$\alpha$ were tested in comparison with prednisolone, in the presence of target cells to which BsMAb bind.

### 3.5.1 [methyl-$^{3}$H] Thymidine activation assays

For this purpose, four different constellations of BsMAb and target cells were analyzed. CD19$^{+}$ SKW6.4 cells, FLT3$^{+}$ NALM-16 cells, Endoglin$^{+}$ HUVECs, and PSMA$^{+}$ 22RV1 cells were incubated with the respective BsMAb, and thymidine incorporation assays were performed.

The results showed that neither blockade of CD18 or CD11a on T cells, nor CD54 on target cells impair T cell proliferation in any of the four described constellations. In contrast, TNF$\alpha$ blockade could be shown to reduce on-target T cell activation by 0 to 40%. Notably, blockade of CD2 led to a reduction of on-target activation by 30 to 50%. Prednisolone almost completely blocked any T cell activation triggered by a TAA $\times$ CD3 BsMAb and TAA$^{+}$ cells (Fig. 3.8).

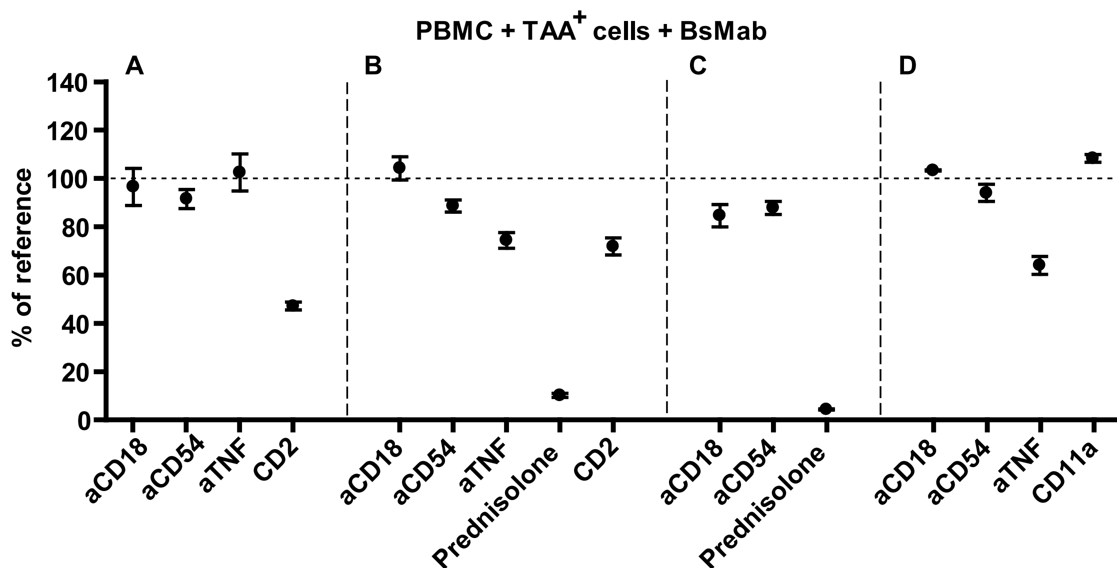


**Figure 3.8:** ***Inhibition of BsMAb-induced on-target T cell activation*** *Freshly isolated PBMCs from healthy donors were cocultivated with TAA$^{+}$ cells and corresponding TAA×CD3 BsMAb at a concentration of 1 $\mu$g/ml and different blocking reagents at a concentration of 1 $\mu$g/ml for two plus one days and incorporated radioactivity was determined.* ***A*** *FLT3$^{+}$ NALM-16 + FLT3×CD3* ***B*** *CD19$^{+}$ SKW6.4 + CD19×CD3* ***C*** *PSMA$^{+}$ 22RV1 + PSMA×CD3* ***D*** *Endoglin$^{+}$ HUVECs + Endoglin×CD3. Representative results from one out of three experiments with PBMCs from different donors are shown.*

### 3.5.2 FACS-based lysis assay

The following lysis assay was performed to examine whether blocking agents interfere with the killing of leukemic blasts by autologous immune cells. PBMCs containing leukemic blasts were drawn from a leukemia patient and cocultivated with a FLT3xCD3 antibody and several blocking agents for 72 hours. FACS analysis was performed to determine reduction of the blast portion and T cell activation. As this assay is conducted using autologous immune cells and leukemic blasts, the experiment is as close as possible to *in vivo* conditions during clinical application.

It was shown that a FLT3 × CD3 antibody is capable of reducing the percentage of leukemic blasts in human PBMCs. After three days, the blast portion was reduced from 50% to 10%. The required dose to yield this effect is about 20 ng/ml. A PSMA × CD3 BsMAb served as the negative control and was not capable of inducing killing of tumor cells.

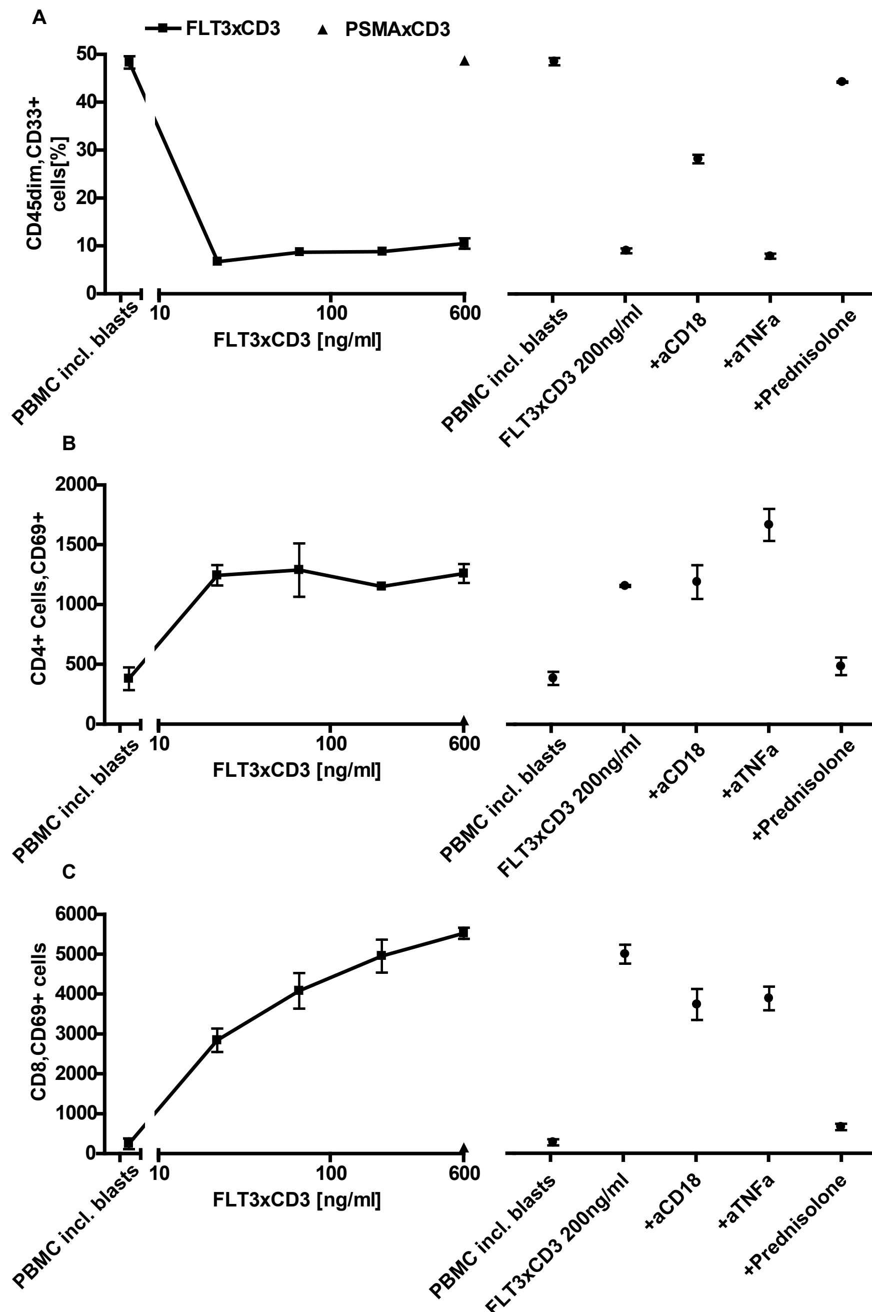


**Figure 3.9:** ***Impairment of the killing of leukemia cells by autologous immune cells*** *500,000 PBMCs including leukemic blasts were drawn from a patient and cocultivated with a FLT3xCD3 antibody at a concentration of 1$\mu$g/ml and several blocking agents for 72 hours. After that, FACS analysis was performed using beads to determine absolute cell numbers.* **A** *Leukemic blasts were defined as CD45dim, CD33$^+$, CD117$^+$.* ***B&C*** *CD69 expression on T cells. Representative results from one out of two experiments with PBMCs from different patients are shown.*

In the next step, different blocking reagents were tested for kill-inhibiting properties. Notably, blockade of CD18 on T cells impaired tumor cell killing, but did not lead to a reduced expression of CD69 on T cells. Addition of aTNF$\alpha$ antibodies did neither impair killing of leukemic blasts nor CD69 expression in this assay, whereas prednisolone caused a complete blocking of BsMAb-induced tumor cell killing and CD69 expression on T cells (Fig. 3.9).

### 3.5.3 IFN$\gamma$ ELISA

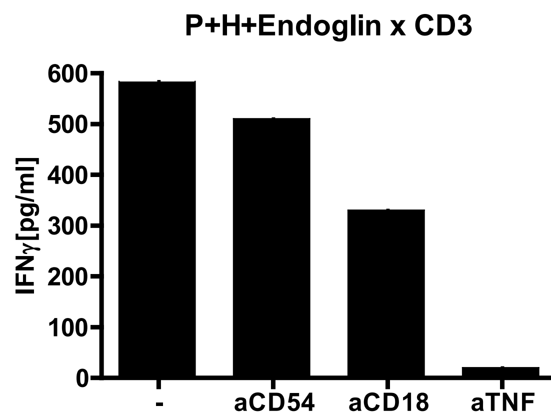


**Figure 3.10:** ***Inhibition of on-target induced IFN$\gamma$ release*** *PBMCs from a healthy donor were incubated with Endoglin$^+$ HUVECs, a Endoglin$\times$CD3 BsMAb at a concentration of 1ug/ml and different blocking antibodies at a concentration of 1$\mu$g/ml for 48 hours. After that, IFN$\gamma$ release was determined using an ELISA. Mean values of triplicate samples are indicated.*

The following ELISA was conducted to assess, whether different blocking reagents reduce the desired IFN$\gamma$ release that was induced by BsMAb-triggered on-target T cell activation.

The results showed that incubation of PBMCs together with Endoglin$^+$ HUVECs and an Endoglin$\times$CD3 antibody led to a release of IFN$\gamma$ at a concentration of 600 ng/ml. Addition of aCD54 antibodies impaired IFN$\gamma$ release only slightly. In contrast, aCD18 antibodies reduced IFN$\gamma$ release by almost 50%, and addition of aTNF$\alpha$ antibodies blocked IFN$\gamma$ distribution completely (Fig. 3.10).

### 3.5.4 XCELLigence lysis assays

XCELLigence assays are impedance-based lysis assays that allow real-time assessment of lysis of adhering tumor cells by immune cells. Adherent tumor cells were seeded in a 96-well E-Plate and cultivated for 20 hours. Then, PBMCs and antibodies were added. Assessment of cell indices (viable tumor cells) was performed every 15 minutes for 96 hours.

The following experiment was conducted to assess, if different blocking reagents impair killing of PSMA$^+$ 22RV1 cells by T cells in the presence of a PSMA$\times$CD3 BsMAb.

It was shown that neither aCD18 antibodies nor aTNF$\alpha$ antibodies inhibit lysis of tumor cells in an XCELLigence assay. In contrast, prednisolone almost completely blocks killing of the tumor cells (Fig. 3.11).

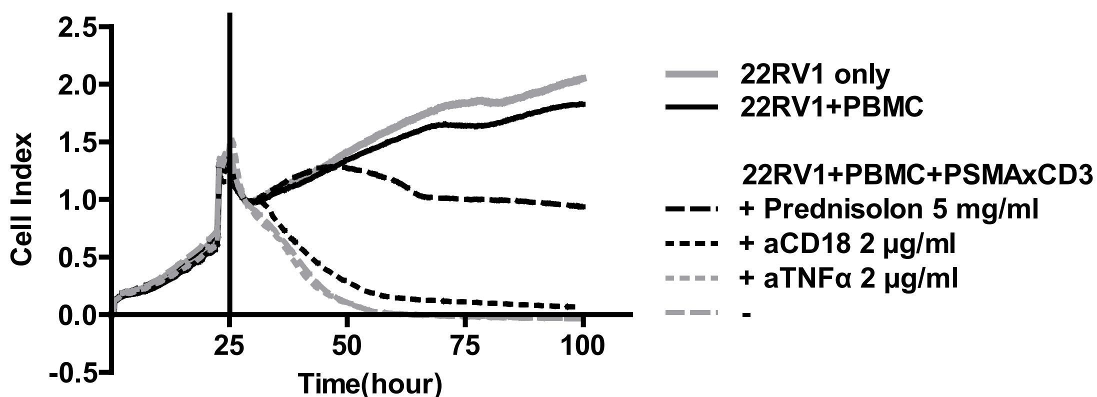


**Figure 3.11:** ***Real-time assessment of tumor cell death mediated by BsMAb*** *22RV1(PSMA$^+$) adherent tumor cells were seeded in a 96-well E-Plate and cultivated for 20 hours. Then, PBMCs and a PSMA$\times$CD3 BsMAb as well as different blocking reagents were added at a concentration of 1 $\mu$g/ml. Assessment of cell indices (viable tumor cells) was performed every 15 minutes for ca. 96 hours. Each curve represents the mean value of triplicate samples.*

### 3.5.5 Target-cell dependency of on-target blockade

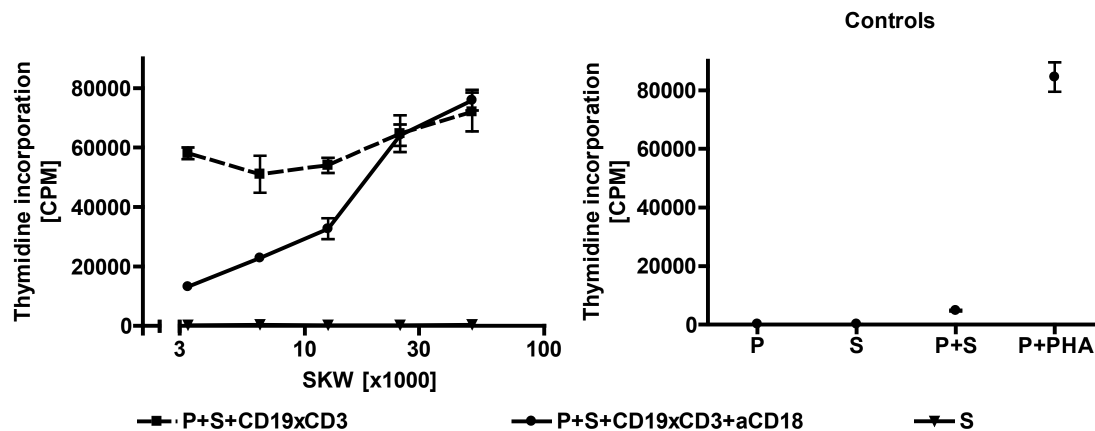


**Figure 3.12:** ***On-target blockade in the presence of varius amounts of target cells*** *100,000 freshly isolated **P**BMCs from healthy donors were cocultivated with different numbers of CD19$^+$ **S**KW6.4 cells and a CD19×CD3 antibody at a concentration of 1 μg/ml for two plus one days. 50% of all samples contained an aCD18 antibody at a concentration of 1μg/ml. Incorporated thymidine was determined. Phytohemagglutinin (PHA) at a concentration of 10 mg/ml was used as a positive control. Mean values and standard deviations of triplicate samples are indicated.*

In the following experiment, the target-cell dependence of on-target impairment was assessed. For this purpose, thymidine incorporation assays were performed using 100,000 PBMCs and differing numbers of CD19$^+$ SKW6.4 cells, a CD19×CD3 BsMAb, and an aCD18 antibody. It could be shown that CD18 blockade impairs on-target activation only at E:T ratios above 3:1. At lower ratios, e.g., 2:1, no blockade of on-target activation could be observed (Fig. 3.12).

In summary, aCD18 and aTNF$\alpha$ antibodies impaired tumor cell killing in different experimental settings only slightly, with the reduction of tumor cell killing by aCD18 antibodies being most pronounced in a FACS-based lysis assay. Anti-CD2 antibodies and prednisolone showed significant reduction of both on-target T cell activation and tumor cell killing, which are undesired effects upon clinical application.

## 3.6 Induction of leukocyte adhesion through off-target stimulation

### 3.6.1 Adhesion assay

The following experiments addressed the induction of leukocyte adhesion through off-target stimulation by BsMAb and the underlying mechanisms. To this end, an adhesion assay was established.

Freshly isolated PBMCs were stimulated with a PSMA$\times$CD3 antibody and plated on a HUVEC monolayer together with blocking antibodies. After an incubation of three hours, the number of adhering CD45$^+$ lymphocytes was determined.

The results suggest that a bispecific PSMA$\times$CD3 antibody is capable of inducing T cell adhesion to an endothelial cell layer. Antibodies against CD18 could inhibit this adhesion completely. The resulting adhesion rate was even lower than the spontaneous adhesion of CD45$^+$ cells to HUVECs. Antibodies directed to CD2 were found to be less effective in reducing off-target induced T cell adhesion (Fig. 3.13).

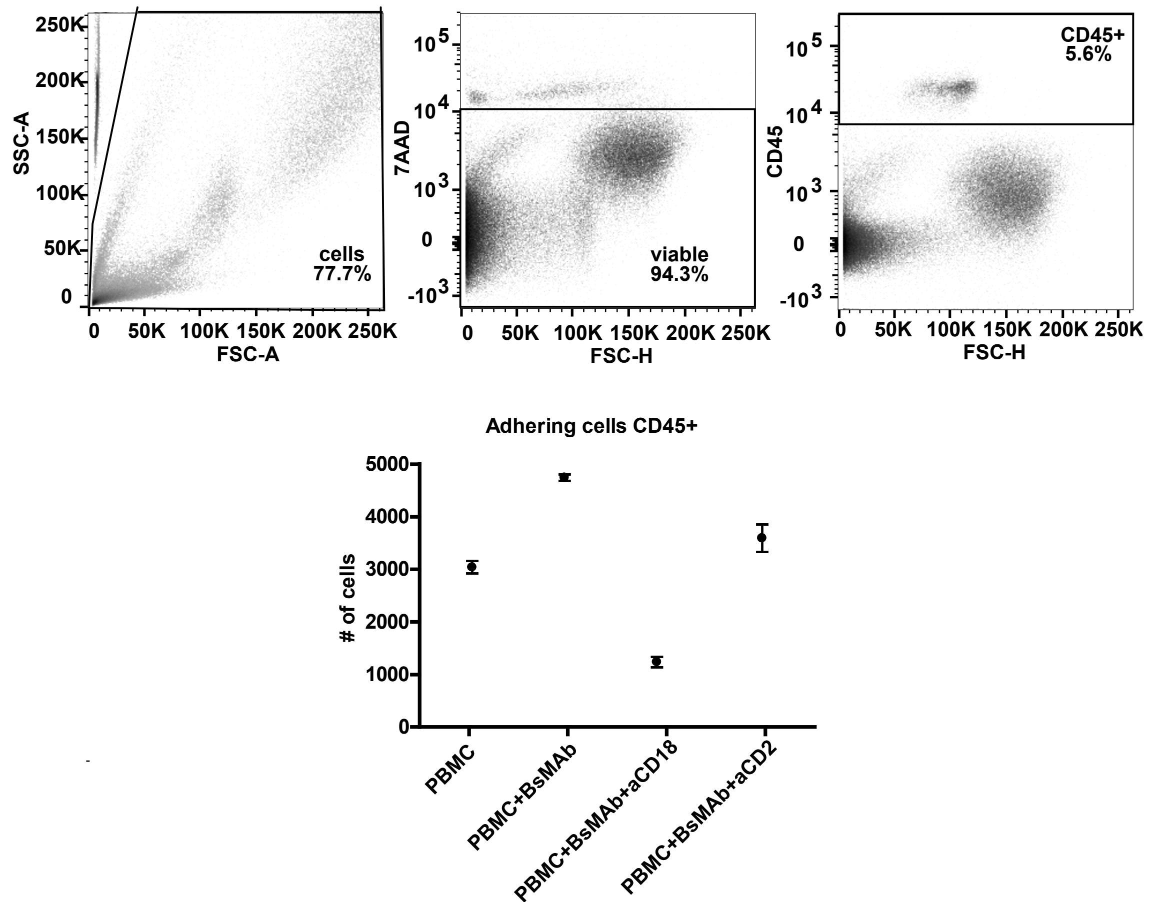


**Figure 3.13:** ***Leukocyte adhesion after off-target stimulation*** *Freshly isolated PBMCs were stimulated with a PSMA×CD3 antibody at a concentration of 1μg/ml for one hour. Subsequently, they were plated on a HUVEC monolayer together with blocking antibodies at a concentration of 1 μg/ml. After an incubation of three hours, non-adherent cells were removed. HUVECs and adhering cells were collected and flow cytometry was performed to determine the number of adhering cells.*

# 4 Discussion

## 4.1 General considerations

Bispecific monoclonal antibodies directed against CD3 mark a very promising class of therapeutic agents that is able to activate T cells against defined target cells, e.g., tumor cells. Multiple clinical trials proved the therapeutic efficacy of such molecules, e.g., in patients suffering from lymphatic leukemia, however at the cost of severe side effects at rather low antibody doses [J. Tibben et al. 1993] [Kroesen et al. 1994] [Topp et al. 2011]. These include fever, chills, headache, hyperthermia, hypotension, and gastrointestinal symptoms, which are induced by massive release of cytokines such as TNF$\alpha$, IFN$\gamma$ and IL-2. The cytokine release syndrome occurs only a few minutes after intravenous application of the BsMAb, thus suggesting that endothelial cells release cytokines in response to BsMAb. This effect is considered an off-target activation of the immune system. While the BsMAb blinatumomab is being activated by CD19$^+$ B cells circulating in the blood, other BsMAb face a similar problem, even though their target antigen is apparently not present in the blood stream. These observations suggest that BsMAb are capable of activating T cells in the absence of target cells at least to some extent, which would explain the side effects seen with BsMAb. The results presented in this work further support this hypothesis.

Assuming, that off-target activation plays a major role in the development of cytokine release syndromes, a better understanding of this phenomenon is of utmost importance.

## 4.2 A bispecific PSMA x CD3 antibody is capable of inducing T cell proliferation due to off-target stimulation

In this work, it could be shown, that a bispecific PSMA$\times$CD3 antibody, NP-CU, is capable of inducing T cell activation in the absence of target cells, which resulted in CD69 expression on the T cells, IFN$\gamma$ release and proliferation. These findings suggest that every BsMAb with a CD3 binding site faces this problem.

Notably, T cell proliferation in thymidine incorporation assays required the presence of BsMAb at doses of 300 ng/ml, whereas CD69 expression was induced by BsMAb at a concentration of 100 ng/ml. These findings suggest that flow cytometry-based activation assays are more sensitive than thymidine incorporation assays.

The formation of aggregates is a common challenge during the production of "complicated" recombinant molecules, such as BsMAb. Size exclusion chromatography performed by Dipl.-Biol. Fabian Vogt showed no evidence of aggregates after purification of NP-CU. Thus it is unlikely that aggregates account for the off-target activation observed in this work. However, further tests for aggregates after a certain storage time should be performed to rule out any late formation of aggregates.

Another possible source of unspecific T cell activation are endotoxins. Moreover, the batch of antibody used in this work was shown to contain no significant amount of endotoxins, ruling out an endotoxin-induced phenomenon.

## 4.3 Stimulating bystander cells enhance off-target activation of T cells

It was demonstrated that certain lymphoid cell lines are capable of enhancing off-target T cell activation in the presence of BsMAb, but not in the absence of such antibodies. It is tentative to speculate that *in-vivo*, cells of the lymph node compartment could exert such stimulatory activity.

Human umbilical vein endothelial cells (HUVECs) have also been shown to augment off-target T cell activation. The findings in this work are consistent with those presented by Molema et al. [Molema et al. 2000]. Our findings suggest that BsMAb-coated T cells in the blood stream are activated by SBCs and thus induce further cytokine release by immune cells as well as endothelial cells. As endothelial cells are most likely the first SBCs encountered by BsMAb after application, further research should focus on the effect of BsMAb on these cells.

The activating properties of SBCs could be shown repeatedly with PBMCs from different donors, and various cell types serving as SBCs, such as SKW6.4, JY and HUVECs. Several other cell lines, e.g., melanoma and glioblastoma cells, did not exhibit such properties. These results clearly show that the stimulatory effect was not due to allogenic reactivity.

## 4.4 The mechanism of off-target activation

The main goal of the subsequent experiments was the identification of costimulatory molecules on SBCs that enhance off-target activation, in order to eventually diminish this effect by antagonistic antibodies. CD40 was the only molecule that was shown to be present on SBCs, but not on non-stimulatory cell lines. However, in blocking experiments, anti-CD40 antibodies failed to exhibit anti-stimulatory properties. The CD40 antibody used in this work was characterized as blocking by the supplier Biolegend. However, the antagonistic properties of the antibody were not confirmed by functional assays. It is therefore still possible, that CD40 plays an important role in the induction of off-target activation, a hypothesis that should be tested in additional experiments.

Likewise, all antibodies directed against costimulatory molecules were characterized as antagonistic by the supplier Biolegend. Thus, the conclusion that not blockable molecules are not responsible for off-target activation, appears to be valid. However, it should be noted that the antagonistic properties of the antibodies used in this work have not been confirmed by own independent functional assays.

ICOS-LICOS interaction was described as a possible cause for dramatic undesired T cell activation, as observed after application of the CD28 MAb TGN1412 [Weissmüller

et al. 2012]. In this work, LICOS was found on both stimulating and non-stimulating cell lines, thus suggesting that this interaction does not play a major role in the induction of off-target activation by BsMAb.

ICAM-1 (CD54) was found to be present on both stimulating and non-stimulating cell lines and is known to be the ligand for LFA-1 (CD11a:CD18). ICAM-1 upregulation, however, occurs rapidly after stimulation of endothelial cells with cytokines such as TNF$\alpha$. This mode of action could explain the leukopenia as well as the overwhelming cytokine response after the application of BsMAb [Molema et al. 2000].

## 4.5 Blocking of defined costimulatory molecules can reduce off-target activation

From what has been said above, it is likely, that ICAM-1/LFA-1 interaction marks a very important step in the induction of off-target activation by BsMAb. This is confirmed by the finding that the most significant reduction of off-target activation was exerted by antibodies against CD18. In experimental settings with both SKW6.4 and HUVECs, anti-CD18 antibodies blocked the undesired T cell activation completely. Application of anti-CD54 antibodies resulted in a reduction of off-target activation that was less pronounced than that observed with anti-CD18 antibodies.

An important difference between the assays with SKW6.4 and HUVECs as SBCs is the influence of anti-TNF$\alpha$ antibodies. Off-target activation triggered by HUVECs was markedly reduced by infliximab, whereas TNF$\alpha$ blockade did not impair SKW6.4-triggered activation. These findings suggest that both TNF$\alpha$-dependent and independent mechanisms are involved in the induction of off-target activation, with endothelial cells being more susceptible to stimulation by TNF$\alpha$.

Furthermore, blockade of CD2 could markedly inhibit off-target activation, suggesting that the interaction of CD2 with CD58 plays a role in the induction of this phenomenon.

## 4.6 Inhibition of on-target activation by blocking reagents

Inhibition of off-target activation by blocking agents could diminish the side effects seen with BsMAb. However, these agents should not impair BsMAb-induced tumor cell killing. The aim of the subsequent experiments was to test anti-CD18 and anti-CD2 antibodies, TNF$\alpha$ blockers, and steroids for any suppressive effects on on-target T cell activation induced by BsMAb in the presence of target cells. To this end, various immunological assays were conducted.

No impairment of T cell activation and tumor cell killing by anti-CD18 antibodies was observed in standard experimental settings such as XCELLigence lysis assays and thymidine incorporation assays. The latter was performed using three different cell lines and three different BsMAb.

However, the experimental setting that resembles the conditions *in-vivo* most closely is a flow cytometry-based lysis assay with PBMCs isolated from leukemic patients. For example, autologous immune cells and leukemic blasts from a patient at effector-target ratios « 1 resemble *in-vivo* conditions of an AML patient suffering from blast crisis. In this assay, anti-CD18 antibodies reduced T cell activation and tumor cell lysis markedly. The very low effector target ratios could explain the susceptibility of tumor cell killing to CD18 blockade in this setting.

Using thymidine incorporation assays, it was shown that the effect of CD18 blockade on on-target T cell activation is target cell-dependent: anti-CD18 antibodies block on-target activation, only if the target antigen is present at a low concentration. This phenomenon could be clinically relevant, since tumor-associated antigens are often upregulated at the tumor site, which means that on-target, but off-tumor activation can be reduced by this antibody, but not on-tumor activation.

It is evident that the application of prednisolone at rather low doses totally diminished anti-tumor activity of BsMAb-triggered T cells. These findings are not consistent with the results presented by Brandl et al.. In this publication, prednisolone exerted no negative influence on tumor cell killing [Brandl et al. 2007]. As corticosteroids are a well-characterised group of immunosuppressant drugs, it appears likely that these drugs impair

BsMAb-induced tumor cell killing *in-vivo*.

A main goal of this work was the identification of antibodies that could replace steroids as first-choice medication for the prevention of off-target T cell activation. As anti-CD2 antibodies effectively impaired on-target T cell activation, they do not appear suitable as a possible medication for the prevention of off-target activation. In contrast, anti-TNF$\alpha$ antibodies as well as anti-CD18 antibodies showed no significant impairment of tumor cell killing in a standard experimental setting, which makes them promising therapeutical options for the prevention of off-target T cell activation.

## 4.7 BsMAb-induced off-target activation induces leukocyte adhesion

Leukopenia in patients that received BsMAb is a phenomenon that could be observed repeatedly [Kontermann 2005]. The most likely reason for that is a transient adhesion of leukocytes to endothelial cells. In preliminary adhesion assays, it could be shown that the BsMAb NP-CU induces adhesion of CD45$^{+}$ cells to a layer of HUVECs. As anti-CD18 antibodies were shown to diminish this effect, it is likely that ICAM-1/LFA-1 interaction plays a major role in the induction of leukocyte adhesion by BsMAb. Further studies should aim at a better understanding of this phenomenon. It remains an open question whether ICAM-1/LFA-1 interaction is the first activating step in a chain of signals that eventually leads to excessive cytokine release, or rather a result of previous cytokine release by T cells.

## 4.8 Clinical application of anti-CD18 and anti-TNF$\alpha$ antibodies

The anti-TNF$\alpha$ antibody used in this work, infliximab, is an authorized pharmaceutical drug for the treatment of inflammatory bowel disease. Infliximab can be safely applied and does not cause severe side effects. Off-label use of infliximab should therefore be considered an option to prevent cytokine release in patients receiving BsMAb.

In the 1990s, different phase 1 and 2 clinical trials with anti-CD18 antibodies were conducted, but the antibodies failed to prove their therapeutic potential in the prevention of myocardial damage following ischemia, hemorrhagic shock and multiple sclerosis. However, it could be shown that anti-CD18 antibodies can be safely applied in patients, resulting in sufficient plasma levels [Baran et al. 2001]. The anticipated mode of action was the prevention of leukocyte migration towards the site of inflammation in order to avoid destruction of ischemic tissue. Such antibodies include Rovelizumab (LeukArrest®), developed by Icos, and Erlizumab, developed by Genentech/Roche.

As an anti-CD18 antibody aims at integrin $\beta 2$, a target that is similar to the target bound by natalizumab, a specific side effect of natalizumab will be discussed here. Natalizumab binds to integrin $\alpha 4$, thus preventing autoreactive T cells from extravasation and subsequent destruction of myelin sheaths in patients suffering from multiple sclerosis. Natalizumab was approved in 2004, but later withdrawn from the market because of multiple cases of progressive multifocal leukencephalopathy caused by JC virus. The antibody was later reapproved by the EMA as monotherapy in JC virus negative patients [Yousry et al. 2006]. Induction of similar side effects by an anti-CD18 antibody appears to be unlikely. First, the anti-CD18 antibody would only be applied once, as a replacement for prednisolone, whereas cases of PML attributed to Natalizumab were only observed in patients that received repeated doses [Bloomgren et al. 2012]. Second, the dosage needed to prevent cytokine release is most probably lower than the doses applied in the clinical trials with Natalizumab.

## 4.9 Outlook

Further research is needed to (1) minimize the off-target properties of BsMAb and (2) identify therapeuticals that suppress off-target T cell activation and subsequent cytokine release, without affecting the desired on-target activity.

Minimization of off-target activation by BsMAb should include complete humanization of therapeutic BsMAbs, characterization of different antibody formats regarding off-target activation, including the use of various CD3-binding epitopes such as OKT3, UCHT-1, and others.

In this work, anti-CD18 antibodies as well as anti-TNF$\alpha$ antibodies were characterized as promising agents for the prevention of off-target T cell activation. *In-vivo* studies are necessary to further confirm the blocking properties of these agents. Possible experimental designs include the use of CD18 knockout mice.

The data presented in this work suggests that CD18 knockout mice are less susceptible to off-target T cell activation. Thus, *in-vivo* studies could be conducted to find out, whether the respective blocking agents impair tumor cell killing or extravasation of immune cells at the tumor site.

An alternative would be the use of surrogate antibodies containing a CD3 antibody, which is reactive with the mouse molecule. Those reagents, and in particular their specific side effects, could be then studied in immunocompetent mice.

# Summary

Recently, bispecific monoclonal antibodies (BsMAb) yielded promising results regarding the treatment of various malignant diseases. However, the serum levels that can be safely achieved in humans remain suboptimal in terms of saturation of the tumor-associated antigen (TAA), as the application of higher doses results in systemic cytokine release and transient leukopenia. Off-target activation of circulating immune cells, stimulated by the BsMAb, could explain most of the side effects observed after in-vivo application of BsMAb.

In this work, immunoassays were conducted, which showed that BsMAb are capable of inducing T cell proliferation in the absence of target cells. Furthermore, it was demonstrated that human umbilical vein endothelial cells (HUVECs) as well as various lymphoid cell lines markedly amplify this effect. They are acting as stimulating bystander cells (SBCs). Several lymphoid and non-lymphoid cell lines did not exhibit this property, thus ruling out allogeneic effects.

Functional assays were conducted to characterize blocking antibodies directed against costimulatory and adhesion molecules on both stimulating bystander cells and immune cells, that are capable of reducing off-target activation. Antibodies against CD54, CD2, and TNF$\alpha$ reduced off-target activation markedly, whereas antibodies directed against CD18 as well as prednisolone blocked it completely.

Further experiments addressed the effect of blocking reagents on the induction of on-target activation and subsequent killing of tumor cells by T cells after stimulation with BsMAb. Prednisolone and aCD2 antibodies impaired tumor cell killing markedly, while only minor reduction was observed with aCD18 and aTNF$\alpha$ antibodies.

Using an adhesion assay, it was shown that PBMCs adhere to a HUVEC layer to a higher extent when stimulated with BsMAb compared to unstimulated PBMCs. Antibodies directed against CD18 could greatly reduce this phenomenon.

In summary, aCD18 and aTNF$\alpha$ antbodies appear to be promising agents for the prevention of cytokine release after application of BsMAb. Eventually, they could replace prednisolone as first line drug, since prednisolone exerted a pronounced inhibition of BsMAb-induced tumor cell killing. However, further *in-vivo* assays need to be conducted to further characterize the blocking properties of aCD18 and aTNF$\alpha$ blocking antibodies and possible side effects, such as a higher susceptibility to infection.

# Zusammenfassung


In den letzten Jahren erzielten bispezifische monoklonale Antikörper (BsMAb) bemerkenswerte Erfolge in der Behandlung von malignen Erkrankungen. Die in Menschen erreichbaren Serumkonzentrationen dieser Antikörper sind jedoch nicht ausreichend, um die anvisierten Tumorantigene (TAA) zu sättigen. Der Grund dafür ist das Auftreten eines Zytokinfreisetzungssyndroms und einer transienten Leukopenie. Die Aktivierung von zirkulierenden Immunzellen durch BsMAb abseits von Tumorzellen würde die meisten Nebeneffekte erklären, die bisher beobachtet wurden.

In dieser Arbeit wurden Immunassays durchgeführt, die zeigten, dass BsMAb T-Zell-Proliferation in der Abwesenheit von Zielzellen induzieren können. Außerdem konnte gezeigt werden, dass humane Nabelschnurvenenendothelzellen (HUVECs) sowie verschiedene lymphatische Zelllinien diesen Effekt verstärken. Deswegen wurden sie im weiteren als stimulierende Bystanderzellen (SBCs) bezeichnet. Andere lymphatische und nicht-lymphatische Zellen verstärkten die unspezifische Aktivierung nicht, wodurch ein rein allogener Effekt ausgeschlossen werden konnte.

Blockierende Antikörper, die gegen bestimmte Zytokine, kostimulierende und Adhäsionsmoleküle auf Immunzellen und stimulierenden Nebenzellen gerichtet waren, konnten die unspezifische Aktivierung reduzieren. So konnten Antikörper gegen CD54, CD2 oder TNF$\alpha$ den Effekt deutlich eindämmen. Antikörper gegen CD18 sowie das Steroid Prednisolon konnten die unspezifische Aktivierung vollständig unterbinden.

Weitere Experimente wurden durchgeführt, um den Einfluss der blockierenden Antikörper auf die BsMAb-induzierte T-Zell-Aktivierung, sowie das nachfolgende Killing von Tumorzellen zu untersuchen. So blockierten Prednisolon und Antikörper gegen CD2 die Aktivierung und Tumorzelllyse vollständig, während Anti-CD18-Antikörper und Anti-TNF$\alpha$-Antikörper kaum negative Effekte hatten.

Mittels eines Adhäsionsassays konnte gezeigt werden, dass PBMCs vermehrt an Endothelzellen (HUVECs) haften, wenn sie vorher mit BsMAb stimuliert wurden. Dieser Effekt konnte mit Antikörpern gegen CD18 unterbrochen werden.

Im Endeffekt stellen Antikörper gegen CD18 und TNF$\alpha$ mögliche Medikamente zur Verhinderung von Zytokinfreisetzungssyndromen dar. Nach eingehender Charakterisierung könnten beide Prednisolon als Therapeutikum der Wahl für diese Indikation ersetzen. Letzteres hatte die Anti-Tumor-Aktivität der T-Zellen in den hier durchgeführten Experimenten deutlich eingeschränkt. Nichtsdestotrotz sollten weitere *in-vivo* Assays durchgeführt werden, um Antikörper gegen CD18 und TNF$\alpha$ weiter zu charakterisieren und mögliche Nebenwirkungen zu erkennen, zum Beispiel eine höhere Anfälligkeit für Infektionen.

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# Erklärung zum Eigenanteil der Dissertationsschrift

Die Arbeit wurde im Interfakultären Institut für Zellbiologie, Abteilung Immunologie unter Betreuung von Prof. Dr. Gundram Jung durchgeführt.

Die Konzeption der Arbeit erfolgte durch Prof. Dr. Gundram Jung.

Sämtliche Versuche wurden nach Einarbeitung durch das Labormitglied Dipl.-Biol. Fabian Vogt von mir eigenständig durchgeführt.

Die statistische Auswertung erfolgte eigenständig durch mich.

Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den 01.11.2016

Joseph Kauer

# Own Publications

Jung G, Salih H, Vogt F, Kauer J.

"*Use of blocking-reagents for reducing unspecific T cell-activation*"

Patent Publication number: EP 3029067 A1

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