ASSESSMENT OF THE ANTIGEN-PRESENTING FUNCTION OF MURINE B CELLS IN VIVO: INDUCTION OF ANTI-TUMOUR IMMUNITY

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Zusammenfassung

B-Zellen sind bisher hauptsächlich für ihre Funktion als Antikörper-produzierende Zellen in der humoralen Immunantwort bekannt. In letzter Zeit jedoch stehen die Antikörper-unabhängigen Funktionen von B-Zellen im Fokus des Interesses. Hier leistet die Antigenpräsentation durch B-Zellen nicht nur einen entscheidenden Beitrag zur Induktion von schützenden Immunantworten, sondern ist im Gegensatz dazu auch beteiligt an schädigenden Immunreaktionen wie Autoimmunerkrankungen. Antigenpräsentation durch B-Zellen ist gekennzeichnet durch die Fähigkeit, B-Zell-Rezeptor-abhängig und -unabhängig Antigene aufzunehmen, zu prozessieren und über MHC-Klasse-II Komplexe zu präsentieren. Während einer Immunantwort interagieren T-Helferzellen über die Expression von CD40-Ligand (CD40L) mit B-Zellen, welche daraufhin aktiviert werden, proliferieren und sich differenzieren.

Diese CD40-aktivierten B-Zellen (CD40-B) sind professionelle Antigen-präsentierende Zellen, vergleichbar mit dendritischen Zellen (DC) und weisen ebenso eine hohe Expression an ko-stimulatorischen und MHC-Molekülen auf. Desweiteren sind CD40-B Zellen nicht nur in der Lage naive $CD4^+$ und $CD8^+$ T-Zellen zu primen, vielmehr induzieren sie auch sekundäre Gedächtnis-T-Zellantworten *in vitro*. Außerdem exprimieren sie vollständig die „lymph node homing triad" (CD62L, CCR7/CXCR4 und LFA-1) und induzieren T-Zell-Chemotaxis *in vitro*. Kürzlich konnten wir zudem zeigen, dass murine CD40-B Zellen (mCD40B) spezifisch in sekundäre lymphoide Organe einwandern. Im Unterschied zu dendritischen Zellen jedoch sind CD40-aktivierte B-Zellen in nahezu unbegrenzter Anzahl mit einer Reinheit von über 95 % für Vakzinierungen verfügbar. Somit können mit CD40-B als zellulärem Adjuvans hohe Impf-Dosen und -Frequenzen realisiert werden, die als äußert wichtig erachtet werden um eine Tumorkontrolle zu erreichen. Allerdings waren B-Zell-basierte Adjuvantien, die *in vivo* in Tiermodellen getestet wurden, bisher meist nicht in der Lage funktionelle und schützende zytotoxische T-Zellantworten zu induzieren.

Im Rahmen dieser Arbeit stelle ich die Entwicklung eines wirksamen mCD40B-Zell-basierten Maus-Vakzinierungsmodells vor. Dieses Model ist durchaus geeignet um *in vivo* zu untersuchen, wie mCD40B-Zellen präventive anti-Tumorantworten induzieren.

So etablierten wir ein System zur Stimulation von murinen B-Zellen, das auf einer wirksamen B-Zellaktivierung durch Ko-Kultivierung mit membranständigem CD40L und Interleukin-4 basiert. Mithilfe dieser Technik ist es möglich hoch-aktivierte mCD40B-Zellen bis zu fünffach und mit einer Reinheit von über 90 % innerhalb von 14 Tagen anzureichern. Wir identifizierten geeignete mCD40B Subpopulationen, die funktionell näher charakterisiert wurden. Obwohl ihre Fähigkeit zur T-Zellstimulation *in vitro* der von murinen dendritischen Zellen unterlegen war, konnten wir zeigen, dass Antigen-beladene mCD40B-Zellen *in vivo* effektiv spezifische zytotoxische T-Zellantworten gegen virales und Modell- Peptidantigen induzieren. Wir konnten die Injektionsroute, die Zelldosis und die Anzahl der Impfwiederholungen als jene Parameter identifizieren, die einen gewichtigen Einfluss auf die Induktion der T-Zellantwort durch mCD40B-Zellen haben. Unter Berücksichtigung dieser Parameter im Vakzinierungsalgorithmus konnte in der vorliegenden Arbeit gezeigt werden, dass mCD40B-Zellen *in vivo* eine Zytolyse von Antigen-spezifischen Zielzellen induzieren, die vergleichbar ist mit der von murinen dendritischen Zellen. Schließlich wurde der Vakzinierungsalgorithmus unter Verwendung von Tumorantigen-beladenen mCD40B-Zellen erfolgreich in einer präventiven Impfung gegen das B16-Melanom angewandt. Zusammenfassend weisen hoch-aktivierte mCD40B-Zellen in der Tat Antigen-präsentierende Eigenschaften auf, die hinreichend sind um eine schützende Immunität gegen das B16-Melanom zu induzieren.

Abstract

B cells have mainly been recognized for their antibody-secreting function in humoral immune responses. Lately, the focus of interest has moved to the antibody-independent functions of B cells. In that context, it has become clear that antigen presentation by B cells is critically involved in both physiologic immune responses as well as pathologic immune reaction such as autoimmune diseases. Antigen presentation by B cells is characterised by their ability to take-up, process and present antigen via MHC-II in a B cell receptor dependent and independent fashion. Furthermore, during immune responses armed helper T cells interact with B cells by expression of CD40 Ligand (CD40L), which thereby become activated and in turn start to proliferate and differentiate.

CD40-activated B cells (CD40-B) are professional antigen-presenting cells comparable to dendritic cells (DC) exhibiting a high expression of costimulatory and MHC molecules. CD40-B cells were shown to not only prime naïve $CD4^+$ and $CD8^+$ T cells efficiently, but also expand memory T cells *in vitro*. Furthermore they express the full lymph node homing triad (CD62L, CCR7/CXCR4 and LFA-1) and were shown to induce T-cell chemotaxis *in vitro*. Recently, we demonstrated that murine CD40B cells (mCD40B) specifically migrate to secondary lymphoid organs *in vivo*. Importantly, these cells are available at virtually unlimited amounts at a purity > 95% for high-dose, high-frequency vaccination considered crucial for the control of cancer. However, most B cell-based vaccines tested so far failed to induce functional and protective cytotoxic T lymphocyte (CTL) responses in animal models *in vivo*.

Within this work I present the successful development of a functional murine mCD40B-based vaccination model. This model is indeed suitable to study the ability of mCD40B cells to induce protective anti-tumour responses *in vivo*.

We established a murine B-cell stimulation system based on potent B-cell activation maintained by co-cultivation with membrane-bound CD40 ligand and interleukin-4. By using this technique highly activated mCD40Bs expand more than 5-fold with purity above 90% within 14 days. Hence, we identified appropriate mCD40B subsets and further characterised them on a functional level. Despite their T-cell stimulatory capacity *in vitro* being inferior to murine dendritic cell (mDC) subsets, we demonstrated that vaccination with antigen-loaded mCD40B effectively induces specific CTL responses against viral and model peptide antigen *in vivo*. We clearly identified injection route, cell dose and vaccination repetitions as parameters exerting dominant influence on the effective induction of specific T cell responses by mCD40B. Incorporating these parameters into the vaccination algorithm, I showed that mCD40B induce *in vivo* cytolysis of antigen-specific target cells comparable to mDC. Finally, by using tumour antigen-loaded mCD40B we successfully transferred our vaccination algorithm to preventive immunization against B16 melanoma. In summary, highly activated mCD40B cells indeed exhibit antigen-presenting capacity sufficient to induce protective anti-tumour immunity in B16 melanoma.

List of Abbreviations

Table 1: List of Abbreviations

Acronym	Expansion
aa	amino acid
aAPC	artificial antigen-presenting cell
ADCC	antibody-dependent cell-mediated cytolysis
AIDS	Acquired immunodeficiency syndrome
APC	antigen-presenting cell
β-ME	β-Mercaptoethanol
BCR	B-cell receptor
BSA	bovine serum albumin
CAR	chimeric antigen receptor
CD40-B	CD40-activated B cell
CD40L	CD40 ligand
CDC	complement-mediated cytotoxicity
cDNA	complementary DNA
CEA	carcinoembryonic antigen
CLP	common lymphoid progenitor
CTL	cytotoxic T cell
CpG-ODN	oligodeoxynucleotides with non-methylated cytosine-guanine motifs
CR2/1	complement receptor 2/1
CRPC	castrate-resistant prostate cancer
CsA	Cyclosporin A
CVCTWG	Cancer Vaccine Clinical Trial Working Group
DC	dendritic cell
DMSO	dimethyl sulfoxide
EBNA-1	Epstein-Barr virus nuclear antigen 1
ECM	extracellular matrix
FACS	Fluorescence Activated Cell Sorting
FasL	Fas ligand
FBS	foetal bovine serum
FcR	Fc-Receptor
FO	follicular
GC	germinal centre
GM-CSF	granulocyte/macrophage colony-stimulating factor
H&E	hematoxylin and eosin
HEV	high endothelial venules
HGFR	haematopoietic growth factor receptor
HPV	human papillomavirus
HSC	hematopoietic stem cell

Acronym	Expansion
HTL	helper T cells
IFA	Incomplete Freund´s Adjuvant
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ip	intraperitoneal
iv	intravenous
LPS	lipopolysaccharide
LT	lymphotoxine
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MLR	Mixed Lymphocyte Reaction
NC	negative control
NEAA	non-essential amino acid
NK	natural killer cell
NKT	natural killer T cell
mCD40B	murine CD40-activated B cell
mDC	murine dendritic cell
mRNA	messenger ribonucleic acid
MZ	marginal zone
PAP	phosphatase antigen
PC	positive control
PBS	phosphate-buffered saline
PGE-2	prostaglandin E2
PMA	Phorbol 12-Myristate 13-Acetate
PSA	prostate-specific antigen
RAG	recombination activating gene
RT	Room temperature
sc	subcutaneous
SD	standard deviations
SLO	secondary lymphoid organ
TAA	tumour-associated antigen
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TGF	transforming growth factor
TNF	tumour necrosis factor
TLR	Toll-like receptor
Treg	regulatory T cell
TRP	tyrosinase-related protein
TSA	tumour-specific antigen
VEGF	vascular endothelial growth factor

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INTRODUCTION

1. INTRODUCTION

This study wants to contribute to the current understanding of B-cell function other than in humoral immune responses. In particular, the role of murine CD40-activated B cells (CD40-B) as model of professional antigen-presenting cells alternative to dendritic cells (DC) is analysed.
In order to establish how the immunological characteristics of CD40-B cells affect the induction of specific T cell responses against cancer, the basic principles of cancer immunology are discussed in detail (section 1.1.). Due to their immune-stimulatory capacity CD40-B cells are recognized as cellular adjuvant. Therefore, current concepts of targeted cancer immunotherapy are subsequently summarized (section 1.2.). Thereafter, I set up how the model of CD40-CD40 ligand interaction equips B cells with antigen-presenting function and report on the current knowledge in this regard (section 1.3.). Finally, the key questions and aims leading to the present study are identified (section 1.4.).

1.1. Basic Principles of Cancer Immunology

By definition immunology deals with the complex defence system that protects an organism differentiating self from foreign and neutralizing pathogenic organisms or substances. To date a growing body of knowledge about the complex interactions of immunological active organs, tissues, cells and signalling molecules comprising the immune system exists. Our understanding is based on the principles of innate and adaptive immunity and their cellular players inducing cellular and humoral immune responses.

1.1.1. Overview of the Immune system

On the one hand cells of the innate immune system build the first line of defence. Macrophages equipped with specialized receptors recognize pathogen and hence induce phagocytosis of pathogens and release of cytokines and chemokines like tumour necrosis factor-α (TNF-α) and interleukin-12 (IL-12) which drive inflammation and the attraction of further leukocytes (Mantovani A. and Sica A., 2010 1; Ma X., 2001 2). Furthermore macrophages are activated through interferon-γ (IFN-γ) and CD40 ligand (CD40L) by armed $CD4^+$ helper T cells triggering production of toxic nitrogen or oxygen intermediates, thereby exhibiting direct antimicrobial activity (Dedon P.C. and Tannenbaum S.R., 2004 3). Macrophage-derived cytokines or release of IFNs activates natural killer (NK) cells which recognize transformed cells lacking MHC molecules. Their cytotoxic activity is tightly regulated by stimulatory and inhibitory receptors and induced by release of lytic granules. On the other hand NK cells and macrophages recognize immunoglobulin G (IgG) -coated pathogens by binding to their Fcγ-receptors leading to antibody-dependent cell-mediated cytolysis (ADCC) or phagocytosis of infected cells (Herberman et al., 1986 4). In ADCC apoptosis of infected cells is induced via release of perforin and granzymes.
Natural killer T cells (NKT) are considered as specialized $CD4^+$ T cell subset and a remainder of an evolutionary primitive form of the immune system. They co-express a characteristic NK receptor (NK1.1) and a T-cell receptor. NKT cells recognize antigens presented by the MHC-like molecule

CD1 expressed by most of the antigen-presenting cells. Upon CD1-antigen recognition they immediately start secretion of IFN-γ or IL-4 thereby probably polarizing type 1 or 2 T-cell responses (Berzins et al., 2011 5).
On the other hand T cells are the principal effectors of the adaptive immune responses, mainly mediated by $CD8^+$ cytotoxic T cells (CTL) and $CD4^+$ helper T cells (HTL). Professional antigen-presenting cells (APCs) take up antigen directly in the blood-stream or in tissue and migrate to lymph nodes or spleen where they present it to T cells. This interaction drives the initiation of antigen-specific adaptive immune responses. In order to activate naïve T cells two signals are necessary: First, antigen presentation via MHC molecule on APC recognized by a specific T-cell receptor (TCR) on the T cell; and second, costimulation via B7 receptors (CD80 and CD86) on activated APC and CD28 on armed T cell (Bretscher P.A., 1999 6; Bevan M.J., 2004 7).

1.1.1.1. CD8+ cytotoxic T cells

As the potent effectors of adaptive immune responses $CD8^+$ T cells are able to recognize and eliminate cells presenting 8-10 amino acid long peptides arising from proteasomal degradation of intracellular proteins bound to MHC-I (Klein et al., 2000 8). Naïve $CD8^+$ T cells become initially activated ("primed") when antigen presented by activated APC is recognized via MHC-I. Then $CD8^+$ T cells rapidly expand and differentiate into effector T cells which migrate to the periphery. There they function by production of cytokines such as IFN-γ as well as the apoptosis inducing effector molecules perforin and granzyme B. Additional up-regulation of Fas ligand (FasL) on CTLs mediates direct cytolysis of target cells. Furthermore memory T cells are established which respond faster and develop more efficiently into effector cells upon re-exposure of antigen (Weninger et al., 2002 9). Co-stimulatory agents and cytokines therefore represent key strategies for the modulation of tumour-directed T cell responses.

1.1.1.2. CD4+ helper T cells

$CD4^+$ helper T cells (HTL) recognize 10-34 amino acid long peptides from exogenous proteins, endocytosed and digested intracellularly in endosome-lysosome compartments and presented on MHC-class II molecules (MHC-II)(Klein et al., 2000 8). Cytokine release during initial stage of immune response drives the differentiation of HTL into two major classes:
Type 1 cytokine-secreting $CD4^+$ helper T cells (T_h1 cells) provide essential help for activation and expansion of $CD8^+$ T-cell responses. T_h1 cells develop in the presence of IL-12 and are characterized by secretion IFN-γ and TNF-β favouring cellular immune responses. They activate APCs mainly through engagement of CD40 ligand expressed on $CD4^+$ T cells and CD40 on APCs (Bourgeois et al., 2002 10). These CD40-activated APCs in turn up-regulate MHC and co-stimulatory molecules and are able to directly drive $CD8^+$ T cell responses (Lanzavecchia A., 1998 11). Upon activation by IL-4 and IL-6 T_h2 cells secrete IL-10 and transforming growth factor-β (TGF-β) thereby effectively activating B cells in humoral responses and inhibiting T_h1 driven cellular responses. Furthermore antigen nature has an influence on type of HTL-response induced: Whereas a strong interaction

and high density of antigen tend to promote T_h1-like response, weak interaction or low density predominantly causes T_h2-like responses (Fishman M.A. and Perelson A.S., 1999 12).

1.1.1.3. Immunological Tolerance

The absence of an immune response to a particular set of self-antigens shows that the immune system does discriminate between self and non-self. There are two mechanism involved in the development of immunological tolerance.

In thymus-dependent central tolerance developing T cells are selected for their recognition pattern (positive selection) and affinity (negative selection), mainly resulting in a T-cell repertoire recognizing self MHC molecules complexed to foreign peptides (Hogquist et al., 2005 13). But also auto-reactive T cells that escape thymic deletion recognizing cryptic or subdominant epitopes of self-antigens can develop (Farris et al., 2000 14).

In peripheral tolerance absent costimulatory signal results in incomplete activation of APCs due to deficient expression of B7 receptors (CD80, CD86). Thus, T cells specific for certain tissue antigens not selected for in thymus become insufficiently activated and are then tolerized by antigen recognition and do not induce autoimmune responses (Albert et al., 2001 15).

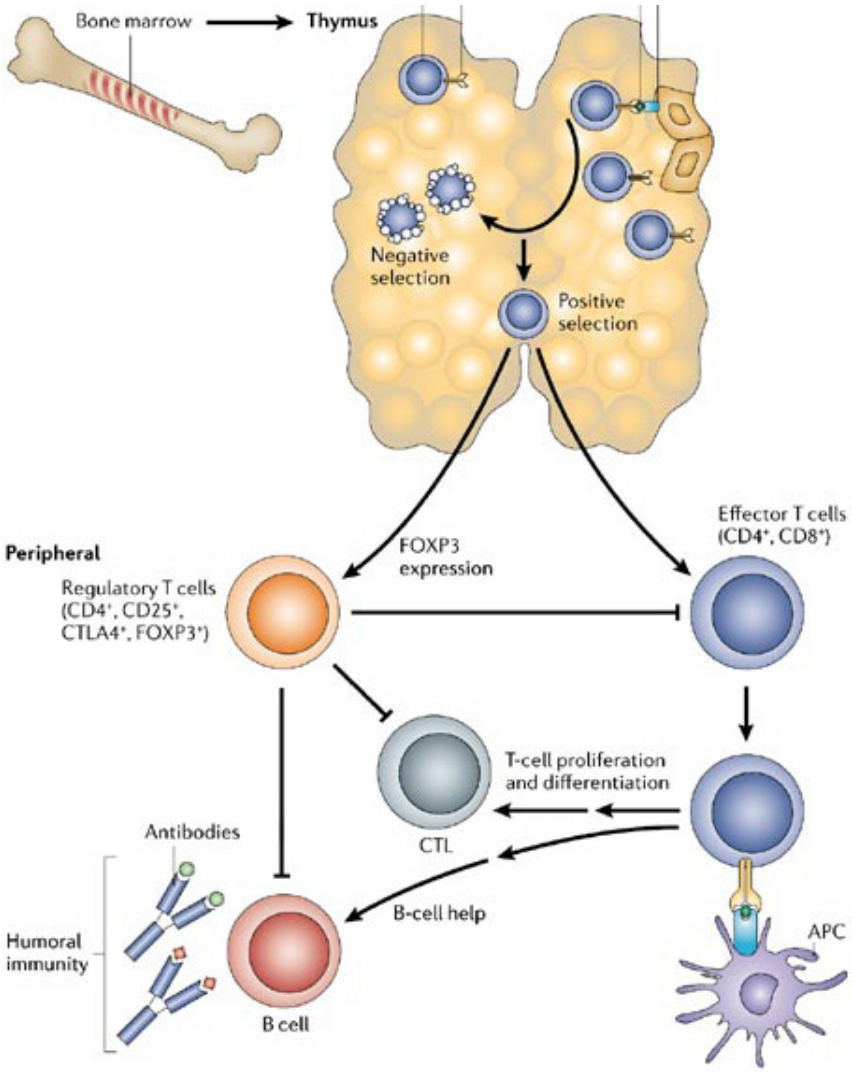


Figure 1: Central and peripheral tolerance mechanisms

The upper panel shows thymic central tolerance mechanisms due to negative and positive selection of self-reactive thymocytes giving rise to CD4+ and CD8+ effector T cells and - depicted in the lower panel - regulatory T cells which are the key mediators of peripheral tolerance (adopted from Gregersen et al., 2006 16).

Recently, the involvement of regulatory T cells (Treg), which are capable of suppressing T-cell responses through direct contact with APCs or through production of anti-inflammatory cytokines, in the induction of tolerance has been recognized (Nomura et al., 2005 17; Shevach E. M., 2009 18).

1.1.1.4. Lymphopoiesis and Humoral Function of B cells

Diverse roles of B cells in immune responses have been discovered with functions in the innate and adaptive branches of the immune system as well as interacting with many other cell types. A short overview of the main B-cell developmental stages and immunological functions will be described in the next section.
B cells develop in the bone marrow in continuous developmental stages from hematopoietic stem cells (HSC) to common lymphoid progenitors (CLP), pro B cells and pre B cells. These stages are characterized by immunoglobulin gene rearrangement providing diversity of the B-cell antigen receptor (BCR) repertoire as well as by changes in gene expression and surface phenotype (Osmond et al., 1990 19; Hardy et al., 1991 20; Hardy R.R., 2003 21). Furthermore, dysfunctional or auto-reactive B cells recognising self-antigens are eliminated by receptor editing (Melchers et al., 1995 22; Hardy et al., 2000 23). Immature B cells then leave bone marrow and enter the spleen where transitional (T1 and T2) are followed by mature B cell stages giving rise to naïve functional B cells. Past this step in maturation when expression of IgM isotype is accompanied by IgD on the surface of B cells, engagement of the BCR by an antigen leads to terminal differentiation (Osmond et al., 1998 24; Rolink et al., 1999 25). Three subsets of naïve B cells are generally distinguished due to their location: marginal zone (MZ), follicular (FO) and B-1 B cells.

B-1 B cells locate mainly in the pleural and peritoneal cavities (Kantor et al., 1993 26). Because of their mainly foetal origin B-1 B cells may represent a separate lineage from MZ and FO B cells (Martin F. and Kearney J.F. 2001 27; Allman et al., 2004 28). B-1 B cells are involved in very early stages of thymus-independent immune responses against particulate bacterial antigens, like lipopolysaccharide (LPS) and other Toll-like receptor (TLR) -ligands. Upon BCR-signalling they can, together with MZ B cells, differentiate into short-lived IgM-producing plasmablasts responsible for the majority of “natural” IgM in the serum (Martin F. and Kearney J.F. 2000 29; Hardy R.R and Hayakawa K., 2001 30).

Marginal zone B cells are located to the marginal sinus in the spleen which borders the red and the white pulp and do not re-circulate after differentiation (Martin F and Kearney J.F. 2002 31). Due to their location MZ B cells like MZ macrophages are able to respond early after infection as they are into close contact with blood-borne antigens and pathogens, thereby linking innate and adaptive immunity (Kraal G., 1992 32). Following stimulation of BCR and TLRs or TLRs alone, they migrate to the T-B border of the lymphoid follicle where they can present antigen to $CD4^+$ T cells (Attanavanich K. and Kearney J.F., 2004 33). Upon migration into the red pulp these MZ B cells will differentiate into plasma cells secreting large amounts of IgM (Martin et al., 2001 34). Alternatively, after a

combinatorial encounter of antigen and complement along with subsequent engagement of BCR and complement receptor CR2/1 (CD21/CD35) MZ B cells are able to transfer antigen to follicular DC leading to downstream T-cell activation (Whipple et al., 2004 35; Ferguson et al., 2004 36). As largely B-1 and MZ B cells do not form germinal centres and therefore do not undergo somatic hypermutation simply low-affinity plasma cells are formed. This is in contrast to functional roles of follicular B cells.

Follicular B cells make up the biggest B cell population re-circulating through blood and lymph to B cell follicles of lymph nodes, spleen and Peyer´s patches mediating humoral B cell functions (Allman D. and Pillai S., 2008 37). Herein, interaction of cognate antigen with specific BCR will lead to cross-linking of BCR and BCR-dependent signal transduction, initiating receptor-mediated endocytosis of the antigen, antigen processing in endosomes and its presentation onto MHC-II molecules (Lanzavecchia A., 1990 38). Upon antigen-encounter B cells change their chemokine-receptor profile and migrate to the B-T boarder where activated $CD4^+$ T cells with corresponding TCR can bind to the MHC-antigen complex. Clustering of T and B cells after cognate antigen-recognition is also referred to as “immunological synapse” describing the highly organized spatial and temporal processes of signal transduction upon antigen-recognition (Grakoui et al., 1999 39; Pereira et al., 2010 40). Upon building of the “primary focus” some activated B cells differentiate into plasmablasts with initiation of antibody synthesis and further into short-lived plasma cells producing large amounts of secreted IgM antibody (Jacob et al., 1991 41). Furthermore some activated B cells migrate to lymphoid follicles and form germinal centres (GC) there (Thorbecke et al., 1993 42; Carter R.H. and Myers R., 2008 43). Here activated B cells built the light and dark zones composed of strongly proliferating centroblasts as well as centrocytes undergoing somatic hypermutation, affinity maturation and isotype switching resulting in long-lived plasma cells producing affinity-matured antibody (mainly IgG, with some IgE and IgA), and memory B cells (Coico et al., 1983 44; Ziegner et al., 1994 45). These are released from the GC, thus providing a more effective subsequent secondary immune response. Upon cognate antigen re-exposure memory B cells react more quickly and start producing large amounts of high-affinity antibodies that neutralize their antigen upon binding. Furthermore antibodies trigger complement-mediated cytotoxicity (CDC), enable lysis of coated pathogens by ADCC and mediate apoptosis by disrupting oncogenic signals. (Rolink A. and Melchers F., 1991 46; Rajewsky K., 1992 47; Mackay C.R., 1993 48).

1.1.1.5. Antigen Presentation by Dendritic cells

An immune response will only be generated when antigen is presented by an activated APC, such as dendritic cell, macrophage or B cell (Cassell et al., 1994 49; Askew et al., 1995 50; Banchereau et al., 1998 51). This happens in secondary lymphoid organs (SLO) like spleen, lymph nodes, tonsils and Peyer´s patches where the organ architecture brings antigen and APC into close contact with T cells. It is accepted that DCs play a critical role in inducing primary T-cell responses, thus permitting establishment of immunological memory (Steinman R.M., 1991 52; Hart D.N.J., 1997 53; Banchereau et al., 2005 54; Palucka et al., 2011 55).

After the first report on dendritic cells by Steinman and Cohn with a round, branching morphology (Steinman et al., 1973 56) different subtypes arising from $CD34^+$ bone marrow progenitors have been identified: (A) $CD11c^+$ myeloid DC give rise to Langerhans cells localized in epidermis, interstitial DC localized within other tissue than skin and (B) $CD11c^-$ lymphoid DC resulting in plasmacytoid DC and (C) follicular DC assumedly derived from mesenchymal progenitors with different phenotype as well as innate and adaptive immune functions (Shortman et al., 2002 57; Steinman et al., 2010 58). Progenitor DCs develop in the bone marrow and migrate through the blood into peripheral tissues where they encounter antigen. There immature DCs develop and efficiently capture antigen by several uptake mechanisms such as phagocytosis, macropinocytosis and receptor-mediated endocytosis. Upon antigen processing DC mature by means of inflammatory stimuli and CD40-CD40L interaction and migrate through the afferent lymphatics to the draining lymphoid organs where they present antigen to T cells. Thereby cellular immune responses are induced involving both $CD4^+$ helper T cells (T_h1) and $CD8^+$ cytotoxic T cells. Furthermore, besides inducing cellular immunity DCs elicit humoral immune responses by activation of naïve and memory B cells. Finally DC are also able to activate cells of the innate branch of immunity, such as NK cells and NKT cells as well as non-antigen specific macrophages and eosinophils (Banchereau et al., 1998 51 and 2000 59; Steinman R.M., 2008 60). Hence, “nature´s adjuvant” dendritic cells drive all arms of the immune system and have therefore been intensively studied for use in vaccination against cancer.

1.1.2. Cancer Immunosurveillance

Already at the beginning of the 19th century Paul Ehrlich suggested that the immune system could recognize and eliminate neoplastic cells (Ehrlich P., 1909 61). 50 years later Burnet and Thomas formulated the "cancer immunosurveillance hypothesis" which stated that a cellular immune reaction might protect from neoplastic disease (Thomas L., 1959 62 and 1982 63; Burnet F.M., 1970 64 and 1971 65). Due to a lack of direct experimental evidence this hypothesis remained highly controversial. Only during the last 15 years researchers have been able to show that the immune system indeed plays a vital role in the control of neoplastic growth.

Based on tumour formation experiments in mice in the 90s three fundamental mechanisms of anti-tumour immunity were discovered: First, the contribution of endogenously expressed cytokine IFN-γ to protection against growth of tumours (Kaplan et al., 1998 66; Street et al., 2001 67). Second, perforin-mediated cytotoxicity of effector lymphocytes was shown to control tumour development (van den Broek et al., 1996 68; Street et al., 2001 67). And third, by using immune-deficient RAG-2 knockout mice lacking mature lymphocytes their protective role against tumour formation was demonstrated (Shankaran et al., 2001 69).

Further pieces of evidence for immune surveillance derive from observations made in humans. On the one hand an increased incidence of oncogenic virus infections and B-cell lymphomas in transplant patients treated with immunosuppressive drugs and AIDS patients was found (Grulich et al., 2007 70). On the other hand a number of bacterial infections were shown to be cancer-associated and several cancer-causing viruses have been identified (Mager D.L., 2006 71; Sarid R. and Gao S.-J., 2011 72).

1.1.3. Cancer Immunoediting

To describe the complex interactions that occur between a developing tumour and the immune system the "cancer immunosurveillance" hypothesis was extended from a simple host-protective role of the immune system to a more complex process in which the tumour is imprinted by its immunological environment, termed "cancer immunoediting" (Dunn et al., 2002 73 and 2004 74,75). This process can lead to three possible outcomes. (A) Either the tumour is completely eliminated by the innate and adaptive arms of the immune system (elimination phase), or (B) in case of elimination failure a dynamic equilibrium between tumour and immune system (equilibrium phase) is kept, when due to selection pressure through heterogeneity and genetic instability tumour cells can sustain immune attack. (C) The third phase is reached when these unstable, mutated tumour cells become resistant to immune detection (escape phase) and malignant disease develops.

In the light of these findings Hanahan and Weinberg added immune surveillance to their definition of the "Hallmarks of cancer" (Hanahan et al., 2000 76 and 2011 77). Furthermore, recently the involvement of the tumour microenvironment to the tumour-formation process has been identified. Herein various cell types located in the tumour microenvironment build up tumour stroma where a cross-talk of immune and inflammatory cells, blood and lymph vessels and nerves takes place. Fibroblasts secreting growth factors and chemokines support angiogenesis, and altered ex-

tracellular matrix (ECM) provides additional oncogenic signals enhancing cancer-cell proliferation and invasion (Liotta et al., 2001 78).

1.1.4. Tumour-mediated Immune Escape

Mechanisms underlying tumour evasion are in focus of interest to break tolerance to tumour antigens and to induce anti-tumour responses. Several tolerance mechanisms by which tumours evade destruction by the immune system (immune-escape) have been discovered.
Active tolerance mechanisms induced by the tumour (figure 2) include the down-regulation of MHC-molecules or the tumour-antigen itself leading to impaired T-cell recognition (Ferrone et al., 1995 79). Secretion of immunosuppressive anti-inflammatory cytokines such as TGF-β, IL-10 and expression of co-inhibitory molecules such as apoptosis-inducing Fas-Ligand were shown to induce apoptosis of tumour-reactive T cells and immune evasion (Chen et al., 1994 80, Walker et al., 1998 81; Teicher et al., 2007 82).

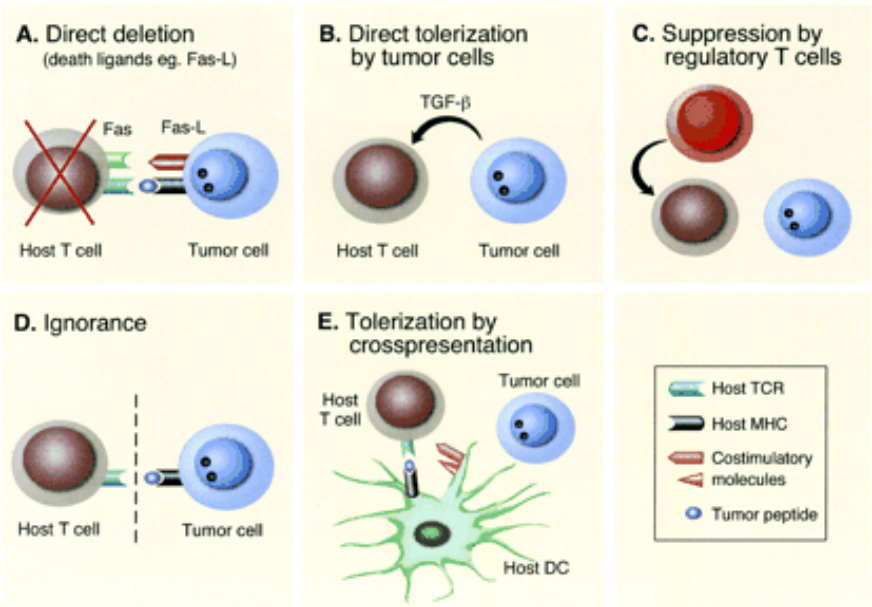


Figure 2: Tumour-mediated immune evasion

Tumour cells may escape immune recognition through (A) direct deletion by engagement of death-inducing ligands, (B) direct tolerization by secretion of immunosuppressive cytokines, (C) suppression of tumour-reactive T cells by Treg, (D) spatial separation and / or (E) tolerization of T cells by cross-presentation of tumour-derived antigens (adopted from Mapara et al., 2004 83).

At last, for the successful treatment of cancer the key step is to prime T lymphocytes resulting in the induction of an effective immune response finally leading to tumour destruction.

1.2. Targeted Cancer Immunotherapy

Already in the 1890s William Coley pioneered the field of immunotherapy. He supposed a causal link between streptococcal infection and tumour regression in some of his patients, leading him to the first documented attempts to treat cancer with mixed bacterial vaccines, so-called Coley´s toxins (Coley W.B., 1893 84). Later it´s use was replaced by the promising advent of chemotherapy and radiation therapy. Given that cancer resists conventional surgery and nonsurgical treatment options and still remains major cause of death worldwide, the development of novel prophylactic and therapeutic strategies is required. More than one century of growing body of knowledge about the complex cellular and molecular interplay between immune system and tumour and the identification of tumour-associated antigens (TAA) have led to the development of targeted cellular immunotherapeutic strategies to face malignant tumour (reviewed in Baxevanis et al., 2009 85).

1.2.1. Tumour Antigens

The link between specific immune responses and targeted cancer immunotherapy was established with the first identification of tumour antigens recognized by $CD8^+$ cytotoxic T cells in human melanoma (van der Bruggen et al., 1991 86). During the last 20 years a large array of immunogenic tumour antigens has been identified and characterised (van der Bruggen et al., 2011 87). Furthermore to support and accelerate the development of therapeutically effective cancer vaccines the American National Cancer Institute (NCI) has developed a priority ranking of 75 cancer vaccine target antigens through defining and weighting relevant or 'ideal' cancer antigen criteria as the following (in descending order): therapeutic function, immunogenicity, oncogenicity, specificity, expression level and percent antigen-positive cells, stem cell expression, number of patients with antigen-positive cancers, number of antigenic epitopes and cellular location of expression (Cheever et al., 2009 88). Basically, two primary groups can be distinguished: tumour-specific antigens and tumour-associated antigens (Graziano et al., 2005 89).

1.2.1.1. Tumour-specific antigens

Tumour-specific antigens (TSA) result from modification in genes of tumour cells that may be implicated with tumoural transformation (Carbone et al., 2005 90). They arise from genetic alterations like point-mutations, deletions or chromosomal translocations (Bielas et al., 2006 91). Since they are unique for an individual patient their usefulness for standard immunotherapy is limited, because most are highly patient-specific and therefore manufacture of such a vaccine would be labour-intensive and costly (Sensi et al. 2006 92). Then again, shared tumour-specific antigens have been isolated found among several tumours of patients which are derived from oncogenes and tumour suppressor genes. For example, oncogenic *ras* being mutated in 30-40% of all colorectal carcinomas, and the most prominent tumour suppressor p53 being mutated in 60-70 % of all cancers (Bos et al., 1989 93 and Chiba et al., 1990 94, respectively). Other tumour-specific

antigens are virus-associated proteins, like the oncoproteins E6 and E7 from the human papillomavirus (HPV) expressed in cervical cancer, or like EBNA-1, the Epstein-Barr virus nuclear antigen expressed by Burkitt´s lymphoma and nasopharyngeal carcinoma cells (zur Hausen H., 2002 95; Hislop et al., 2007 96). Being foreign and therefore highly immunogenic viral oncoproteins do not bear the risk to induce tolerance or autoimmunity reactions after vaccination.

1.2.1.2. Tumour-associated antigens

Tumour-associated antigens are tumour-antigens expressed differently by cancer cells and most normal cells. Herein, the first group consists of proteins encoded by genes usually expressed exclusively in primitive germs cells of the testes. Because these cells do not express MHC molecules, antigenic peptides should not be presented to T cells. In cancer cells those germ line genes can be aberrantly activated (Caballero et al., 2009 97). Therefore such antigens can be considered as strictly tumour-specific. The genes encoding such antigens have also been named cancer-testis genes including for example the melanoma-antigen family (MAGE) of proteins.
The second group contains differentiation antigens only expressed in certain types of tissue in embryogenesis or in particular stages of differentiation. Examples are Melan-A, gp100, tyrosinase and tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2) which are expressed by both healthy melanocytes and melanoma cells. Therefore, their use as targets for cancer immunotherapy may result in auto-immunity towards the corresponding normal tissue. Other well-known group members are prostate-specific antigen (PSA) in prostate carcinoma and carcinoembryonic antigen-related cell adhesion molecule (CEA) in gut carcinoma.
The third group of tumour-associated antigens is characterised by expression in normal tissue and overexpression in tumour cells for example due to gene amplification. Prominent group members are HER2/neu and telomerase, which are overexpressed in several tumour entities. As those antigens are expressed in normal tissue only at very low level a minimal amount of peptide is required to elicit a T-cell response and auto-immunity should not be induced (van der Bruggen et al., 2011 87).
Besides identifying immunogenic tumour antigens as targets for cancer immunotherapy and choosing the right platform in which to deliver these, it is essential to break self-tolerance and minimize contrary tumour-escape mechanisms. Two basic immunotherapeutic approaches for the manipulation of the immune system to regain effective anti-tumouricidal properties can be distinguished.

1.2.2. Passive Immunotherapy

On the one hand, passive strategies aim at stimulating the patient´s immune system through adoptive transfer of anti-tumour antibodies, antibody-derivatives or cytolytic effector cells, like T cells, NK cells or NKT cells (Rosenberg et al., 1986 98; Wu et al., 2003 99; Motohashi et al., 2006 100; An Z. et al., 2011 101; Chhabra A., 2011 102). There are several monoclonal antibodies that have been approved by the US Food and Drug Administration (FDA) for the use in human. For example antibodies like Herceptin directed against specific antigens aberrantly expressed on tumour cells (ErbB2 in breast cancer) lead to the destruction target cells by CDC and ADCC. Further monoclonal antibodies block the action of inhibitory receptors (e.g. anti-CTLA-4 and anti-PD-1) or trigger activation of effector lymphocytes (e.g. anti-4-1-BB). Moreover other lethal principles are coupled to antibodies, like toxins, radionuclides or enzymes (Zarour H.M. and Ferrone S, 2011 103). In adoptive T cell transfer tumour-specific T cells are re-infused into the lympho-depleted patient to induce anti-tumour immunity. They derive from patients enriched *ex vivo* by either multiple *in vitro* stimulations with tumour antigen or by genetic engineering to introduce tumour antigen-specific TCR (Baxevanis C.N. and Papamichail M., 2004 104; June C.H., 2007 105). Furthermore chimeric antigen receptors (CAR) represent a promising technology where genetic constructs are introduced into lymphocytes containing an antigen-recognizing antibody linked to a T-cell signalling domain. Thereby T cell specificity and effector activity are redirected against target cells recently showing positive results in early clinical studies (Porter et al., 2011 106; Kalos et al., 2011 107).

1.2.3. Active Immunotherapy

On the other hand, active immunotherapeutic approaches aim at induction of live-long immunity through eliciting a specific *in vivo* anti-tumour response by vaccination (Dougan et al., 2009 108). Herein, the adaptive immune system is specifically activated through the induction of cellular immune responses, for example by $CD8^+$ and $CD4^+$ T cell recognition of peptides presented by MHC class I and II, respectively. Peptides may be presented to T cells after processing of intracellular antigens or after APCs engulf extracellular antigens, process them, and present peptides on the host´s endogenous MHC molecules via cross-presentation. Different treatment modalities including the injection of inactivated autologous cancer cells, as well as the injection of tumour antigen, protein, peptide, or nucleic acid delivered alone, cellularly or genetically, with or without adjuvant are possible.

Strategies comprise non-cellular vaccines with purified tumour-antigen or synthetic peptide as well as vaccines containing autologous tumour lysates or irradiated tumour cells. However these approaches involve the combination with adjuvants. Such agents composed of aluminium-salts or oily emulsions augment the immune response to an antigen unspecifically, for example through tissue irritation and attraction of immune cells or through prolonging the time of antigen release (*depot-effect*).

Other cellular vaccination strategies directly use the immunological properties of APCs to induce

tumour-specific T cells - the main advantage being selective with only little toxicity (Ribas et al., 2003 109; Berzofsky et al., 2004 110; Rosenberg 2004 111; Hinz et al., 2006 112). Most effort is undertaken using dendritic cells as APCs in various settings. As an alternative artificial APCs (aAPC) or B cells are investigated (Schultze et al., 1997 113 and 2004 114; Banchereau et al., 2005 54; Mason et al., 2008 115; Butler et al., 2010 116). Strategies with APCs loaded with TAA aim at inducing effective cellular and antibody-mediated responses specific for antigens selectively expressed by the tumour and at avoiding unintended tissue damage as seen by other conventional approaches such as chemotherapy (Grabbe et al., 1995 117; Ward et al., 2003 118; Banchereau et al., 2005 54). To this end challenges to be met have been identified as finding immunogenic tumour antigens, overcoming tolerance and generating professional antigen-presenting cells that effectively activate T-cell immunity.

1.2.4. Dendritic cell Vaccination

In mice vaccinations with bone-marrow-derived *ex vivo* matured antigen-loaded DCs have already been shown to induce protective and therapeutic anti-tumour immunity (Mayordomo et al., 1995 119; Zitvogel et al., 1996 120; Gilboa et al., 1998 121). Several strategies to select immunogenic, tumour-specific antigen targets and to deliver the antigens to DCs have been explored. Thus tumour antigens are isolated from cDNA-libraries or from peptides eluted from the tumour cell surface and DC are loaded by simply incubation with antigen ("pulsing") in forms of peptides or proteins (Parkhurst et el., 1996 122; Li et al., 2002 123), by transfecting with mRNA or plasmid DNA (Boczkowski et al., 1996 124) or by transducing with viral vectors (Ribas et al., 2002 125). Further DC vaccination strategies include tumour-DC fusions, loading DC with dying tumour cells (Albert et al., 1998 126; Berard et al., 2000 127) and enhancing the immune-stimulatory context in which the vaccines are delivered, for example by targeting DCs *in vivo* with antibodies specific for DC surface molecules like DEC205 or DC-SIGN (Gilboa E., 1999 128; Fong et al., 2000 129).
In order to further improve DC-based immunotherapeutic approaches processes exploited by tumours to down-regulate effective immune responses elicited by DC vaccines are investigated; for example the targeting of endogenous DCs in tumour-bearing patients by tumour-derived factors like IL-10, TGF-β, VEGF (vascular endothelial growth factor), IL-6 and PGE-2 (prostaglandin E2) leading to a subsequent delay in maturation and impaired development of effector T-cell functions (Almand et al., 2001 130; Inaba et al., 2003 131, Pinzon-Charry et al., 2005 132).
With the discovery of methods to generate human autologous DCs *ex vivo* for example by differentiating $CD34^+$ progenitors with granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 into immature DC and maturing them or terminally differentiating them by triggering toll-like receptors for example with CpG-ODN or PolyIC and tumour-necrosis factor receptors (TNF-R) with LPS, tumour-necrosis factor-α (TNF-α) or CD40 ligation, clinical studies using DC as vaccines were facilitated (Caux et al., 1992 133; Romani et al., 1994 134; Sallusto et al., 1994 135). These revealed the importance of the maturation status of DCs and the importance of the particular subset of DCs being targeted. It was demonstrated that antigen-presentation by immature DC subsets induces undesired antigen-specific tolerance mechanisms impeding effec-

tive anti-tumour responses. Moreover different DC-maturation programs elicited by treatment with various cytokine combinations also result in differential effects on T-cell function (figure 3). So by generation of different DC subsets distinct types of immune responses can be induced, for example favourable induction of tumour-specific $CD8^+$ T cells, T_h1 polarization and limited induction of Treg as well as a deleterious T_h2 directed T-cell response, T-cell anergy, T-cell deletion and the development of Treg further limiting DC application (Steinman et al., 2003 136; Mailliard et al., 2004 137; Liu et al., 2009 138).

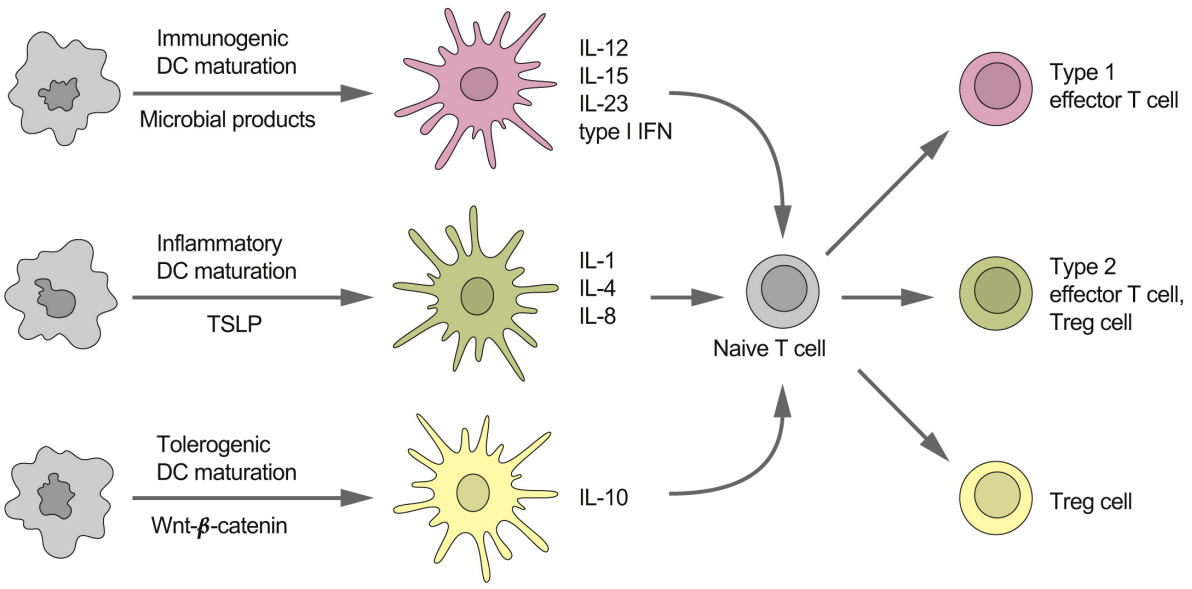


Figure 3: Dendritic cell activation stimuli and subsequent type of T-cell response

Activation of DCs by immunogenic, inflammatory or tolerogenic stimuli leads to their maturation and subsequent release of distinct cytokine patterns. Depending on the signal the type of the elicited adaptive immune response will be determined. TSLP: thymic stroma lymphopoietin (adopted from Palucka et al., 2011 139).

Another challenge regarding the quality of immune responses elicited by DCs is represented by their capacity to migrate to the T cell-rich areas of secondary lymphoid organs, to closely interact with T cells and thereby influence the homing properties of the effector cells (Morse et al., 1999 140; Verdijk et al., 2008 141). Thus besides DC subset, maturation status, dose and frequency of vaccinations, the injection route (intravenous vs. non-intravenous routes: subcutaneous, intradermal, intralymphatic, intranodal) is considered an important variable that has to be adjusted to improve immunotherapy (Mullins et al., 2003 142 and Palucka et al., 2005 143, Draube et al., 2011 144). As intralymphatic and intranodal injections are technically challenging and still only less than 4 % of the administered DC reach the regional lymph nodes, intradermal and intravenous injections are preferred in clinical studies (Ridolfi et al., 2004 145; Quillien et al., 2005 146),

whereas in mice subcutaneous injection is effective in both targeting DC to draining lymph nodes and inducing immune responses (Eggert et al., 1999 147; Okada et al., 2001 148).

During the last decade few hundred clinical trials have been performed using DCs as cellular adjuvants in cancer immunotherapy with low induction of clinical responses. In 2005 the Cancer Vaccine Clinical Trial Working Group (CVCTWG) of experts from academia, biotech and pharmaceutical industry as well as regulatory bodies from Europe and America elaborated a *clinical development paradigm for cancer vaccines and related biologics* addressing end points for clinical trials, trial designs and statistical methods, technical and developmental challenges as well as combination therapy to set up a guideline meeting the specific requirements of therapeutic cancer vaccines (Hoos et al., 2007 149). The major feasibility hurdles have been identified as the reliable production of functioning DC preparations as it is difficult to quality control the levels of antigens being processed and presented by DC. Furthermore ensuring the proper maturation status and the appropriate DC subset are critical for ensuring that DCs are capable of migrating into secondary lymphoid organs to prime T cells. DC vaccinations are costly in terms of labour and money as autologous DC preparations have to be generated individually for each patient.

Nevertheless, recently the first autologous DC-based therapeutic cancer vaccine, Sipuleucel-T (PROVENGE) based on *ex vivo* generated DC activated with a recombinant PAP-GM-CSF fusion protein (PA2024) used to target prostate acid phosphatase antigen (PAP) on prostate cancer cells has been approved by the US Food and Drug Administration (FDA) prolonging the median survival of men with advanced castrate-resistant prostate cancer (CRPC) by 4.1 months (Cheever M.A. and Higano C.S., 2011 150). On the other hand strategies targeting DC *in vivo* are pushed forward recently. While GVAX® consisting of two prostate cancer cell lines transfected with GM-CSF failed to exceed standard therapy in the phase III study, other strategies aiming at immune activation by targeting costimulatory molecules like B7.1, ICAM-1 and Lfa-3 (targeted in PROSTVAC®) seem to be promising (Galsky et al., 2011 151; Heery et al., 2011 152). Furthermore, anti-CTLA-4 monoclonal antibody Ipilimumab blocking immune inhibitory pathway leading to T-cell inactivation, has shown to be effective in immunotherapeutic treatment of metastatic melanoma (Cameron et al., 2011 153). Thus, combinatorial strategies with immunomodulatory agents and DCs together with conventional strategies like radiation and chemotherapy are considered to provide new therapeutic approaches and improve the therapeutic efficacy of vaccination strategies against cancer (Palucka et al., 2010 154 ; Le et al., 2010 155). An overview of DC-based therapeutic vaccination strategies is given in figure 4.

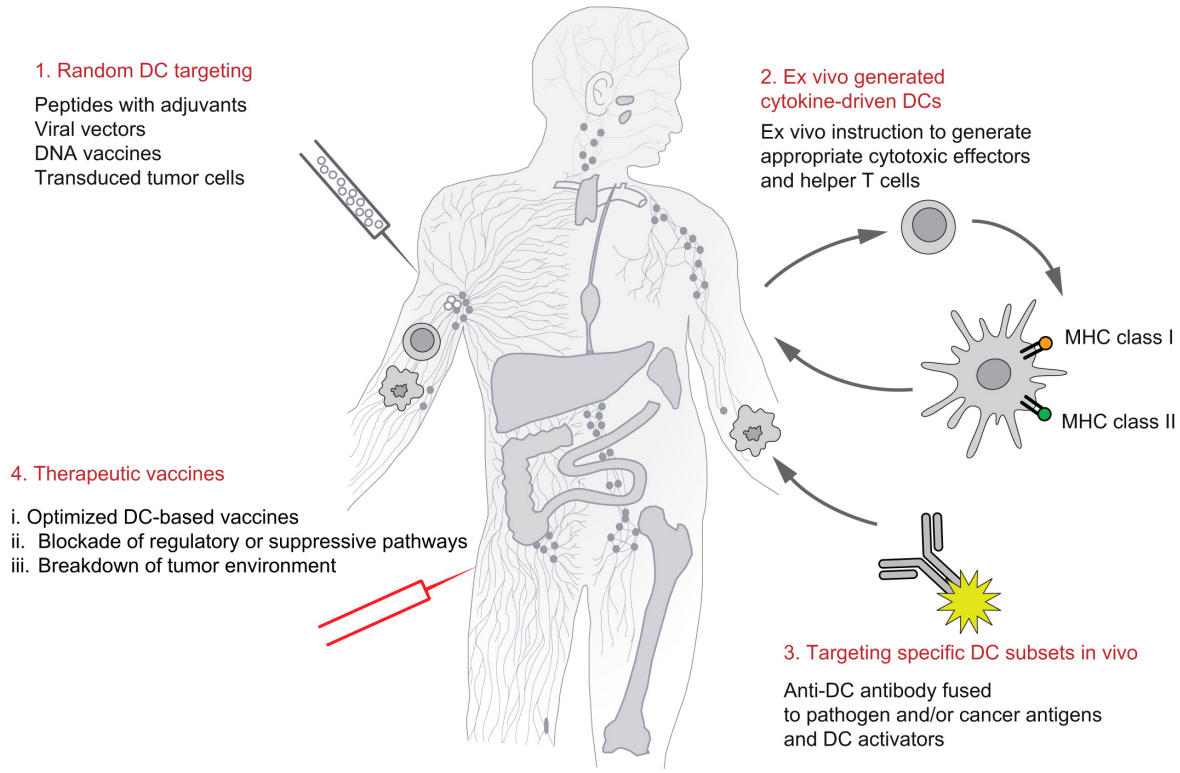


Figure 4: Therapeutic vaccination strategies with DC

1) Antigen-based vaccination randomly targeting DC *in vivo*. 2) *Ex vivo*-generation of antigen-loaded DC infused back to the patient. 3) Specific DC-targeting *in vivo* and 4) optimized combinatorial DC approaches (adopted from Palucka et al., 2011 139).

1.3. B cells as Antigen-Presenting cells

Besides their role as producers of antibody in humoral immune responses B cells are increasingly recognized as antigen-presenting cells which play a crucial role in the initiation of T cell responses. Even though dendritic cells are thought to be the most important APCs, it has become clear that antigen-presentation by B cells plays an important role under certain circumstances. Compared to DCs B cells are less efficient at capturing antigens by either adsorption, or pinocytosis, or Fc-Receptor (FcR)-mediated up-take (Chesnut et al., 1982 156; Grey et al., 1982 157; Batista F.D. and Neuberger M.S., 1998 158; Rodriguez-Pinto D., 2005 159). Despite the fact that lymph-borne soluble antigens enter the lymph node via the afferent lymph vessels at the subcapsular sinus and diffuse directly into the follicle where the B cells reside (Pape et a., 2007 160), antigen-presentation to B cells is usually thought to be mediated through cross-presentation by other professional APCs that take up antigens unspecifically through generic receptors, for example subcapsular-sinus macrophages in the lymph nodes and follicular DCs (FDCs) in lymph nodes and spleen (Wykes et al., 1998 161; Qi et al., 2006 162; Carrasco Y. and Batista F., 2007 163; Phan et al., 2007 164).
In contrast, specific BCR-mediated antigen up-take processing of antigen in the endosomal compartment leads to an export of MHC class II-peptide complexes to the B-cell surface. As mentioned before sufficient T cell activation occurs when naïve T cells receive two signals from APCs: signal 1) recognition of the MHC-peptide complex by TCR and signal 2) engagement of the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) on APCs via interaction with CD28 on T cells (Bretscher P.A., 1999 6). However, the expression of co-stimulatory molecules on APCs needs to be induced first and in turn is triggered by the reciprocal interaction of CD40L (CD154, gp39) expressed on activated T cells with CD40 receptor expressed on APCs, such as B cells (van Kooten C. and Banchereau J, 2000 165).

1.3.1. CD40-CD40L Interaction

The well recognized model of CD40-CD40L interaction has been extensively investigated on molecular and functional levels and shown to regulate diverse cell-mediated immune functions (Banchereau et al., 1994 166; Foy et al., 1996 167; Grewal I.S. and Flavell R.A., 1996 168; van Kooten C. and Banchereau J., 1996 169; Noelle R.J., 1996 170; Mackey et al., 1998 171): CD40 is a cell surface receptor that belongs to the TNF-receptor family. First identified in 1985 on B cells, engagement of CD40 receptor on B cells was shown to play an important role in B-cell survival and proliferation, GC and memory B-cell formation, and immunoglobulin isotype switching, functions which are abrogated in patients suffering from hyper-IgM syndrome due to defects in CD40-CD40L interaction. CD40-activation of B cells exhibits directly effects on cytokine production as well as on expression of adhesion, costimulatory and MHC class I and II molecules. CD40 was demonstrated to be also critical for the maturation of DCs, and CD40 expression on endothelial cells, fibroblasts and epithelial cells was shown to be involved in inflammatory responses, autoimmunity and tolerance induction. Thus CD40-CD40L interaction is playing a more general role in cellular immune regulation.

The CD40L homotrimer is transiently expressed on mature $CD4^+$, $CD8^+$ and γδ T cells and its ligation through cross-linked CD40 enhances cytokine production of T cells. Furthermore, different CD40L mRNA species have been identified in monocytes, DCs, B cells, NK cells and further more. CD40 and CD40L have also been described in soluble forms enabling a cytokine-like action at distant sites and CD40-CD40L interactions can result in bidirectional stimulatory signalling of B and T cells. Thus, besides its role in humoral immunity CD40-CD40L interaction has also been investigated for its essential role in the initiation of antigen-specific T-cell responses. There is no doubt that CD40-CD40L interaction is essential for effective *in vivo* priming, expansion and maturation of $CD4^+$ T cells into effector cells (Grewal et al., 1995 172; van Essen et al., 1995 173; Grewal I.S. and Flavell R.A., 1996 174; Scaria et al., 1997 175). Furthermore this interaction plays an important role for the establishment of memory CTL and for providing CD4-dependent help for cross-priming of $CD8^+$ cytotoxic T cells (Borrow et al., 1996 176; Bennett et al., 1997 177; Bennett et al., 1998 178; Schoenberger et al., 1998 179; Bourgeois C. and Tanchot C., 2003 180).

Pharmacologic manipulation of the CD40-CD40L pathway seems a promising tool in the treatment of different immunological diseases. On the one hand a specific interference of uncontrolled immune reactions, like inflammation, autoimmunity and allo-graft rejection can be achieved by using blocking anti-CD40L antibodies (Larsen et al., 1996 181; Kirk et al., 1997 182; Kirk et al., 2001 183; Pearson et al., 2002 184; Grammer et al., 2003 185; Sidiropoulos P.I. and Boumpas D.T., 2004 186). On the other hand the amplification of CD40-CD40L interactions via agonistic CD40-antibodies results in direct anti-proliferative anti-tumour effects by binding to CD40 expressed on tumour cells or mediates cellular immunity required for effective tumour protection (Mackey et al., 1998 187; Diehl et al., 1999 188; Tong A.W. and Stone M.J., 2003 189; Watanabe et al., 2003 190; Eliopoulos A.G. and Lawrence S.Y., 2004 191; Vonderheide et al., 2007 192). Thus, triggering CD40 on APCs displays an attractive approach to manipulate their capacity to prime and expand antigen-specific $CD4^+$ and $CD8^+$ T cells.

1.3.2. CD40-activated B cells

Based on the observation that long-term human B cell lines could be established by triggering CD40 and IL-4 (Banchereau et al., 1991 193 and 194) the group of Lee M. Nadler first reported that CD40-activated human B cells can serve as an alternative source of highly efficient APC to induce allogeneic $CD4^+$ and $CD8^+$ T cell responses and to generate autologous tumour-antigen specific T cells *in vitro,* comparable to dendritic cells (Schultze et al., 1997 113). Furthermore, upon CD40 ligation and IL-4 signalling these B cells strongly expand from small amounts of peripheral blood, up-regulate co-stimulatory, MHC as well as adhesion molecules, and show increased antigen presentation capacity *in vitro,* which was confirmed by Lapointe et al. in 2003 (Lapointe et al., 2003 195). Thus, in addition to implementing CD40-stimulated B lymphocytes in the context of tumour-antigen discovery (SEREX) and the *ex vivo* generation of T cells for adoptive immunotherapy (Kondo et al., 2002 196; Fujiwara et al., 2005 197; Ivanov et al., 2005 198) the clinical application of CD40-activated B cells as antigen-presenting cells in therapeutic vaccination strategies appears

promising (von Bergwelt-Baildon et al., 2002 199; Coughlin et al., 2004 200; Yoon et al., 2005 201; Van den Bosch et al., 2005 202; Shen et al., 2008 203; Wu et al., 2010 204).

To this aim, diverse *ex vivo* strategies to manipulate CD40-CD40L interactions in order to activate B-lymphocytes and evaluate their potential as APC in cellular therapies have been developed (reviewed in Néron et al., 2011 205). Besides triggering CD40 with agonistic monoclonal anti-CD40 antibodies, usage of recombinant soluble CD40L proteins, soluble CD40L membrane fractions as well as CD40L expressing feeder cell lines have been tested alone or in combination with other stimuli and an impact of signal strength (cross-linking) and nature (soluble vs. membrane bound) on B-cell activation has been proposed (Fanslow et al., 1994 206; Néron et al., 2005 207). Furthermore different antigen-loading strategies have been tested showing that CD40-activated B cells can be pulsed with peptide, protein, tumour lysates, transfected with DNA or RNA, or transduced with viral vectors (Kondo et al., 2002 196; von Bergwelt-Baildon et al., 2002 199; Lapointe et al., 2003 195; Coughlin et al., 2004 200; Ahmadi et al., 2008 208).

Several studies have proven that antigen-presenting activated B cells induce effective expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells *in vitro*, however also $CD4^+$ or $CD8^+$ and $CD25^+FoxP3^+$ Treg can be expanded with resting or suboptimally activated B cells emphasising the need for the application of optimal activated B cell formulations in the right setting (Tu et al., 2007 209; Zheng et al., 2009 210; Zheng et al., 2010 211). In addition, the capacity of activated B cells to migrate *in vivo* and home to secondary lymphoid organs has been under examination by us and others (unpublished data N. Klein-Gonzáles; Guo et al., 2009 212). We could show that human CD40-activated B cells express the full lymph node homing triad of CD62L, CCR7/CXCR4 and LFA-1, and induce T-cell chemotaxis, requirements necessary for successful T-cell interaction (von Bergwelt-Baildon et al., 2006 213). Furthermore when properly activated GFP-labelled CD40-B cells were injected intravenously (iv) into recipient mice, we were able to find significant amounts in peripheral lymph nodes and spleen. There, CD40-B cells localized in the B cell zones of the splenic white pulp and in the B/T boundary close to the T cell zone in spleen and lymph nodes. In contrast, when administered subcutaneously the number of CD40-B cells found in SLO was significantly lower than when injected iv (unpublished data N. Klein-Gonzáles).

Finally, very few in studies have been conducted so far investigating the *in vivo* antigen-presenting capacity of CD40-activated B cells and their participation in anti-tumour immunity. In 2004 Ritchie et al. showed that mice immunized with B cells activated with membrane fractions containing CD40 ligand and loaded with the MHC-I binding peptide $LCMV_{33-41}$ [KAVYNFATM] injected iv could induce a delay in tumour growth of the LCMV-transfected tumour cell line LL-LCMV in a preventive setting. Furthermore, this effect was not only due to cross-dressing of antigen from CD40-B cells to other antigen-presenting cells but also through direct APC mechanisms (Ritchie et al., 2004 214). Still, the contribution of B cells as antigen-presenting cells to induction of antigen-specific T- cell responses is discussed controversially.

1.4. Purpose of this study

While we have detailed knowledge about the antibody-dependent functions of B cells, comparatively little is known about the antibody-independent functions, such as antigen presentation and their contribution to the induction of primary immune responses *in vivo*. In order to elicit a functional immune response, antigen-presentation to and co-stimulation of T cells are prerequisites of professional APCs – which have shown to be fulfilled by properly activated B cells *in vitro*, too. In order to study antigen-presentation by B cells *in vivo* this study aimed at the development of a murine model which enabled the examination of antigen-presentation by activated B cells.

The first step was to develop a cell culture system for the generation of murine CD40-activated B cells (mCD40B) based on murine CD40-ligand expressing feeder cells (tmuCD40L HeLa), in order to test whether the strength and nature of the applied CD40 stimulus and cultivation conditions were able to drive B cells through their differentiation program to generate suitable stimulators of immune responses. Second, mCD40B cultivation conditions were optimized on the basis of yield, purity and cell activation status and the immune stimulatory efficacy of mCD40B compared to state-of-the-art murine dendritic cells (mDCs) was evaluated *in vitro*. With the intention to find the main parameters impacting the strength of the elicited immune-response *in vivo* a murine vaccination model was developed based on the loading of mCD40B with foreign antigen by means of peptide-pulsing. Furthermore, the specific immune response elicited by mCD40B loaded with peptide was characterised phenotypically and functionally by assessing cytokine secretion and by measuring cytotoxic potential against target cells, respectively. Finally, as a proof-of-principle and to investigate whether vaccination with mCD40B is able to overcome immune evasion and to prime T cells, antigen-presentation by mCD40B was studied in a murine melanoma model.

Taken together, this work therefore investigates antigen presentation by B cells particularly in the context of tumour immunotherapy with the aim to provide further insights into the mechanisms of the APC function of B cells and their therapeutic manipulation.

MATERIAL & METHODS

2. MATERIAL & METHODS

2.1. Chemicals

For preparation of buffers and solutions chemicals of analytically purity were used exclusively.

Table 2: Chemicals

Name	Manufacturer
Acetic Acid	Roth
β-Mercaptoethanol (β-ME)	Sigma
Bovine Serum Albumin (BSA)	Sigma
5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE; CFSE) - mixed isomers	Invitrogen
Cyclosporin A (CsA)	Sigma
CpG-ODN D-SLIM	Mologen
Dimethyl sulfoxide (DMSO)	Sigma
Ethanol	Roth
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Foetal Bovine Serum (FBS) (Lot 5SB0018)	Lonza
VLE-FBS (very low endotoxin) (Lot 6SB0007)	Lonza
Gentamicin	PAA
Golgi Stop (Monensin)	BD
Hydrochloric Acid (HCl)	Roth
Hygromycin	PAA
Ionomycin	Sigma
Isopropanol	Roth
LPS (derived from E.coli 055:B5)	Sigma-Aldrich
Pancoll Mouse (density 1.086 g/mL)	PAN Biotech
Paraformaldehyde (PFA)	Sigma
Phorbol 12-Myristate 13-Acetate (PMA)	Sigma
Sodium Azide (NaN3)	Sigma
Sodium Chloride (NaCl)	Roth
Sodium Hydroxide (NaOH)	Roth
Trypan Blue Stain 0.4 %	Gibco
Trypsin-EDTA	Gibco

2.1.1. Antibodies

Table 3: Anti-Mouse Antibodies

Antigen [clone]	Species / Ig class	Labelling	Manufacturer
B220 (CD45R)	Rat IgG2a, κ	FITC	BD Pharmingen
CD3e	Hamster IgG1, κ	APC-Cy7	BD Pharmingen
CD4	Rat IgG2a, κ	PE-Cy7	BD Pharmingen
CD8a	Rat IgG2a, κ	PerCP	BD Pharmingen
CD11b	Rat IgG2b, κ	APC	BD Pharmingen
CD11c	Hamster IgG1, λ2	PE	BD Pharmingen
CD16/CD32	Rat IgG2b, κ	unlabelled	BD Pharmingen
CD19	Rat IgG2a, κ	PE-Cy7	BD Pharmingen
CD25	Rat IgG2b, κ	APC	BD Pharmingen
CD25	Rat IgG1, κ	PE	Immunotools
CD40 [HM40-3]	Hamster IgM, κ	unlabelled	BD Pharmingen
CD69	Hamster IgG	PE	Caltag Laboratories
CD80	Hamster IgG2a, κ	FITC	BD Pharmingen
CD83	Rat IgG1, κ	APC	BD Pharmingen
CD86	Rat IgG2a, κ	PE	BD Pharmingen
CD154 (CD40L)	Hamster IgG3, κ	PE	BD Pharmingen
H-2Db (MHC-I)	Mouse IgG2b, κ	PE	BD Pharmingen
I-Ab (MHC-II)	Mouse IgG2a, κ	FITC	BD Pharmingen
IFN-γ	Rat IgG1, κ	PE	BD Pharmingen
Vβ 5.1, 5.2 TCR	Mouse IgG1, κ	PE	BD Pharmingen

Table 4: Isotype Controls

Species / Ig class	Labelling	Manufacturer
Rat IgG1, κ	APC-Cy7	BD Pharmingen
Hamster IgG1, λ2	PE	BD Pharmingen
Rat IgG 2a	FITC	Caltag
Rat IgG 2a	PE	Caltag
Rat IgG 2a	PE-Cy7	Caltag
Rat IgG 2b, κ	APC	BD Pharmingen

Table 5: Secondary anti-Mouse Antibodies and Streptavidin Conjugates

Antigen [clone]	Species / Ig class	Labelling	Manufacturer
B220 (CD45R)	Rat IgG2a, κ	Biotin	BD Pharmingen
CD3e	Hamster IgG1, κ	Biotin	BD Pharmingen
CD19	Rat IgG2a, κ	Biotin	BD Pharmingen
CD34 [RAM34]	Rat IgG2a, κ	Biotin	eBioscience
Streptavidin		PE	BD Pharmingen

2.1.2. Peptides

Table 6: MHC class I Peptides

Name (amino acid position)	Purity	Peptide sequence (N-terminal->C-terminal)	Manufacturer
LCMV (aa33-41)	> 98 %	KAVYNFATM	Thermo Scientific
OVA (aa257-264)	> 97 %	SIINFEKL	Thermo Scientific
TRP-2 (aa180-188)	> 99 %	SVYDFFVWL	Thermo Scientific

2.1.3. Recombinant Cytokines

Table 7: Recombinant Cytokines

Cytokine	Species (Specific Activity U/mg)	Manufacturer
GM-CSF	Mouse (5 x 10e5)	Immunotools
Interleukin-4	Mouse (5 x 10e5)	Immunotools

2.1.4. Buffers & Solutions

Table 8: Buffers and solutions

Name		Composition / Manufacturer
Bone marrow Isolation	Buffer 1	1x PBS 1 % VLE-FBS 1mM EDTA
	Buffer 2	1x PBS 1 mM EDTA
	Buffer 3	1x PBS 5 % VLE-FBS 1 mM EDTA
Cell Fix	1x	BD Biosciences
Cell WASH		BD Biosciences
Dulbecco´s PBS	1x	PAA
EasySep Purification buffer		1x PBS 2 % FBS 1 mM EDTA
Erylysis	Buffer 1	0.2 % NaCl pH 7.4
	Buffer 2	1.6 % NaCl pH 7.4

Name		Composition / Manufacturer
FACS Flow		BD Biosciences
FACS Staining buffer		1xPBS 2 % FBS 0.1 % Sodium Azide pH 7.4
HEPES Buffer	1 mM	PAA
MEM NEAA	1x	Gibco

2.1.5. cell culture media

Table 9: Feeder cell Standard medium

Medium (Manufacturer)	Composition and Supplementation
RPMI 1640 (Gibco)	
	10 % FBS (Lot 5SB0018)
	300 µg/mL L-Glutamine
	10 mM HEPES
	15 µg/mL Gentamicin

Table 10: Feeder cell Selection medium

Medium (Manufacturer)	Composition and Supplementation
RPMI 1640 (Gibco)	
	10 % FBS (Lot 5SB0018)
	300 µg/mL L-Glutamine
	10 mM HEPES
	15 µg/mL Gentamicin
	0.2 mg/mL Hygromycin B

Table 11: mCD40B Washing medium

Medium (Manufacturer)	Composition and Supplementation
DMEM (Gibco)	
	580 µg/mL L-Glutamine
	4.5 mg/mL Glucose
	10 mM HEPES
	15 µg/mL Gentamicin

Table 12: mCD40B Standard medium

Medium (Manufacturer)	Composition and Supplementation
DMEM (Gibco)	
	10 % FBS (Lot 5SB0018)
	580 µg/mL L-Glutamine
	4.5 mg/mL Glucose
	10 mM HEPES
	0.1 mM MEM NEAA
	1 U/mL IL-4
	100 µM/mL β-Mercaptoethanol
	0.63 µg/mL Cyclosporin A
	15 µg/mL Gentamicin

Table 13: mDC Standard medium

Medium (Manufacturer)	Composition and Supplementation
VLE-RPMI 1640 (Biochrom)	
	5 % VLE-FBS (Lot 6SB0007)
	L-Glutamin
	50 µM/mL β-Mercaptoethanol
	15 µg/mL Gentamicin

Table 14: Serum-free Medium for Peptide Pulsing

Medium (Manufacturer)	Composition and Supplementation
AIM V (Gibco)	
	L-Glutamin
	50 µg/mL Streptomycin Sulfate
	10 µg/mL Gentamicin Sulfate

Table 15: B16 Tumour Medium

Medium (Manufacturer)	Composition and Supplementation
DMEM (Gibco)	
	10 % FBS (Lot 5SB0018)
	L-Glutamin
	4.5 g/L Glucose
	100 µM/mL β-Mercaptoethanol
	50 µg/mL Streptomycin Sulfate
	10 µg/mL Gentamicin Sulfate

2.1.6. Kits

Table 16: Kits

Name	Manufacturer
Cytofix/Cytoperm Plus Kit (554715)	BD Biosciences
Easy Sep Mouse CD19 Positive Selection Kit (18754)	Stemcell Technologies
Easy Sep Mouse T Cell Enrichment Kit (19751) Negative Selection	Stemcell Technologies
Easy Sep Mouse Biotin Selection Kit (18556)	Stemcell Technologies

2.1.7. Cell Lines

Table 17: Cell Lines

Name	Supplier
HeLa	DSMZ
tmuCD40L HeLa	Prof. Dr. C. Wendtner
B16.F10	ATCC

2.1.8. Tools & Instrumentation

Table 18: Tools and Instrumentation

Tool / Instrument	Manufacturer
Aristoplan Microscope	Leitz
BAHR-DIGIMESS 150A4 (Digital Caliper)	TSB-shop
BioBeam 8000	Gamma-Service Medical GmbH
FACS Canto	Becton Dickinson
Freezing Chamber	Nalgene NUNC
Kofferdam Punch Pliers Ainsworth	B. Braun Melsungen AG
Mouse Restraining device	G&B Kunststofftechnik
Purple EasySep Magnet	StemCell Technologies
Televal 31 Microscope fitted with	Zeiss
EOS 350D Digital Camera	Canon

2.1.9. Consumables

Table 19: Consumables

Name	Manufacturer
cell scraper 25 cm	Sarstedt
cell strainer 70, 100 µM	BD Falcon
cryo tube 1.0, 1.8 mL	Sarstedt
dish 6-, 12- well (flat bottom)	NUNC
dish 96-well (round bottom)	NUNC
FACS tube 5 mL	BD Falcon
bottle top filter 0.22 µm	BD Falcon
Minisart filter 0.2 µm	Sartorius Stedim Biotech
needle 20 G (0.90 x 70 mm)	B. Braun Melsungen AG
needle 26 G (0.45 x 12 mm)	BD Medical
petri dish (60 x 15 mm)	Sarstedt
pipette tip 5, 10, 25 mL	Sarstedt
pipette tip 10, 200, 1000 µL	Sarstedt
reaction tube 0.5, 1.0, 1.5, 15, 50 mL	Sarstedt
surgical blade Scalpel No. 22	Feather Safety Razor Co.
syringe Injekt-H 1 mL	B. Braun Melsungen AG
syringe Discradit II 2, 5, 10, 20 mL	BD Medical
tissue culture flask 75, 175 cm^2	Sarstedt

2.2. Cell Culture

2.2.1. Counting of cells – Trypan Blue Exclusion Test

Cells were counted using a Neubauer counting chamber and an aliquot 10x diluted and stained in Trypan Blue Stain 0.4 % to differentiate viable from non-viable cells. 10 µl of stained aliquot were applied to the counting chamber and bright, unstained cells were counted viable. Using a light optical microscope with 20 fold magnification four corner quadrants of the Neubauer chamber were counted completely. One big quadrant has a volume of 0.1 µl. The average cell number per quadrant was multiplied by 10e4 giving the amount of cells present in 1 ml of cell suspension. The total cell number was calculated by including the dilution factor and the total volume. The relative growth of cultures during whole cultivation period was assessed by calculating the relative increase between two passages.

2.2.2. Cryopreservation and Thawing of cells

Cells were suspended at a density of 5-10 x 10e6 cells/mL in cold freezing medium (90 % FCS and 10 % DMSO (v/v)) on ice. Cell suspension was aliquoted into cryo tubes, tubes placed in an iso-propanol freezing chamber and slowly frozen at -80 °C. 24-72 h later, tubes were transferred into liquid nitrogen for long-term storage.
Frozen cells were quickly removed from nitrogen and rapidly thawed in a water bath at 37 °C until a small ice clump was left in the vial. Then, cells were transferred dropwise into excess of medium and spun down (200 g, 5 min) to remove DMSO. Finally, cells were suspended in the appropriate medium or solution for further passaging.

2.2.3. Trypsinization of Adherent Cells

Old medium was removed and adherent cells washed with 10 mL of 1x PBS. After removal of PBS cells were detached by adding 4 (6) mL of 1x Trypsin-EDTA in a 75 (175) cm^2 flask and incubated for 10-15 min at 37 °C. Detachment of cells was facilitated by gentle tapping at the flask. In order to stop trypsin digestion 10 mL of FBS containing medium was added to the cells. Then, cells were harvested, washed twice with 1x PBS in excess (265 g, 7 min) and suspended in Standard Medium.

2.2.3.1. Assessment of Cell Morphology by Microscopy

To assess morphology of cell subsets, cultures were completely screened, a representative section was chosen and photographed with a 5x magnification using an inverted phase Zeiss Televal 31 microscope fitted with a Canon EOS 350D digital camera.

2.2.4. Cell Lines

2.2.4.1. Passaging of tmuCD40L HeLa cell line

tmuCD40L HeLa cell line is an adherent human epithelial cell line, which should never become completely confluent. Therefore cell passaging was done twice per week and cultivated for not longer than 6 weeks. Then a new aliquot stored in liquid nitrogen was thawed.
For regularly passaging adherent tmuCD40L HeLa cells were trypsinized (see section 2.2.3.) and suspended in 10 mL of Feeder cell Standard medium in order to determine the cell number (see section 2.2.1.). Finally, 2.5 x 10e6 cells were disseminated in a 75 cm^2 flask in 10 mL Feeder cell Selection medium and incubated at 37 °C and 5 % CO_2.
For generation of murine CD40-activated B (mCD40B) cells the remaining tmuCD40L HeLa cells were lethally irradiated 3 times with 26 Gy (total: 78 Gy) and subsequently plated on a sterile 6-well plate at a concentration of 0.4 x 10e6 cells/well in 2 ml Feeder cell Standard medium and incubated at 37 °C at 5 % CO_2. After 4 to 24 h of incubation tmuCD40L HeLa cells were checked for

adherence using microscope. Adherent feeder cells were used for B-cell stimulation.
tmuCD40L HeLa is a stable transfectant; its expression of CD40L was regularly tested by flow cytometry using PE-conjugated anti-Mouse CD154 (CD40L) antibody.

2.2.4.2. Passaging of B16.F10 melanocytes

B16.F10 is an adherent cell line derived from murine melanoma. In order to ensure genetic stability of the cell line a new aliquot of B16.F10 cells was thawed every 4-6 weeks and monitored for brown pigmentation. Cells were kept in logarithmic growth phase by passaging them with ≤ 50 % confluence twice per week.
For regularly passaging cells were trypsinized according protocol (see section 2.2.3.) except 0.5 % trypsin/EDTA was used, and suspended in 10 mL of B16 Tumour Medium in order to determine the cell number (see section 2.2.1.). Finally, 1 x 10e6 (5 x 10e6) B16.F10 melanocytes were disseminated in a 75 (175) cm^2 flask in 20 (30) mL of B16 Tumour Medium and incubated at 37 °C and 5 % CO_2.

2.2.5. Cell-subset Enrichment

2.2.5.1. Purification of murine Lymphocytes from Blood

A maximum of 50 µL of blood was withdrawn from lateral tail veins. Therefore, mice were fixed in the Mouse Restraining device, target area was pre-warmed using a red light lamp, target area was sterilised with skin disinfectant and tail vein was cut using a surgical blade. Blood sample was collected in 1 mL tube containing heparin. Lymphocytes were purified from erythrocytes by lysis (see section 2.2.5.6.).

2.2.5.2. Purification of murine Lymphocytes from Lymph Nodes

In order to analyse lymphocytic cells from mesenteric or inguinal lymph nodes by FACS single-cell suspensions were prepared. Therefore, dissected organs were stored in mCD40B Washing Medium on ice until further preparation. First, single-cell suspensions from lymph nodes were made by disintegration of lymph nodes by squashing them between two glass slides. Cellular fragments were washed down into a Petri dish on ice, minced with the back side of a syringe plunger and filtered through a 100 µm cell strainer that was wetted with 1 mL of mCD40B Washing medium. Past this step lymphocytes from lymph nodes were purified by density-gradient centrifugation using Pancoll Mouse (see section 2.2.5.3.).

2.2.5.3. Purification of murine Lymphocytes from Spleen

Murine lymphocytes were separated from whole splenocytes by density-gradient centrifugation using Pancoll Mouse. First, spleens from 8-12 weeks old C57BL/6 mice, BALB/c mice or OT-I

mice were minced by passing them through a 100 µm cell strainer that was wetted with 1 ml of mCD40B Washing medium. Second, the cell suspension was spun down at 265 g for 7 min. Third, splenocytes were suspended in 7-8 ml mCD40B Washing medium and layered on top of 5 ml pre-warmed Pancoll Mouse in a 15 mL reaction tube. Fourth, cells were spun down at 450 g for 25 min without brake, at acceleration level 4 and at RT. Finally, the upper layer was removed and the lymphocyte-rich interphase was carefully collected with 20 G Needle and washed afterwards at least once with 1x PBS at 265 g for 10 min.

2.2.5.4. Purification of murine CD3$^+$ T cells

Murine CD3$^+$ T cells were negatively enriched from whole spleen of 8-12 weeks old BALB/c mice using EasySep Mouse T cell enrichment Kit according manufacturer´s protocol. For that purpose spleens were minced by passing them through a 100 µm cell strainer that was wetted with 1 mL EasySep Purification buffer. Cells were spun down at 200 g for 10 min and suspended at 1 x 10e8 cells/mL in EasySep Purification buffer. 1 mL of cell suspension was filled into a sterile 5 mL FACS tube and supplemented with 50 µL rat serum. First, for negative selection 50 µL EasySep Mouse T cell Enrichment Cocktail was added to the suspension, mixed well by pipetting up and down and incubated for 15 min on ice. Second, 100 µL EasySep Biotin Selection Cocktail was added to the suspension, mixed well and incubated for 15 min on ice. Third, 50 µL of thoroughly mixed EasySep Magnetic Nanoparticles were added to the suspension and incubated for 15 min on ice. Finally, the cell suspension was adjusted to a total volume of 2.5 mL, mixed well by pipetting up and down and placed into the Purple EasySep Magnet for 5 min. Thereafter, the desired unlabelled CD3$^+$ T cell fraction was collected by carefully picking up the magnet and pouring off supernatant into another sterile FACS tube leaving one remaining drop in the tube. Enrichment of CD3$^+$ T cells by EasySep Mouse T cell Enrichment Kit yielded in purities of > 90 % CD3$^+$ T cells as determined by flow cytometric analysis.

2.2.5.5. Purification of murine CD34$^+$ bone marrow progenitor cells

Murine CD34$^+$ bone marrow progenitor cells were enriched from bone marrow of hind limbs of C57BL/6 or OT-I mice by positive selection with EasySep Biotin Selection Kit in combination with biotinylated anti-mouse CD34 antibody.

A) Bone marrow Preparation:
Preparation of bone marrow was performed by sterile isolation of hind limbs. First, hind limbs were cut off above hip joint, foot pads were separated below ankle joint and femurs and lower legs divided. Then muscle tissue was removed and bones were saved for further preparation in Petri dish on ice until all remaining limbs were prepared. Second, bone tips were cut off and bone marrow washed out from each side with 26 G needle by thoroughly rinsing 3 times with 4 mL cold Bone marrow Isolation Buffer 1 into a 50 mL tube containing the buffer reservoir. In order to free bone marrow cell suspension from bone fragments cell suspension was filtered through a 100 µm

cell strainer and spun down at 550 g for 5 min at 4 °C. Then supernatant was discarded and cell pellet suspended in 8 mL pre-warmed Buffer 2 by carefully pipetting up and down.
Third, bone marrow progenitors were further separated by density-gradient centrifugation at 450 g for 30 min at RT without break using Pancoll Mouse. Therefore 5 mL pre-warmed Pancoll Mouse was filled in 15 mL Falcon and cell suspension slowly layer on top.
Finally, the upper layer was removed and the lymphocyte-rich interphase was carefully collected with 20 G Needle and washed twice with 20 mL cold Bone marrow Isolation Buffer 2 at 800 g for 10 min at 4 °C. Then cell pellet was suspended in 1 mL cold Bone marrow Isolation Buffer 3 by pipetting it up and down. Finally cells were counted, spun down at 550 g for 5 min at 4 °C, supernatant was removed completely and cells adjusted to 1 x 10e8 cells/mL in cold Bone marrow Isolation Buffer 3.

B) Positive selection of $CD34^+$ bone marrow progenitors with EasySep Mouse Biotin Isolation Kit:
First, 1 mL of the cell suspension containing 1 x 10e8 bone marrow cells was filled into a sterile 5 mL FACS tube, blocked with 10 µL Fc-Receptor Block (CD16/CD32 antibody), labelled with 15 µL biotinylated anti-Mouse CD34 antibody (c = 0.5 mg/mL) and incubated for 15 min at RT. Cells were mixed during incubation by agitating the tube from time to time. Second, unbound antibody was removed by adding 10 mL cold Bone marrow Isolation Buffer 3, spinning down at 250 g for 7 min at 4 °C and completely pipetting off supernatant. Labelled cells were thoroughly suspended in 700 µL cold Bone marrow Isolation Buffer 3 and filtered through a 70 µm cell strainer into a 5 mL FACS tube. Afterwards cell strainer was rinsed with 300 µL cold Bone marrow Isolation Buffer 3 to free cell suspension from clumps. Third, 100 µL EasySep Biotin Selection Cocktail was added to the suspension mixed well and incubated for 15 min at RT. Cells were mixed during incubation by agitating the tube from time to time. Fourth, 50 µL of thoroughly mixed EasySep Magnetic Nanoparticles were added to the suspension and incubated for 10 min at RT. Cells were mixed during incubation by agitating the tube every 2 min. Then, the cell suspension was adjusted to a total volume of 2.5 mL, mixed well by pipetting up and down and placed into the Purple EasySep Magnet for 5 min. Thereafter, the desired labelled $CD34^+$ bone marrow progenitor cell fraction was collected by carefully picking up the magnet, pouring off the supernatant one drop remaining in the tube, taking the tube out of the magnet and washing out the desired cell fraction 3 times with 1 mL Bone marrow Isolation Buffer 3 into a 15 mL tube. $CD34^+$ cell fraction was collected by spinning down at 360 g for 7 min at 4 °C and suspending the cell pellet in 1 mL mDC Standard Medium. Enrichment of $CD34^+$ bone marrow progenitor cells by EasySep Mouse Biotin Isolation Kit yielded in purities of 75-80 % $CD34^+$ cells as determined by flow cytometric analysis.

2.2.5.6. Lysis of Erythrocytes

In order to prepare splenocyte suspensions for lysis of erythrocytes cells from one spleen were spun down at 158 g for 5 min at 4 °C, thoroughly vortexed and stored on ice. Erythrocytes were removed by addition and quickly suspension of 5-10 mL Erylysis Buffer 1, being followed by addition of the same amount of Erylysis Buffer 2 and spinning down at 158 g for 5 min at 4 °C. The

supernatant containing the lysed erythrocytes was removed, cells filtered through 100 µM cell strainer and washed with cold 1x PBS at 158 g for 5 min at 4 °C 1-3 times until the pellet appeared white. Otherwise lysis was repeated.

2.2.6. Generation of mCD40B cells

For in vitro generation of mCD40B cell cultures murine lymphocytes from C57BL/6 or OT-I mice separated from whole splenocyte suspensions by density-gradient centrifugation were used (see section 2.2.5.3.).

2.2.6.1. Initiation of mCD40B cell cultivation (day 0)

First, lymphocytes were washed in excess of mCD40B Washing medium and counted (see sections 2.2.5.3. and 2.2.1.). Second, portions of 30 x 10e6 cells appropriate for plating one 6-well plate were filled into 50 mL tubes and cell numbers were adjusted to 1.25 x 10e6 cells/mL by spinning down at 265 x g for 7 minutes at RT and suspending in mCD40B Standard medium.
In order to ensure sufficient costimulation mCD40B Standard Medium was supplemented with freshly solutions of murine interleukin-4, ß-Mercaptoethanol and Cyclosporin A immediately before use. If not marked differently 1 U/mL of interleukin-4, 100 µM ß-Mercaptoethanol and 0.63 µg/mL Cyclosporin A were added to one mL of mCD40B Standard Medium. Finally, for co-cultivation with tmuCD40L HeLa cells supernatant from the 6-well plates pre-incubated with feeder cells was removed and 4 mL of the lymphocyte suspension (c = 1.25 x 10e6 cells/mL) were gently added to each well of the 6-well plate. At last, the plate was incubated at 37 °C and 5 % CO_2 until re-cultivation.

2.2.6.2. Cultivation of mCD40B cells (day 3 and then twice a week)

Before harvesting mCD40Bs, cell cultures were checked for proper CD40-stimulation by formation of round cell clusters. mCD40B cell clusters were harvested by vigorously pipetting cell suspension up and down with a 10 mL pipette and pooling suspensions in a 50 mL tube. Then, cell suspensions were spun down at 265 x g for 7 min and supernatant completely removed and replaced by 10 mL of mCD40B Washing medium. Viable cells were counted (see section 2.2.1.) and suspended at a concentration of 0.75 x 10e6 cells/mL in new mCD40B Standard Medium freshly supplemented with 1 U/mL of interleukin-4, 100 µM ß-Mercaptoethanol and 0.63 µg/mL Cyclosporin A if not marked differently. For re-cultivation with tmuCD40L HeLa cells supernatant from the 6-well plates pre-incubated with feeder cells was removed and 4 mL of the lymphocyte suspension (c = 0.75 x 10e6 cells/mL) were gently added to each well of the 6-well plate. Finally, the plate was incubated at 37 °C and 5 % CO_2 until re-cultivation after 3-4 days (twice a week) or final cell harvest usually yielding in > 90 % pure $CD19^+$ $CD3^-$ mCD40B cells with a high expression of MHC-molecules class I ($H\text{-}2D^b$), II ($I\text{-}A^b$) and co-stimulatory molecules CD80, CD86 after a total cultivation period of 14 days. (mCD40B cells harvested after 7 days of cultivation are referred to as mCD40B d7, mCD40B from day 14 of cultivation as mCD40B d14.)

2.2.7. Generation of murine dendritic cells

For *in vitro* generation of mDC cultures $CD34^+$ bone marrow progenitors enriched by using anti-Mouse CD34 antibody and EasySep Mouse Biotin Isolation Kit were used (see section 2.2.5.5. A and B).

2.2.7.1. Initiation of mDC cultivation (day 0)

Purified $CD34^+$ bone marrow progenitors were cultivated at 0.25 x 10e6 cells/mL in mDC Standard Medium supplemented with freshly thawed murine GM-CSF(c = 500 U/mL). 2 mL of the cell suspension was applied per well onto 12 well-plates and incubated at 37 °C and 5 % CO_2.

2.2.7.2. Differentiation of mDC (days 2, 4 and 6)

On days 2, 4 and 6 of cultivation period 50 % of medium was exchanged. Therefore, 1 ml of the overlaying medium was carefully removed from each well with a 25 mL pipette and fresh mDC Standard Medium supplemented with 1000 U/mL murine GM-CSF was added with a 25 mL pipette by letting 1 mL of medium run in very slowly without touching the bottom of the well.

2.2.7.3. Maturation of mDC (from day 7 to day 8)

On day 7 of cultivation, for generation of immature mDC cell suspensions were left untouched. Then, on day 8 of culture immature mDC were harvested by using a cell scraper. For maturation of mDC cell suspensions of day 7 were treated by adding 10 ng/mL LPS (DC LPS) or 1 µg/mL anti-Mouse CD40 antibody (DC aCD40Ab) and incubated at 37 °C at 5 % CO_2 for 24 hours.
Purity of mDC cultures was regularly determined by flow cytometric analysis of the $CD11c^+$ $CD11b^+$ and MHC-II^{high} (I-Ab) cell population. Mature mDC generated from bone marrow progenitors were usually > 90% pure $CD11c^+$ $CD11b^+$ MHC-II^{high} cells with high expression of co-stimulatory molecules CD80, CD86, and CD83.

2.3. Phenotypical and Functional Analyses

2.3.1. Fluorescence Activated Cell Sorting (FACS)

Flow cytometry (FACS analysis) based on antibody-antigen reaction coupled to fluorescent dye was used to quantify the expression of surface and intracellular proteins of viable cells. Flow cytometric analysis was performed using a FACS Canto equipped with BD FACSDiva software. Signal spill-over occurring between fluorescence channels were corrected by setting different compensations for multiple colour analyses. Further analyses and interpretation was done using FlowJo software from Tree Star. Mean values of fluorescence intensity (MFI) were determined from gat-

ing on specific cell subsets in histogram plots which were used to plot bar charts. Frequencies of specific cell subsets were determined by gating on specific cell subsets in dot plots. Mean values of frequencies were calculated to plot bar charts.

2.3.1.1. Surface staining

In order to assess phenotype of cell subsets cells were harvested, counted and 5 x 10e5 cells filled in a 5 mL FACS tube. Cells were washed once with 1.5 mL CellWASH by spinning down at 353 g for 5 min at 4 °C. Then, supernatant was discarded completely and cells stained for 15 min at 4 °C in the dark in a volume of 50 µL FACS Staining buffer containing 1 µL of Fc-Block (CD16/CD32) and 0.8 µL of further denoted antibodies. Afterwards, cells were washed again with 1.5 mL CellWASH and finally spun down at 353 g for 5 min at 4 °C. At last, supernatant was discarded and cells suspended in either 200 µL of CellWASH or 200 µL Cell Fix when performing flow cytometric analysis on the same day or within the next week, respectively.

2.3.1.2. Intracellular Cytokine Staining using Cytofix/Cytoperm Kit

Production of intracellular IFN-γ by specific antigen-experienced $CD8^+$cytotoxic T cells was detected by flow cytometry analysis upon re-stimulation of purifed lymphocytes of vaccinated animals with the same peptide. Therefore, spleens from vaccinated mice or control animals were dissected and lymphocytes purified by density-gradient centrifugation (see section 2.2.5.3.). When necessary lysis of erythrocytes was performed additionally (see section 2.2.5.6.). Cells were counted, suspended at 1 x 10e6 cells/mL in Feeder cell Standard Medium and handled on ice thereafter.

Stimulation with PMA and Ionomycin:
In vitro stimulation with PMA and Ionomycin served as positive control. 1 mL of cell suspension was filled into a sterile 5 mL FACS tube and stimulated with 10 ng/ml of PMA and 500 ng/ml of Ionomycin and incubated for 4 hours at 37 °C and 5 % CO_2.

Stimulation with peptide corresponding to immunization:
1 mL of cell suspension was filled into a sterile 5 mL FACS tube and stimulated with 10 µM of corresponding peptide and incubated for 4 hours at 37 °C and 5 % CO_2.

After 1 hour of stimulation with either PMA/Ionomycin or peptide or control incubation was interrupted for addition of 0.66 µl Golgi-Stop per 1 x 10^6 cells. Then, cells were spun down at 353 g for 5 min at 4 °C and stained for expression of surface markers. Therefore, 2 µL of the following antibodies were added: CD4-PE-Cy7, CD8-PerCP, CD25-APC and CD69-PE (see section 2.2.8.1.). Upon surface staining cells were washed with 3 mL of FACS buffer and supernatant discarded completely by stripping it off over a tissue.

Fixation and Permeabilization of cells using Cytofix / Cytoperm Kit:
In order to fix surface stains on cells during further staining procedure, cells were vortexed thoroughly and suspended in 250 µL of Cytofix/Cytoperm solution by pipetting up and down. Cells were fixed by incubation for 20 min on ice in the dark. Then, fixed cells were washed with 3 mL of FACS buffer and suspended in 1 mL of FACS buffer for overnight storage at 4 °C in the refrigerator. The next day fixed cells were spun down and supernatant discarded completely by stripping it off over a tissue. In order to permeabilize cells, they were vortexed thoroughly and suspended in 500 µL of 1x Perm/Wash solution by pipetting up and down. Cells were permeabilized by incubation for 10 min on ice in the dark. Then permeabilized cells were spun down and supernatant discarded completely by stripping it off over a tissue.

Intracellular staining:
Intracellular staining of fixed and permeabilized cells was performed by thoroughly suspending cells in 200 µL of 1x Perm/Wash solution containing 0.25 µg of PE-labelled IFN-γ antibody or matched isotype control. Cells were incubated for 30 min on ice in the dark. Finally, cells were washed two times with 3 ml 1x Perm/Wash solution, suspended in 250 µl of 1x Perm/Wash solution and analysed by flow cytometry immediately after. Frequencies of IFN-γ positive $CD8^+$ T cells were determined by using FlowJo software and mean values calculated from animals treated identical. Bar charts were plotted from mean values of frequencies of IFN-γ positive $CD8^+$ T cells re-stimulated with peptide normalized to corresponding un-stimulated sample of the same animal.

2.3.2. Mixed Lymphocyte Reaction (MLR)

In order to study the APC capacity of mCD40B and mDC antigen-unspecific allogeneic Mixed Lymphocyte Reactions were performed based on the induction of T-cell responses by TCR signalling through binding of alloantigen-MHC complex and T-cell activation through co-stimulation by APCs.

2.3.2.1. CFSE-labelling of $CD3^+$ T cells

Prior to MLR $CD3^+$ T cells were purified from spleen of naïve BALB/c wild type mice (MHC haplotype d) (see section 2.2.5.4.) and stained with fluorescent proliferation marker CFSE. Therefore, $CD3^+$ T cells were suspended in a 50 mL tube at 100 x 10e6 cells/mL in 1x PBS with 5 % FBS and stained by addition of 10 µM CFSE in PBS. Cells were mixed and stained uniformly by agitating the tube constantly for 5 min in the dark. To stop staining reaction 5 mL pure FBS were added and CFSE-labelled T cells were spun down at 265 g for 5 min. Then, CFSE-labelled T cells were washed twice with Feeder Cell Standard Medium and separated in two fractions. One fraction was suspended at 1 x 10e6 cells / mL and plated in triplicates of 100 µL for APC-to-T cell ratios 1:1, 1:10, 1:20, 1:50, 1:100, the other one was suspended at 5 x 10e5 and plated in triplicates of 100 µL for the APC-to-T cell ratio 3:1 onto a 96-well plate. One triplicate was plated with plain Feeder Cell Standard Medium for the APC-to-T cell ratio 0:1.

2.3.2.2. Irradiation and serial dilution of APCs

To measure the dynamics of the allogeneic T-cell response T cells were cultivated together with DC LPS, DC aCD40Ab, mCD40B d14 or mCD40B d7 at the various APC-to-T cell ratios. Therefore, mCD40B and mDC from C57BL/6 wild type mice (MHC haplotype b) where generated suspended at 1 x 10e6 cells/mL in Feeder Cell Standard Medium and irradiated once with 26 Gy to stop them from proliferation. Upon irradiation serial dilution was performed for the indicated APC-to-T cell ratios and 100 µL plated onto T cells. Cells were kept at 37 °C and 5 % CO_2 for 5 days.

2.3.2.3. Flow cytometric analysis of T-cell divisions

CFSE segregated equally between daughter cells upon cell division, resulting in the sequential halving of cellular fluorescence intensity with each successive generation. When analyzed by flow cytometry, this sequential halving of fluorescence was visualized as distinct peaks in the histogram. After 5 days of co-cultivation cells were harvested and stained with the following antibodies: CD3-APC-Cy7, CD4-PE-Cy7, CD8-PerCP, CD25-APC and CD69-PE (see section 2.3.1.1.). The frequencies of proliferating $CD3^+CD25^+CFSE^{low}CD4^+$ and $CD3^+CD25^+CFSE^{low}CD8^+$ T cell subsets was determined by flow cytometry immediately after. In order to plot bar charts mean values were calculated and normalized to intrinsic T cell activity as determined by APC-to-T cell ratio 0:1.

2.4. In vivo Experiments

2.4.1. Mouse strains

Table 20: Used Mouse Strains

Name	Haplotype	Supplier
BALB/c	H2^d	Harlan
C57BL/6N	H2^b	Charles River
OT-I	H2^b	PD Dr. med. F. Tacke, PhD

2.4.2. Breeding and Handling of mice

The experiments were performed in accordance with the national and European guidelines for laboratory animal keeping with permissions from the local government authorities (permission numbers 8.87-50-203.2-K 17, 16/05 and 8.87-51.04.20.09.369). Referring the German animal welfare law the animals were controlled regularly by the proper authorities.
Mice were offspring from breeding pairs originally obtained from suppliers see table 1.3.1. TCR-transgenic OT-I mice were kindly provided by PD Dr. Frank Tacke (University Hospital Aachen, Germany). OT-I mice were routinely monitored for expression of TCR-chain Vβ5 on $CD3^+CD8^+$ T cells

and expressed TCR-chain Vβ5 in more than 90 % of all $CD3^+CD8^+$ T cells.
All mice were housed under specific pathogen-free conditions and used at 8 to 12 weeks of age. In order to dissect organs for further analysis or preparation of single-cell suspensions mice were sacrificed by cervical dislocation.

2.4.3. Toxicity of mCD40B

2.4.3.1. Cell transfer and schedule

In order to assess secondary immunological effects of adoptive mCD40B cell transfer C57BL/6 mice were challenged with pure mCD40B d14 suspended in 1x PBS. Generation of mCD40B cells was done from spleens of C57BL/6 mice (see section 2.2.6.) and cells routinely analysed by FACS. Highly activated > 90 % pure $CD19^+CD3^-$ mCD40B cells were harvested and thoroughly washed in excess of 1x PBS for 3 times by spinning down at 265 g for 5 min at 4 °C. Cells were filtered through a 100 µm cell strainer and separated into two fractions. One fraction was suspended at 1 x 10e7 cell/mL, the other one was suspended at 1 x 10e8 cells/mL and kept on ice until injection. To address long-term toxicity 1 x 10e6 mCD40B cells were injected two times a week for five weeks. Acute toxicity was assessed by injecting mice once with 1 x 10e7 mCD40B cells. Matched groups of mice received PBS buffer only serving as negative control. Injections of 100 µL were performed in three different routes: intraperitoneal injection, intravenous injection into lateral vein and subcutaneous injection into the right flank.

2.4.3.2. Assessment of Behaviour and Weight

The following five weeks mice were observed for clinical symptoms and changes in behaviour such as reduced food and water intake, reduced activity, aggressiveness, difficulties of breathing and moving, increased body temperature and abnormal body posture. After five weeks of observation mice were sacrificed by cervical dislocation, weighed and organs dissected and stored in mCD40B Washing Medium on ice until further analysis.

2.4.3.3. Hematoxylin and Eosin staining procedure

Microscopy was performed at the former laboratory of PD Dr. Claudia Wickenhauser (Institute for Pathology, University Hospital of Cologne). H & E Staining was performed by the technician Silke Kummer.
For standard histo-chemical staining, paraffin-embedded tissues (heart, lung, liver, kidney and spleen) were sectioned (5 µm). First, samples were deparaffinized with xylene (Merck) twice, each 10 min, and rehydrated in descending ethanol series (100, 100, 96, 70, 50), each 5 min. Second, samples were washed with distilled water. Third, samples were stained in Mayer's hematoxylin solution (Merck) for 3 min. Fourth, samples were washed in running tap water and subsequently

washed with distilled water. Fifth, samples were counterstained with eosin 1 % (w/v) solution aqueous (Merck) for 3 min. Sixth, samples were dehydrated in ascending ethanol series, each 5 min. Finally, samples were mounted in Pertex (Medite, Burgdorf, Germany).

2.4.3.4. Histo-pathological analysis

Samples were analyzed with an Aristoplan Microscope under supervision of C. Wickenhauser. Histo-pathological analysis was performed by completely screening of three non-consecutive histological sections of each organ. If present lymphocytic infiltrates were counted. For semi-quantitative analysis mean values of four independent experiments were calculated and assigned to one of three categories: o no infiltrate, + few lymphocytic infiltrates ($1 \leq x \leq 3$) or ++ several lymphocytic infiltrates ($4 \leq x \leq 8$).

2.4.3.5. Assessment of Lymphocytic subsets in Lymph nodes and Spleen

In order to assess the distribution of lymphocytic subsets in mesenteric and inguinal lymph nodes and spleen lymphocytes were enriched and stained for flow cytometric analysis (see sections 2.2.5.2., 2.2.5.3. and 2.3.1.1.).
Therefore, lymph nodes and spleen were dissected and single cell suspensions prepared. Aliquots of cells were stained with following antibodies: B220-FITC, CD3-APC-Cy7, CD4-PE-Cy7 and CD8-PerCP or matched isotype controls and analysed by flow cytometry.

2.4.4. Vaccination Strategies

2.4.4.1. Immunization with Peptide-loaded Cell Subsets

In order to immunize C57BL/6 or OT-I mice with mCD40B or mDC subsets, cells subsets generated from syngeneic mice were exogenously loaded with indicated peptide. Therefore, freshly prepared mCD40B or mDC subsets of given time points or thawed mCD40B subsets that had been co-cultivated for at least one cultivation period of 3-4 days were harvested and washed three times with excess of 1x PBS by spinning down at 265 g for 5 min at 4 °C. Cell subsets were suspended in serum-free AIM V Medium at 1 x 10e6 cells/ml and filled in 50 mL tube. After addition of 10 µM peptide the cells were incubated for three hours at 37 °C and 5 % CO_2. Before injection cells were washed three times with 20 mL of 1x PBS, filtered through a 100 µM cell strainer, suspended and slowly injected in 100 µL of 1x PBS. Irradiation of indicated mCD40B subsets was performed with 26 Gy for one time.

Table 21: Varying Parameters of Immunizations with mCD40B

Vaccination Parameter	Varying Attribute
route of administration	intraperitoneal intravenous (lateral vein) subcutaneous (right flank)
cell dose in 100 µL	1 x 10e5 1 x 10e6 1 x 10e7
vaccination interval	day 0 – 7 day 0 – 7 – 14
mCD40B formulation	mCD40B d7 mCD40B d14 irradiated mCD40B (mCD40B irr)

Peptide-pulsed mDC were injected subcutaneously into the right flank.

2.4.4.2. Immunization with Incomplete Freund´s Adjuvant (IFA)

Immunizations with 100 µL of IFA/peptide emulsions served as positive control. Herein, the final concentration of peptide per injection amounted to 20 µM. Immunomodulatory CpG-ODN (oligodeoxynucleotide with non-methylated cytosine-guanine motifs) with a double stem-loop structure (D-SLIM) were kindly provided by Mologen AG, Berlin. All solutions were always handled on ice.

In order to prepare the IFA/peptide emulsion sufficient for two injections 50 µL of peptide was mixed with 50 µg (17.5 µL) of CpG D-SLIM and adjusted to 250 µL by addition of 182.5 µL 1x PBS, first. Second, 250 µL of IFA were added to the peptide solution and an emulsion formed by strong vortexing for 3 min at 4 °C. Resulting IFA/peptide emulsion was set aside on ice. When resulting emulsion remained in phase syringes were drawn up and used for immunizations. Otherwise vortexing was repeated. Transfer of emulsion into syringes was carried out without attaching the needle to the syringe. This was done immediately prior to injection. Immunizations with IFA/peptide emulsions were injected subcutaneously into the right flank on day 0 and day 7.

2.4.5. In vivo Cytotoxicity Assay

The *in vivo* cytotoxicity assay aims at detection of *in vivo* killing of antigen-specific target cells by recognition through cognate $CD8^+$ cytotoxic T cells induced by immunization. Hence, three to four days after the last vaccination mice were injected with CFSE-labelled target cells from naïve syngeneic mice loaded with peptide corresponding to immunization.

2.4.5.1. Injection of Target cells

Target cell were prepared from single-cell suspension of spleens (see section 2.2.5.3.). When necessary lysis of erythrocytes was performed additionally (see section 2.2.5.6.). Splenocytes were washed two times with 10 mL of 1x PBS by spinning down at 265 g for 5 min at 4 °C, suspended in 10 mL of 1x PBS and counted. In order to label with CFSE splenocyte suspension was adjusted to 100 x 10e6 cells/mL in 1x PBS + 5 % FBS and separated into two equal fractions. One fraction was labelled with low amount of CFSE (2 µM), one with high amount (20 µM) by adding CFSE and constantly agitating the tube for 5 min in the dark. To stop staining reaction 5 mL pure FBS were added and CFSE-labelled target fractions were spun down at 265 g for 5 min. Then, target cells were suspended in 10 mL of 1x PBS and counted. Cells were adjusted to 1 x 10e7 cells/mL in AIM-V Medium for peptide pulsing. The CFSE high fraction (20 µM) was pulsed with 10 µM of peptide corresponding to immunization, the CFSE low fraction (2 µM) left unpulsed. Upon incubation for one hour at 37 °C and 5 % CO_2 both fractions were washed extensively with 20 mL of 1x PBS for three times, filtered through a 100 µm cell strainer, counted and suspended at 10 x 10e7 cells/mL in 1x PBS. Both fractions were mixed 1-to-1 and 100 µL drawn up per syringe for intraperitoneal injection.

2.4.5.2. Detection of Specific Cytolysis by FACS

In order to analyse remaining CFSE-positive target cell fractions from spleens of immunized mice, mice were sacrificed 16-40 hours after target cell injection by cervical dislocation. Then, spleens were dissected, minced through a 100 µM cell strainer and suspended in mCD40B Washing Medium. Cells were handled on ice and in the dark thereafter. Splenocytes were counted immediately after. For flow cytometric analysis 4 x 10e6 cells were filled in a FACS tube, washed, blocked with 4 µL of Fc-Block (CD16/CD32) and labelled with 3 µL of the following antibodies: CD3-APC-Cy7 and CD19-PE-Cy7 for 15 min on ice in the dark. Cells were analysed by FACS immediately. Frequencies of CFSE-positive fractions were determined by using FlowJo software. Unloaded CFSE low splenocytes served as reference. Specific killing of the peptide-loaded CFSE high target cell fraction was determined by calculating the reciprocal value of the ratio of the frequencies of CFSE high and CFSE low fractions of immunized mice and PBS-injected naïve mice. In order to plot bar charts of specific lysis mean values of frequencies normalized to negative control were calculated.

2.4.6. Tumour Formation

Vaccinations were performed according protocol section 2.4.4.1. with minor modifications. In order to examine the effect of preventive vaccinations with mCD40B on inducing anti-tumour responses, C57BL/6 mice were vaccinated with indicated doses of Trp-2-loaded mCD40B d14 injected intraperitoneally for three times in a seven day interval. Alternatively, mice were vaccinated with indicated dose of DC LPS or DC aCD40Ab pulsed with Trp-2 peptide injected subcutaneously into the left flank. Administration of PBS alone (NC) or mCD40B left unpulsed (mCD40B d14-) in-

jected intraperitoneally served as negative controls.
Seven days after the last vaccination B16.F10 tumour cells were prepared for inoculation. Therefore, adherent B16.F10 cells were trypsinized (see section 2.2.4.2.), washed two times with 1x PBS in excess, filtered through a 100 µM cell strainer and counted. For tumour inoculation B16.F10 cells were suspended at 1 x 10e7 cells/mL in 1x PBS and injected subcutaneously into the right flank. Tumour size was determined daily until day 13 after inoculation by measuring tumour diameter in three dimensions using a vernier calliper. Tumour volume was calculated by multiplying single diameters [mm^3]. In order to plot growth curves mean values of diameters were calculated.

2.5. Statistics

Significant differences were calculated by the Student's t -test for paired data. P-values of less than 0.05 were considered statistically significant and marked with asterisks: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Mean values and standard deviations (SD) were calculated from at least 3 experiments with at least three batches per group.

RESULTS

3. RESULTS

3.1. Generation of murine CD40-activated B cells

To develop a model to study B cells as antigen-presenting cells *in vivo* an *in vitro* culture system to generate murine CD40-activated B cells was established in close collaboration with Nela Klein-Gonzáles.

This *in vitro* culture system is based on a cultivation technique initially developed by Dr. J. Banchereau in the early 90s, allowing the long-term growth of human B lymphocytes through engagement of the CD40 receptor by monoclonal antibodies and IL-4 signalling (Banchereau et al., 1991 194). Later, in the mid 90s Dr. L. M. Nadler and J. S. Schultze elaborated the system (Schultze et al., 1997 113) by co-culturing peripheral blood mononuclear cells with irradiated CD40L transfected feeder cells (thuCD40L NIH3T3) in the presence of IL-4 and cyclosporin A. Thus > 80% pure $CD19^+$ $CD3^-$ activated human B cells were generated. These strongly proliferating cells termed "human CD40-activated B cells" expressed high levels of co-stimulatory, MHC and adhesion molecules. Compared to dendritic cells CD40-Bs were likewise highly efficient alloantigen-presenting cells inducing significant T cell proliferation and IFN-γ production *in vitro*.

3.1.1. Establishment of effective murine CD40B culture conditions

In order to assess the function of mCD40B as antigen-presenting cells for adoptive immunotherapy, we established the murine *in vitro* culture. From the human system we had learned that the key proliferation signals would be triggered by CD40 stimulus, IL-4 signalling and the selection of applicable additives and cell culture media (Liebig et al., 2009 215). C57BL/6 (MHC haplotype b) wild type mice were chosen as model strain as it is the most widely used mouse inbred strain, robust and easy to breed. With the spleen being the biggest lymphocyte reservoir in mouse lymphocytes were isolated from it by density gradient centrifugation with specific mouse Pancoll®. Purified lymphocytes were co-cultivated with irradiated murine CD40L expressing feeder cells (tmuCD40L HeLa) providing the CD40 signal. CD40 stimulus and additives, namely recombinant murine IL-4, ß -mercaptoethanol (ß-ME) and CsA were replenished every 3-4 days.

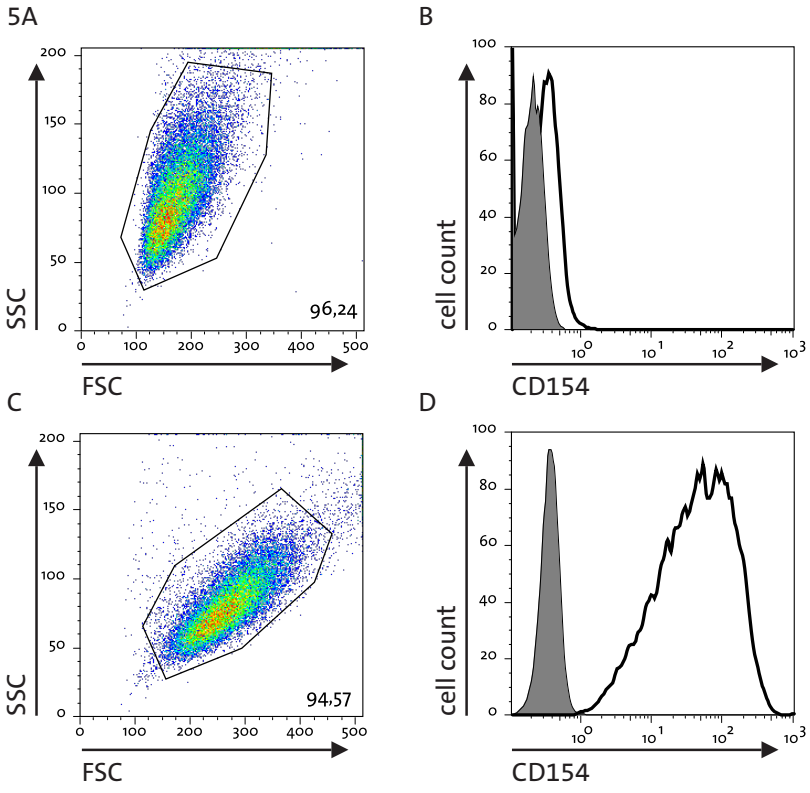
Quantitative analysis of B-cell proliferation was performed by determining the number of viable cells in each culture using the trypan blue exclusion test and a light optical microscope. Purity was assessed by flow cytometric analysis of the $CD19^+$ $CD3^-$ B cell subset in the cultures. To study the B cell activation status the expression levels of MHC ($I\text{-}A^b$ and $H\text{-}2D^b$) and co-stimulatory molecules (CD80 and CD86) were determined. Furthermore up-regulation of adhesion molecules resulting in the morphologic typical formation of homotypic cell clusters was photographically documented.

3.1.1.1. CD40 ligation

The B-cell stimulation system is based on the CD40 receptor – CD40-Ligand (CD40 – CD40L) interaction. CD40 – CD40L interaction naturally occurs when activated $CD4^+$ T cells recognize peptide presented by MHC Class-II on B cells, leading to various effects on both interaction sides (Banchereau et al., 1994 166; Foy et al., 1996 167; Van Kooten et al., 1996 169). On the B cell´s side CD40-activation drives antigen-specific B cells through their differentiation program and prevents the terminal differentiation of activated mature B cells into plasma cells and rescues them from apoptosis. In addition, CD40 activation directly up-regulates cytokine production (IL-6, IL-10, TNF-α, LT-a), expression of adhesion molecules and co-stimulatory receptors (ICAM, CD23, B7.1/CD80, B7.2/CD86) and increases expression of MHC Class-I, MHC Class-II and TAP transporter by B lymphocytes.

As no GMP-grade soluble CD40L with adequate activating and proliferating capacity has been available so far, CD40 activation of B cells was assured by using irradiated recombinant murine CD40 ligand expressing feeder cells (tmuCD40L HeLa) which were kindly provided by Dr. Clemens Wendtner (Mayr et al., 2006 216). HeLa wild type cells served as negative control.

To avoid contamination with non-transfected cells expression of the murine CD40 ligand (muCD40L, CD154) on the transfectants was controlled by flow cytometry regularly. Viable HeLa wild type cells (figure 5A) showed no expression of the ligand (figure 5B) whereas tmuCD40L HeLa transfectants stably expressed CD154 (figures C and D, respectively). (Mean values of fluorescence intensity gated on viable (FSC vs. SSC) cells ± standard deviation (SD); 5B) HeLa wild type 0.38 ± 0.08, n=4, $p < 0.001$ and 1D) tmuCD40L HeLa 47.09 ± 8.82, n=8, $p < 0.001$).



(One representative FACS analysis is shown.)

Figure 5: Phenotype of HeLa wild type cells (A, B) and tmuCD40L HeLa cells (C, D)

Phenotype of HeLa cells was regularly controlled by flow cytometry prior to using them as feeder cells for murine CD40B culture. (A and C) Viable cells were identified by gating FSC vs. SSC. (B and D) Expression of CD40L (CD154) was determined in histogram overlays. Histograms in dark grey represent isotype controls, thick black lines staining with specific antibody anti-mouse CD154-PE.

When splenocytes from C57/BL6 mice were co-cultivated with HeLa wild type cells no proliferation of B cells occurred (figure 6A) whereas murine CD40-activated B cells (mCD40B) proliferated intensively when grown on tmuCD40L HeLa cells (figure 6B). After 14 days of cultivation the relative growth rate of mCD40B was thereby increased on an average 2.6 fold ranging from 1.4 to 4.85 (n=10). Similarly, when splenocytes were cultivated with tmuCD40L HeLa cells mCD40B formed homotypic cell clusters (figure 6D) whereas on Hela wild type cells the cluster formation was inhibited (figure 6C).

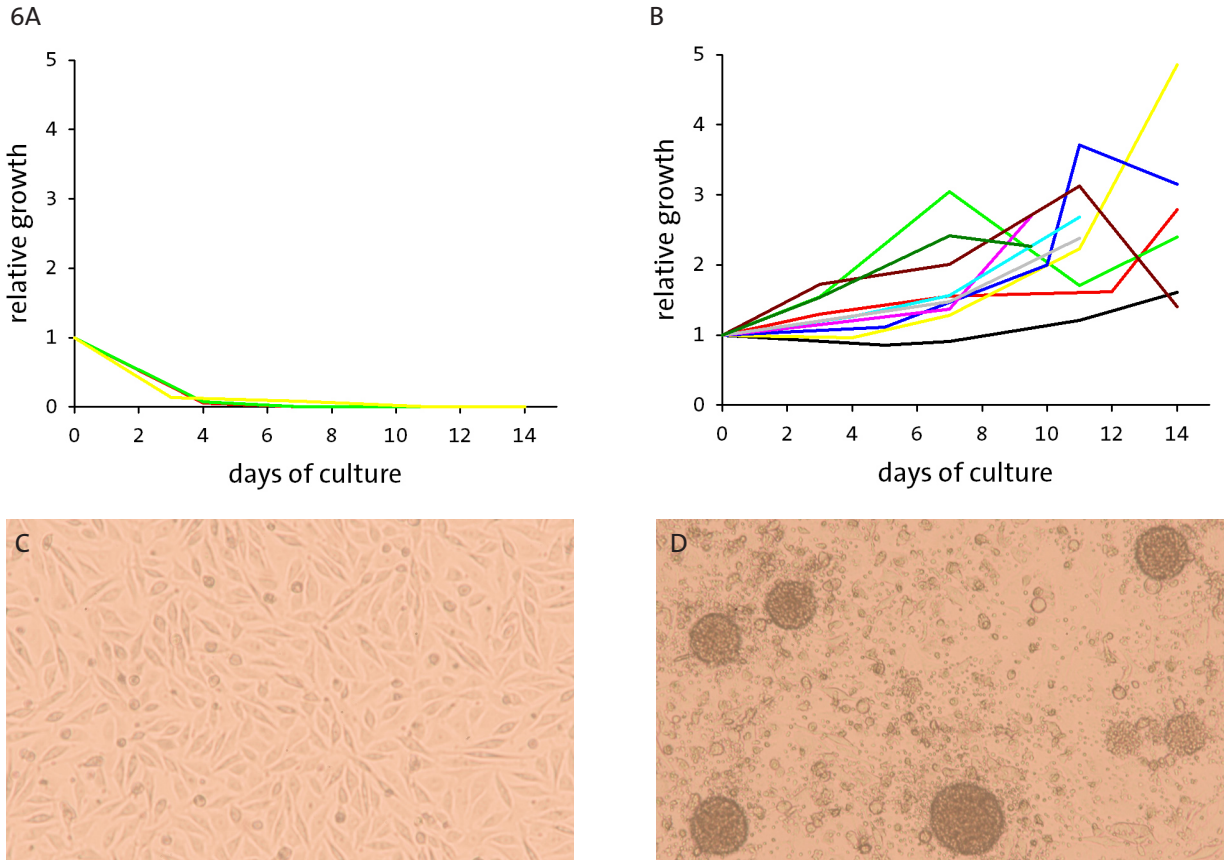


Figure 6: Proliferation and cluster formation of mCD40B cells is dependent on muCD40L expression of HeLa cells

(A and B) The number of viable mCD40B cells was determined every 3-4 days using the trypan blue exclusion test. Until day 14 of culture the relative growth was assessed by calculating the relative increase between two passages of mCD40B cultures grown on HeLa wild type cells (A) or on tmuCD40L HeLa cells (B).

(Growth curves of 10 independently grown mCD40B cultures are shown.)

(C and D) Photographs were taken at a 5 x magnification using a light optical microscope fitted with a digital camera and formation of homotypic B-cell clusters documented using HeLa wild type cells (C) or tmuCD40L HeLa cells (D) as feeder cells.

(One representative picture of at least 10 independent cultures is shown.)

Flow cytometric analysis revealed that the splenocyte suspension initially consisted of 49.3 ± 5.7 % $CD19^+$ B cells and 37 ± 8.2 % $CD3^+$ T cells (mean value ± SD, n=5). During the course of 14 days the mean frequency of $CD19^+$ B cells grown on tmuCD40L HeLa was highly increased to 89.1 ± 4.7 % and 92.7 ± 3.0 % whereas only a small proportion of $CD3^+$ T cells of 6.9 ± 1.8 % and 2.8 ± 2 % remained in the culture on day 7 and day 14, respectively (figure 7A-C). Furthermore analysis of the activation status of $CD19^+$ $CD3^-$ mCD40B over the course of the culture showed a consistent up-regulation of the expression levels of MHC molecules $I\text{-}A^b$ and $H\text{-}2D^b$ (figure 7D, E). A similar effect was observed for the expression levels of co-stimulatory molecules CD80 and CD86 (figure 7F, G) exclusively when B cells were cultivated with tmuCD40L HeLa cells. Therefore, tmuCD40L HeLa were used for generation of murine CD40 activated B cells.

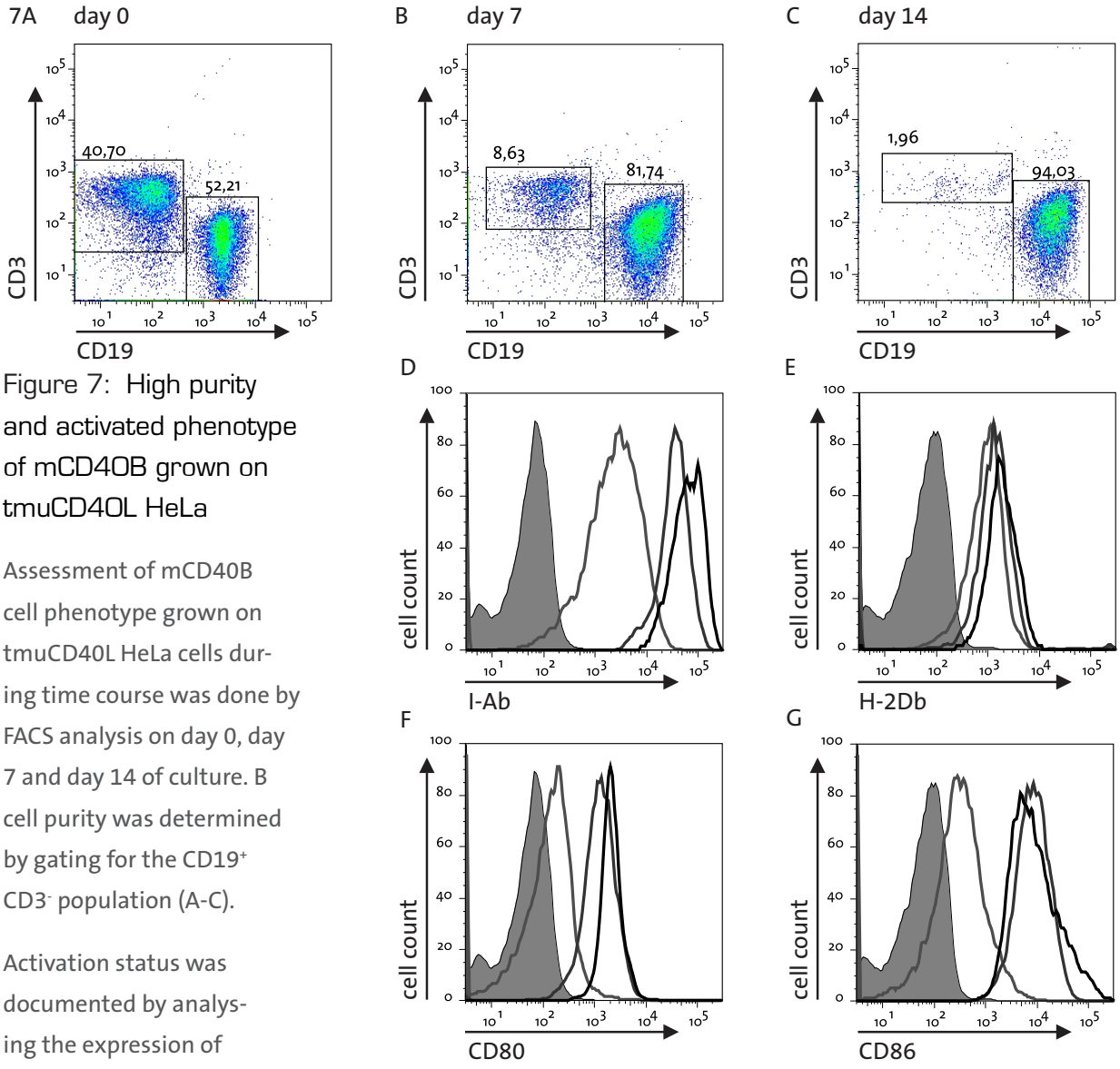


Figure 7: High purity and activated phenotype of mCD40B grown on tmuCD40L HeLa

Assessment of mCD40B cell phenotype grown on tmuCD40L HeLa cells during time course was done by FACS analysis on day 0, day 7 and day 14 of culture. B cell purity was determined by gating for the $CD19^+$ $CD3^-$ population (A-C).

Activation status was documented by analysing the expression of MHC molecules Class-I (H-2Db) and Class-II (I-Ab) (D and E) and the expression of co-stimulatory molecules CD80, CD86 (F, G). Histograms shaded in dark gray represent the isotype controls; lines in light grey stand for specific antibody staining on day 0, line in dark grey on day 7 and black lines represent day 14.

(One representative experiment out of 5 is shown.)

3.1.1.2. Interleukin-4 signalling

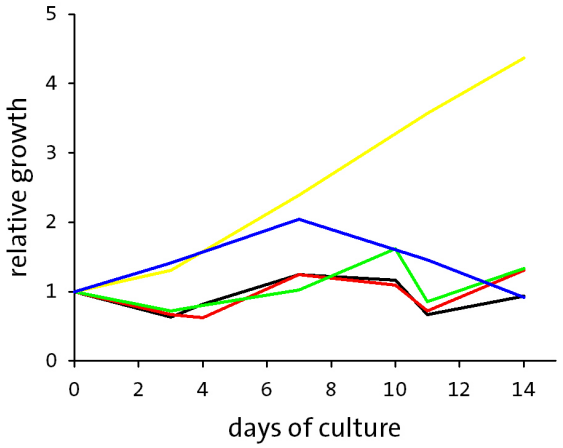
Second, the optimal interleukin-4 (IL-4) concentration applied to co-stimulate murine B cells was determined. IL-4 belongs to the haematopoietic growth factor receptor (HGFR) family and is naturally produced by T_H2 cells, thus providing efficient helper activity for B cells. IL-4 has a wide range of activating effects on B cells such as up-regulation of MHC Class-II expression, induction of proliferation and differentiation of activated B cells as well as regulation of immunoglobulin (Ig) isotype production (Mosmann et al., 1998 217). One common feature of cytokines is their activity at low concentrations. Due to this reason and following the human system for generation of CD40-activated B cells five different IL-4 concentrations [0, 0.2, 0.5, 1 and 2 U/mL] were tested in the murine system to find effective culture conditions.
When splenocytes from C57/BL6 mice were cultivated with tmuCD40L HeLa cells and treated with 0, 0.2 and 0.5 U/mL IL-4 no proliferation of B cells during 14 days of culture occurred (figure 8; black, red and green line, respectively), whereas an initial increase until day 7 of culture was found when murine CD40-activated B cells (mCD40B) were cultivated with 2 U/mL IL-4 (blue line). Best proliferation of mCD40B was obtained when cultivated with 1 U/mL IL-4 (yellow line). After 14 days of culture the relative growth rate of mCD40B was thereby significantly increased 2.8 fold ranging from 1.7 to 4.4 ($p \leq 0.05$, $n = 8$).

Figure 8: mCD40B growth is dependent on IL-4 supplementation

Starting with C57BL/6 splenocytes on irradiated CD40L-expressing feeder cells tmuCD40L-HeLa mCD40B cells were grown in 5 different concentrations of IL-4 depicted by different coloured lines:

[0 U/mL] - black line
[0.2 U/mL] - red line
[0.5 U/mL] - green line
[2 U/mL] - blue line
[1 U/mL] - yellow line

(One representative experiment of 8 is shown.)



Similarly, when splenocytes were cultivated with tmuCD40L HeLa cells with different concentrations of IL-4, arrangement of homotypic cell clusters varied from few small clusters in cultures with no IL-4 (figure 9A) to frayed, dissociated clusters with the concentrations 0.2, 0.5 and 2 U/mL IL-4 (figures 9B, C and E, respectively). In contrast, big, round and circular clusters were observed in mCD40B cultures with 1 U/mL IL-4 (figure 9D).

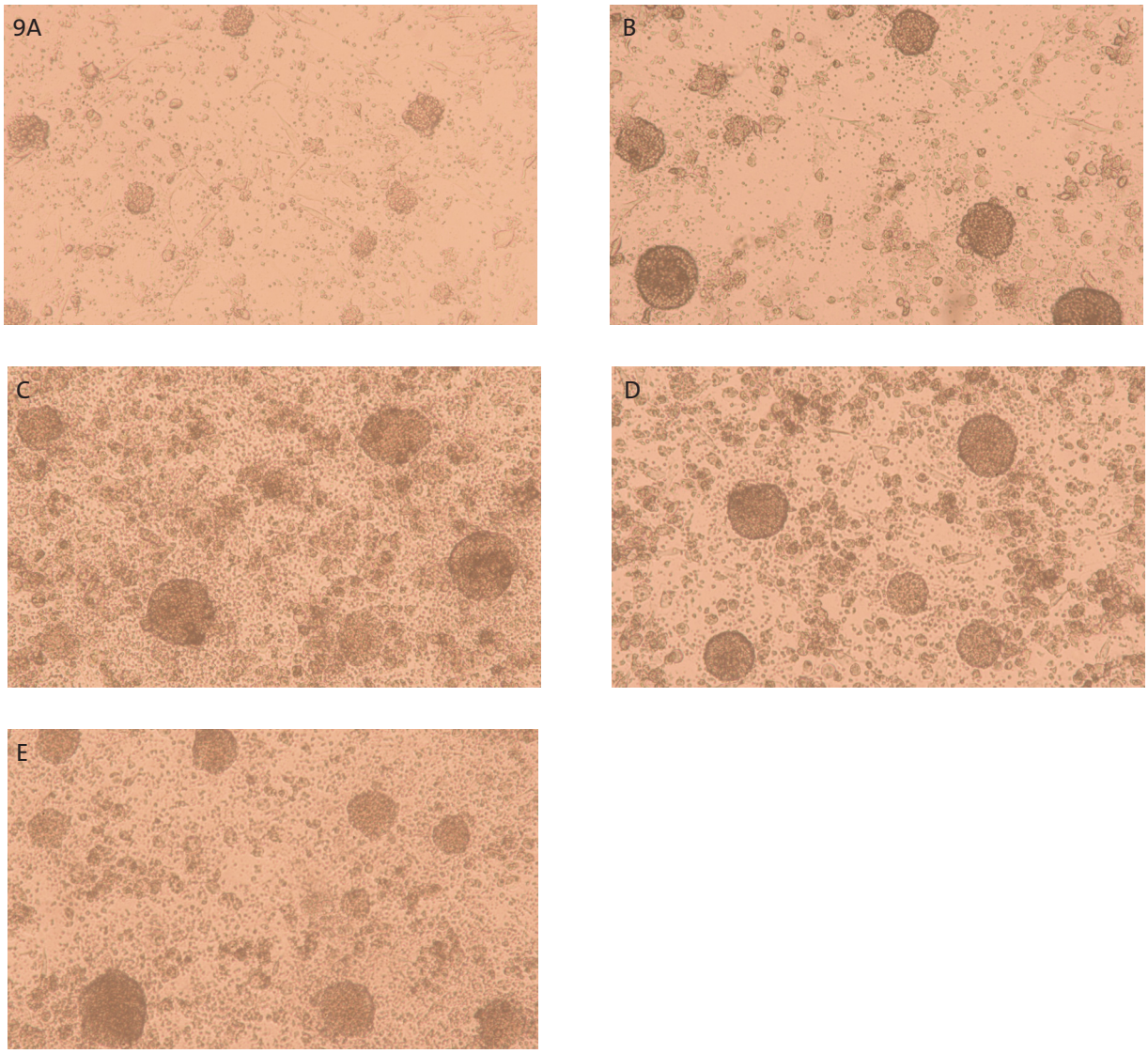


Figure 9: Cluster formation of mCD40B cells is dependent on IL-4 supplementation

Photographs were taken at a 5 x magnification. Formation of homotypic B-cell clusters varies upon IL-4 treatment: omission of IL-4 (A) and treatment with 0.2, 0.5, 1.0 and 2.0 U/mL IL-4 (B, C, D and E).

(One representative picture of at least 8 independent cultures is shown.)

Analysis of the proportions of $CD19^+$ B cells and $CD3^+$ T cells during culture on day 0, day 7 and day 14 by flow cytometry revealed that varying concentrations of IL-4 [0, 0.2, 0.5, 1 and 2 U/mL] had no significant effect on purity of cultures. All cultures revealed a purity of the $CD19^+CD3^-$ population of ≥ 90 % independent from IL-4 treatment. (One representative test series of at least 8 is shown in figure 10A-K). Surprisingly, although omitting IL-4 addition during 14 days of culture a high number of enriched cells were $CD19^+$ B cells (93.5 ± 2.4 %, n=8).

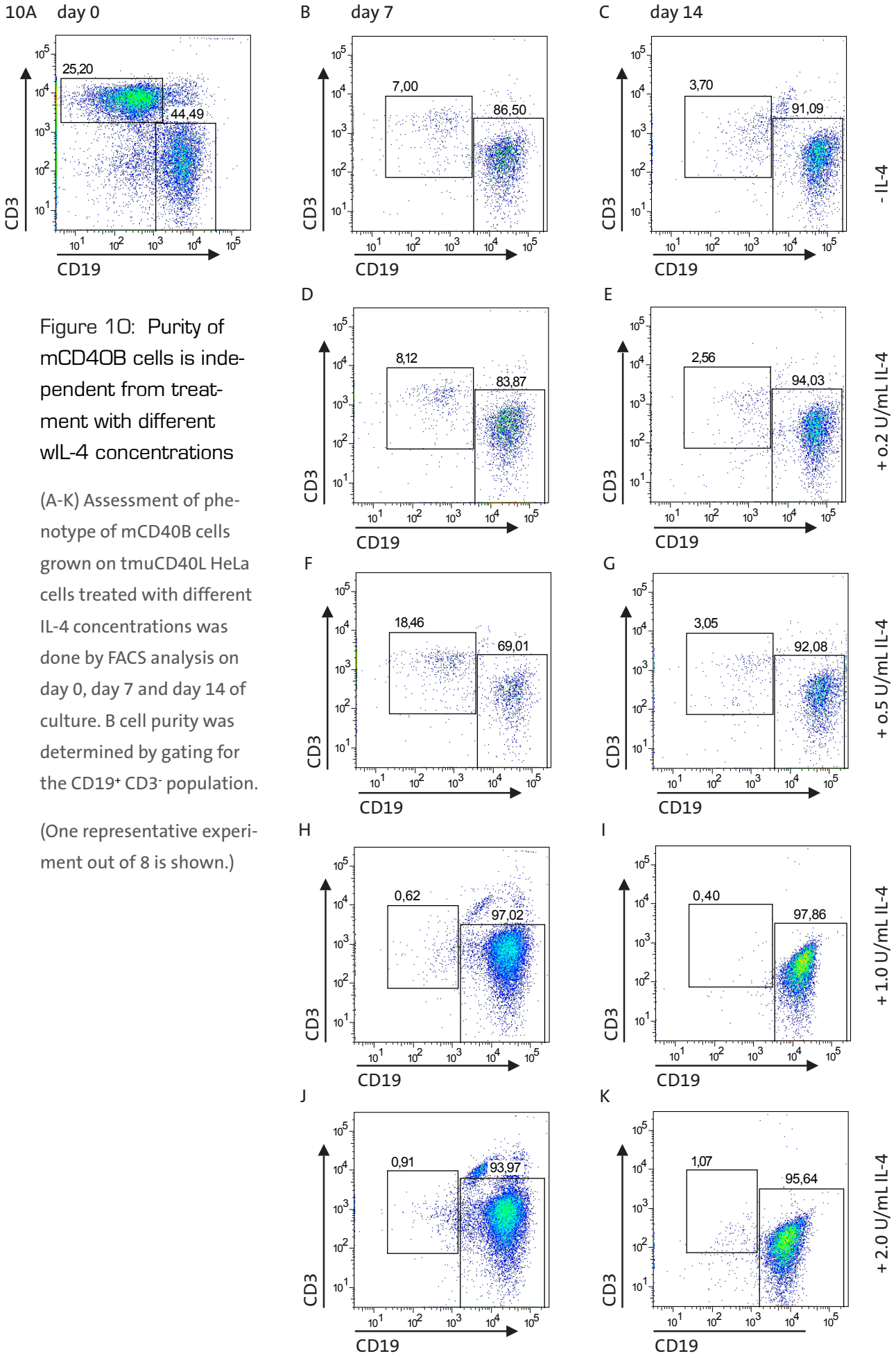


Figure 10: Purity of mCD40B cells is independent from treatment with different wIL-4 concentrations

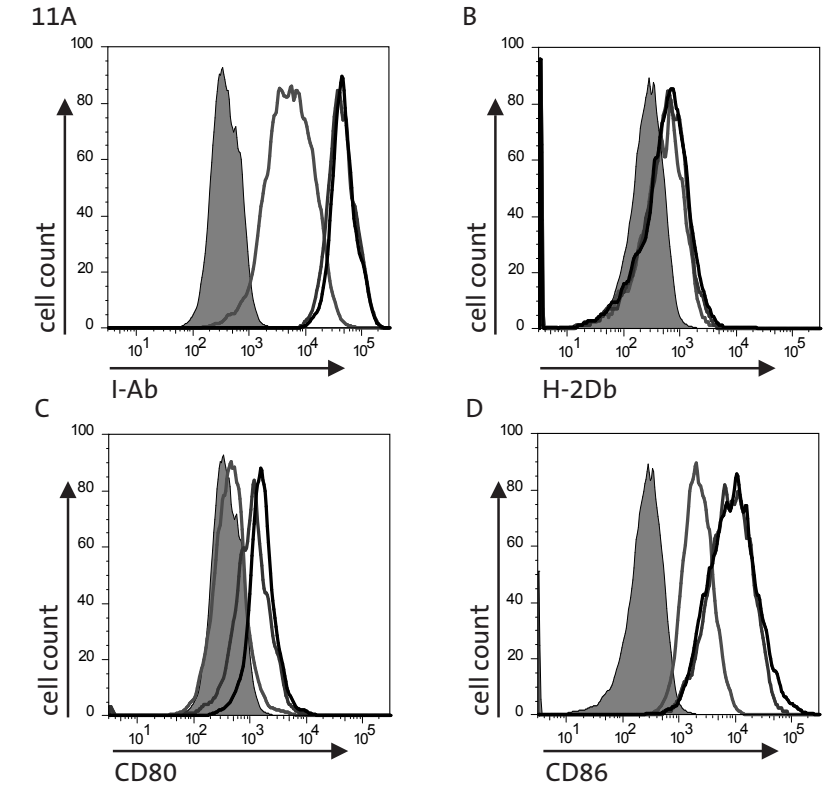
(A-K) Assessment of phenotype of mCD40B cells grown on tmuCD40L HeLa cells treated with different IL-4 concentrations was done by FACS analysis on day 0, day 7 and day 14 of culture. B cell purity was determined by gating for the $CD19^+$ $CD3^-$ population.

(One representative experiment out of 8 is shown.)

Similarly examination of the activation status revealed a consistent up-regulation of MHC molecules I-A^b and H-D^b, and co-stimulatory molecules CD80 and CD86 which was also independent from the concentration of IL-4. (One representative test series of at least 8 is shown in figure 11A-D, 12A-D, 13A-D, 14A-D, 15A-D).
As mCD40B cultures with 1 U/mL IL-4 had a significantly increased proliferation rate and formed big, encircled cell clusters this concentration was chosen for further mCD40B cultures.

Figure 11: Activation status of mCD40B without IL-4 treatment

Phenotypical FACS analysis was performed assessing the expression of MHC molecules Class-I (H-2Db) and Class-II (I-Ab) and the expression of co-stimulatory molecules CD80, CD86 on $CD19^+$ $CD3^-$ mCD40B cells treated with different concentrations of IL-4:
[0 U/mL] - (11A-D)
[0.2 U/mL] - (12A-D)
[0.5 U/mL] - (13A-D)
[1 U/mL] - (14A-D)
[2 U/mL] - (15A-D)



Histograms shaded in dark gray represent the isotype controls; lines in light grey stand for specific antibody staining on day 0, line in dark grey on day 7 and black lines represent day 14.

(One representative experiment out of 8 is shown.)

Figure 12: Activation status of mCD40B with 0.2 U/mL IL-4

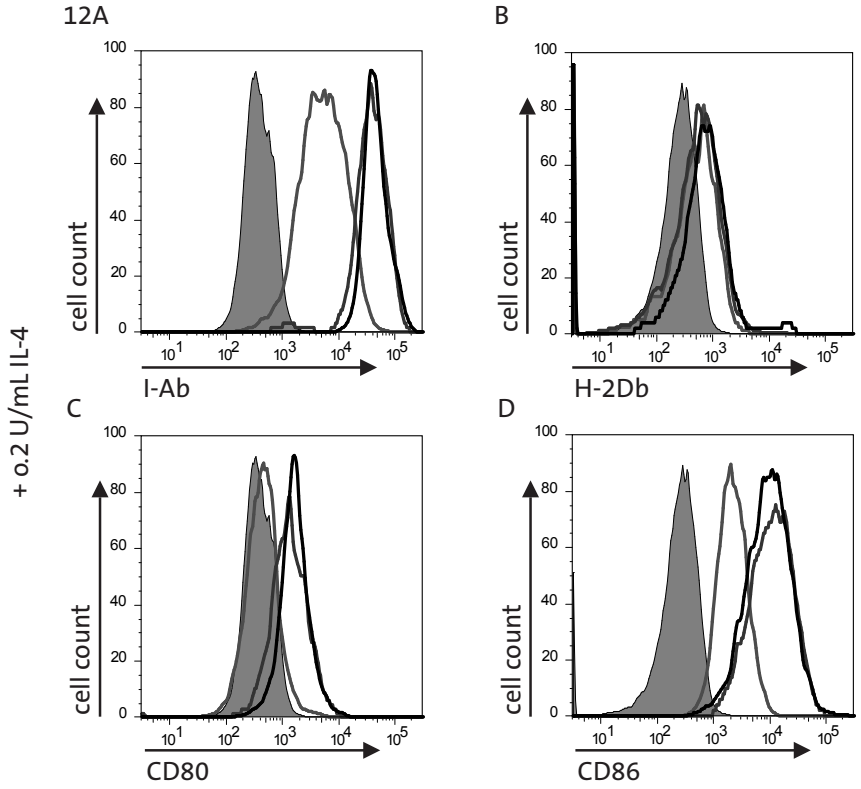


Figure 13: Activation status of mCD40B with 0.5 U/mL IL-4

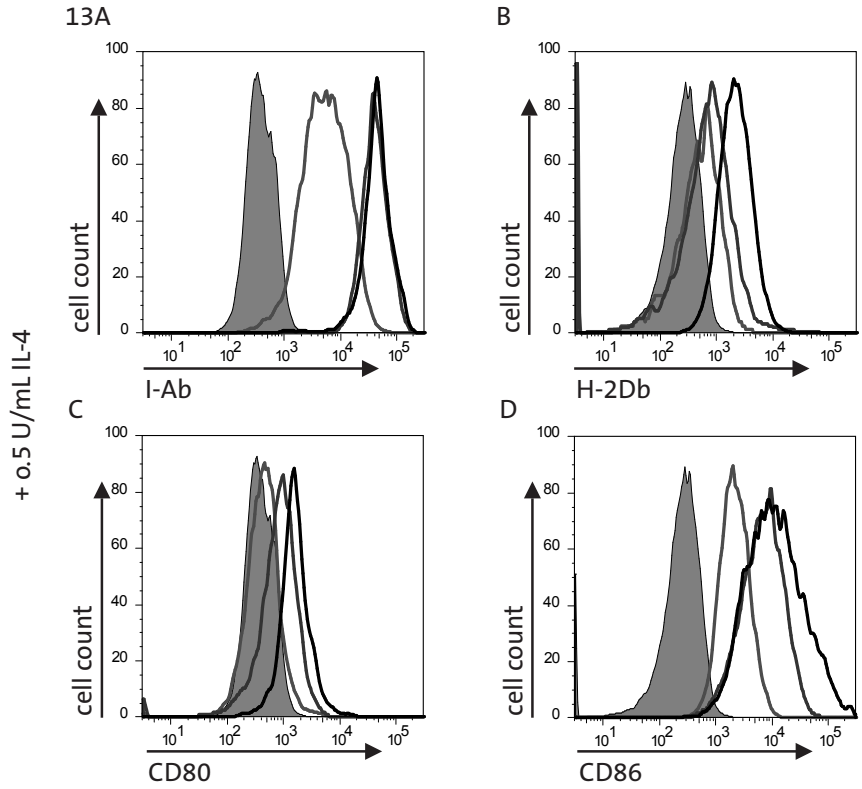


Figure 14: Activation status of mCD40B with 1.0 U/mL IL-4

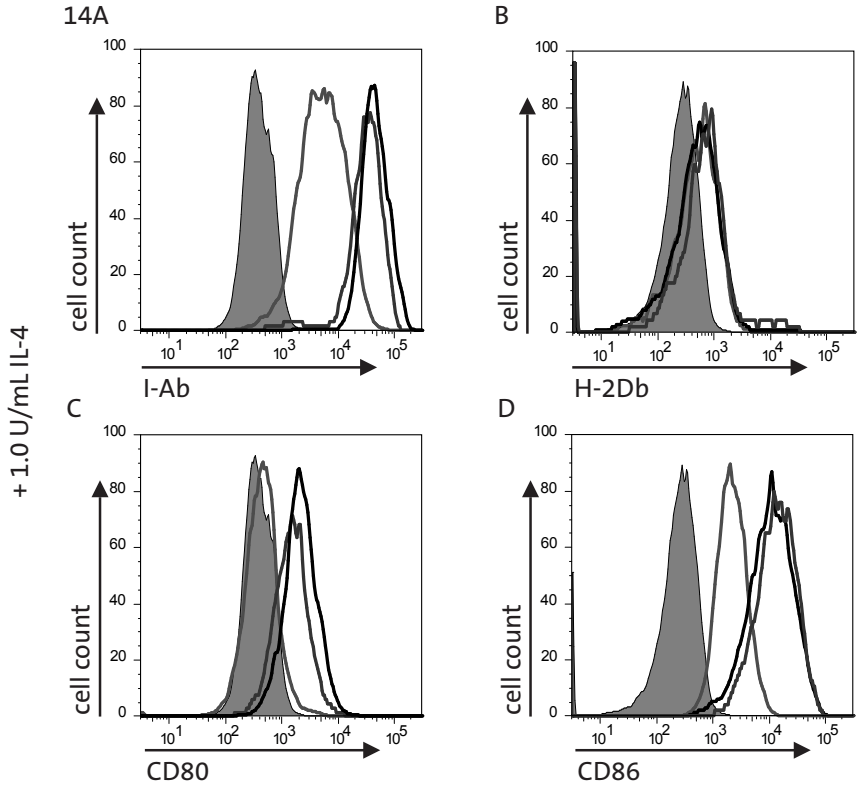
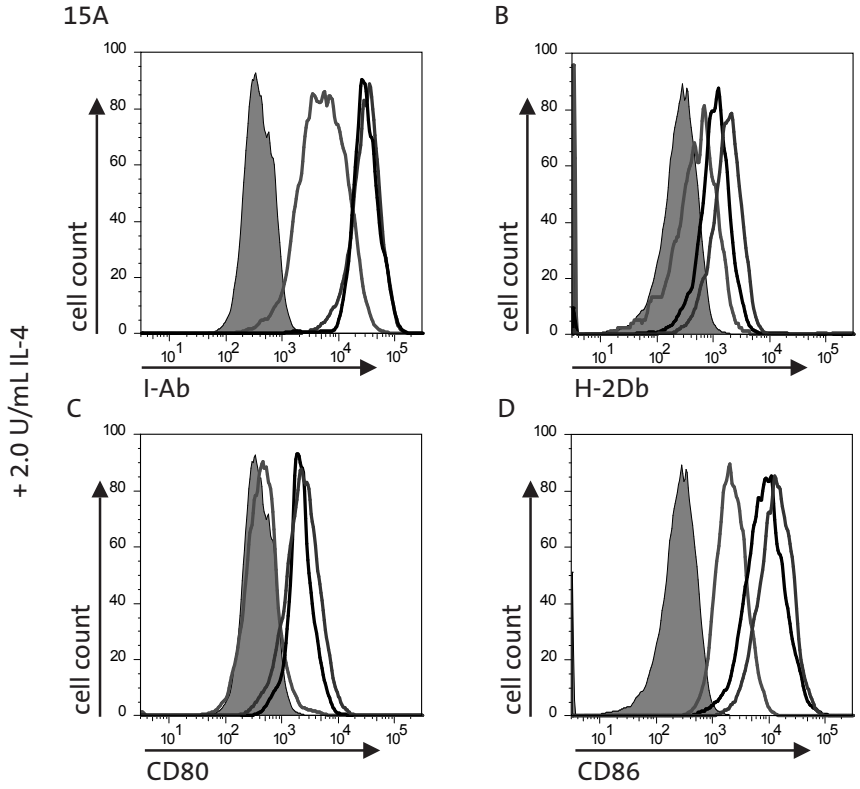


Figure 15: Activation status of mCD40B with 2.0 U/mL IL-4

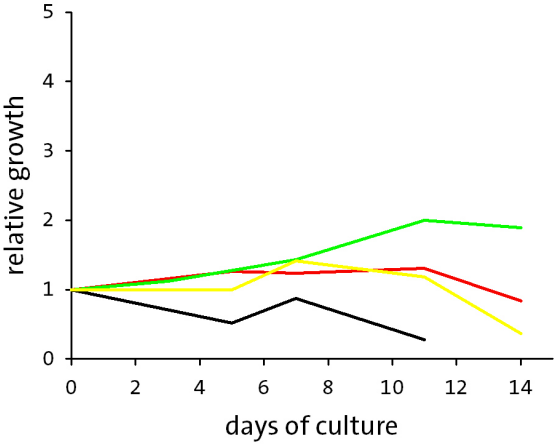


3.1.1.3. ß - Mercaptoethanol supplementation

Third, the optimal concentration of ß-mercaptoethanol (ß-ME) was identified. ß-ME is preferentially used as antioxidant in cell culture and is known to stimulate growth and differentiation of murine cells. Therefore four different concentrations [0, 50, 100 and 200 µM/mL] were tested. When mCD40B culture was conducted and ß-ME was omitted from the culture medium no durable B-cell proliferation was observed (figure 16; black line). Treatment with 50 µM mL ß-ME resulted in a medium proliferation of B cells until day 11 decreasing after further cultivation (red line). Similarly, when mCD40B culture was supplemented with 200 µM/mL ß-ME an initial increase until day 7 of culture was found which was not sustainable thereafter (yellow line). Best proliferation of mCD40B was obtained when cultivated with 100 µM/mL ß-ME (green line). After 14 days of culture the relative growth rate of mCD40B was thereby significantly increased 2.95 fold ranging from 1.9 to 3.7 ($p \leq 0.05$, $n = 8$).

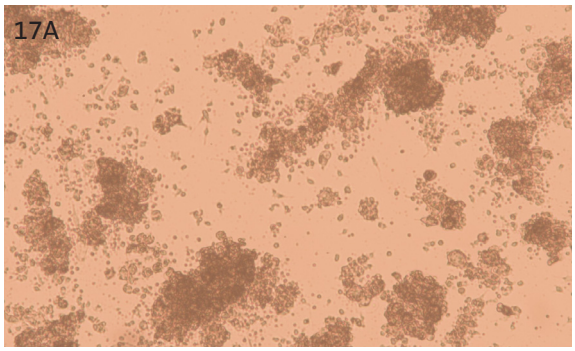
Figure 16: Proliferation of mCD40B cultures is dependent on ß-ME supplementation

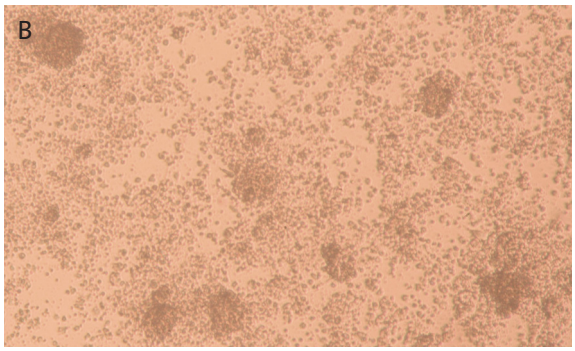
The number of viable mCD40B cells in mCD40B cultures treated with 4 different concentrations of ß-ME was determined until day 14 of culture:
[0 µM/mL] - black line
[50 µM/mL] - red line
[100 µM/mL] - green line
[200 µM/mL] - yellow line



(One representative of at least 8 experiments is shown.)

Cluster formation was prevented when omitting ß-ME from mCD40B culture medium (figure 17A) whereas few frayed and dissociated clusters were observed with 50 and 200 µM/mL ß-ME (figures 17B and D, respectively). Most consistent clusters were found with supplementation of 100 µM/mL culture medium (figure 17C).





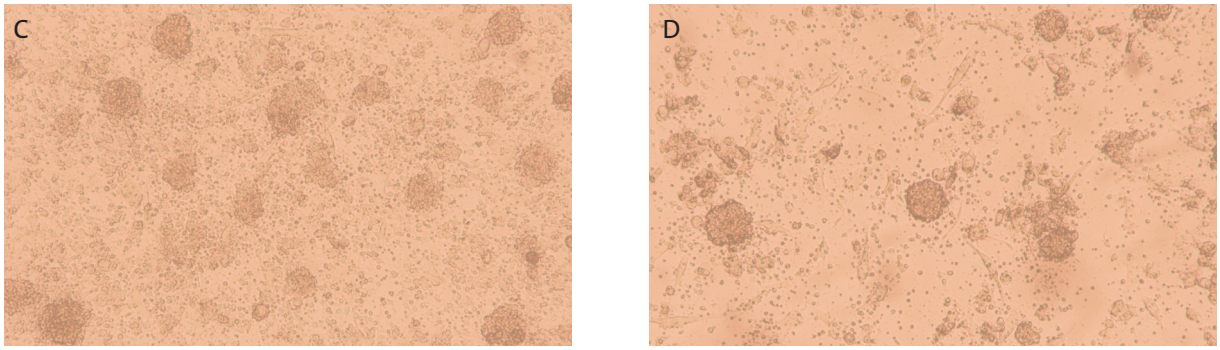
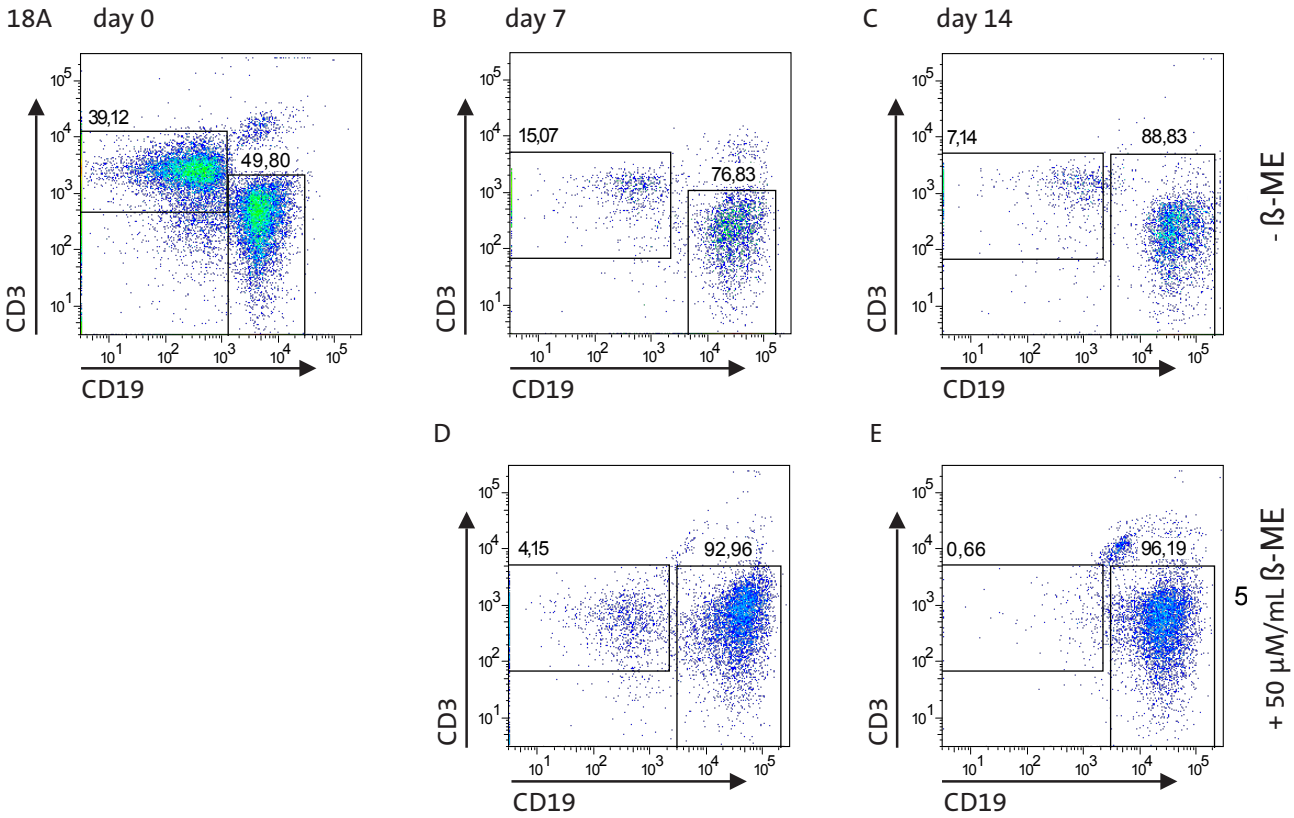


Figure 17: Cluster formation of mCD40B cells is dependent on ß-ME supplementation

Photographs were taken at a 5x magnification and B-cell cluster formation documented after treatment with 4 different ß-ME concentrations:
[0 µM/mL] - (17A) [50 µM/mL] - (17B) [100 µM/mL] - (17C) [200 µM/mL] - (17D)

(One representative picture of at least 8 independent cultures is shown.)

Flow cytometric analysis of the proportions of $CD19^+$ B cells and $CD3^+$ T cells in mCD40B cultures with altering concentrations of ß-ME showed that leaving out ß-ME or supplementing culture with 200 µM / mL resulted in lower $CD19^+$ proportions (85.3 ± 3.6 % and 87.1 ± 2.7 %, respectively, n = 8). In cultures with 50 or 100 µM /mL ß-ME significantly higher proportions of $CD19^+$ mCD40B cells were found after 14 days (97.1 ± 1.3 % and 97.4 ± 1.5 %, respectively, $p \leq 0.01$, n = 8). (One representative test series of at least 8 is shown in figure 18 A-I; see also next page).



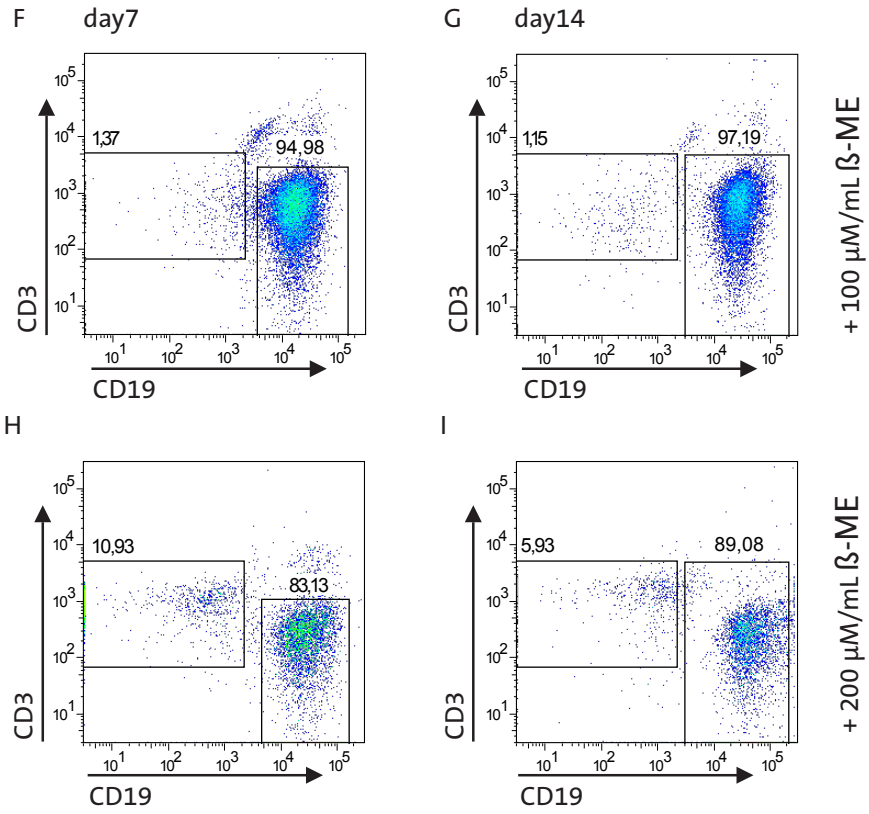


Figure 18: Purity of mCD40B cells is reliant on ß-ME supplementation

Assessment of the proportion of $CD19^{+}$ $CD3^{-}$ B cells in mCD40B cultures supplemented with 4 different concentrations of ß-ME on day 0, day 7 and day 14 of culture by flow cytometry:
[0 µM/mL] - (18A-C)
[50 µM/mL] - (18D, E)
[100 µM/mL] - (18F, G)
[200 µM/mL] - (18 H,I)

(One representative experiment out of 8 is shown.)

Dependent upon ß-ME supplementation the expression of MHC molecules and co-stimulatory molecules varies. The activation status of mCD40B cultivated without ß-ME or with 200 µM / mL was intrinsically lower for MHC molecules $I\text{-}A^{b}$ and $H\text{-}2D^{b}$ as well as CD80 and CD86 whereas supplementation with 50 or 100 µM/mL revealed no significant difference. One representative test series of at least 8 is shown in figures 19 - 22.

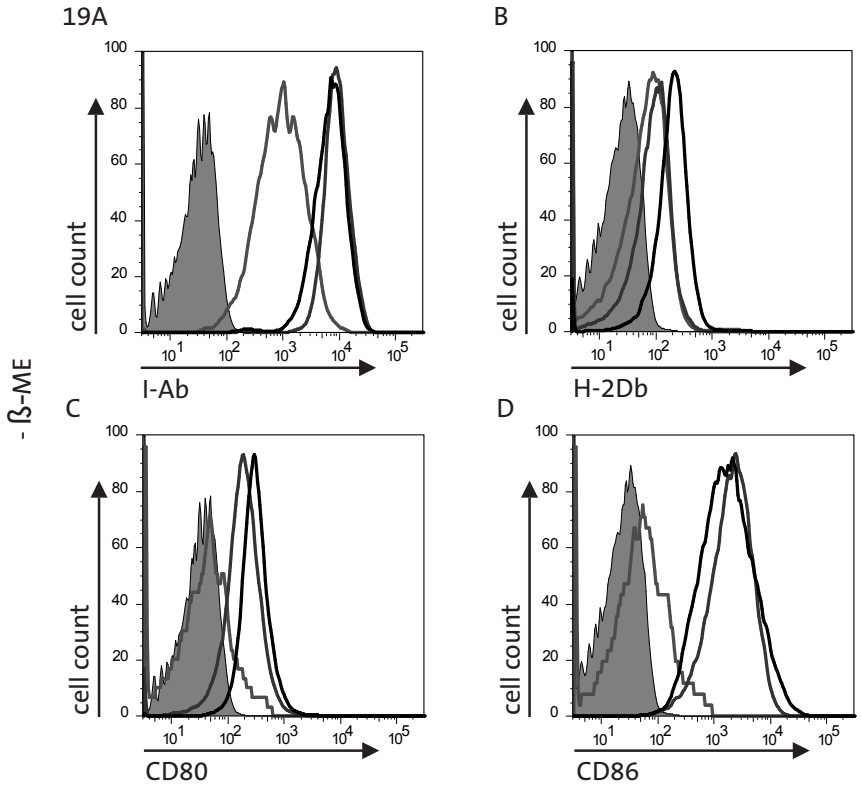


Figure 19: Activation status of mCD40B without ß-ME

Phenotypical FACS analysis was performed assessing the expression of MHC molecules Class-I (H-2Db) and Class-II (I-Ab) and the expression of co-stimulatory molecules CD80, CD86 on $CD19^{+}$ $CD3^{-}$ mCD40B cells treated with different concentrations of ß-ME:

[0 µM/mL] - (19A-D)
[50 µM/mL] - (20A-D)
[100 µM/mL] - (21A-D)
[200 µM/mL] – (22A-D)

Histograms shaded in dark gray represent the isotype controls; lines in light grey stand for specific antibody staining on day 0, line in dark grey on day 7 and black lines represent day 14.

(One representative experiment out of 8 is shown.)

Figure 20: Activation status of mCD40B with 50 µM/mL ß-ME

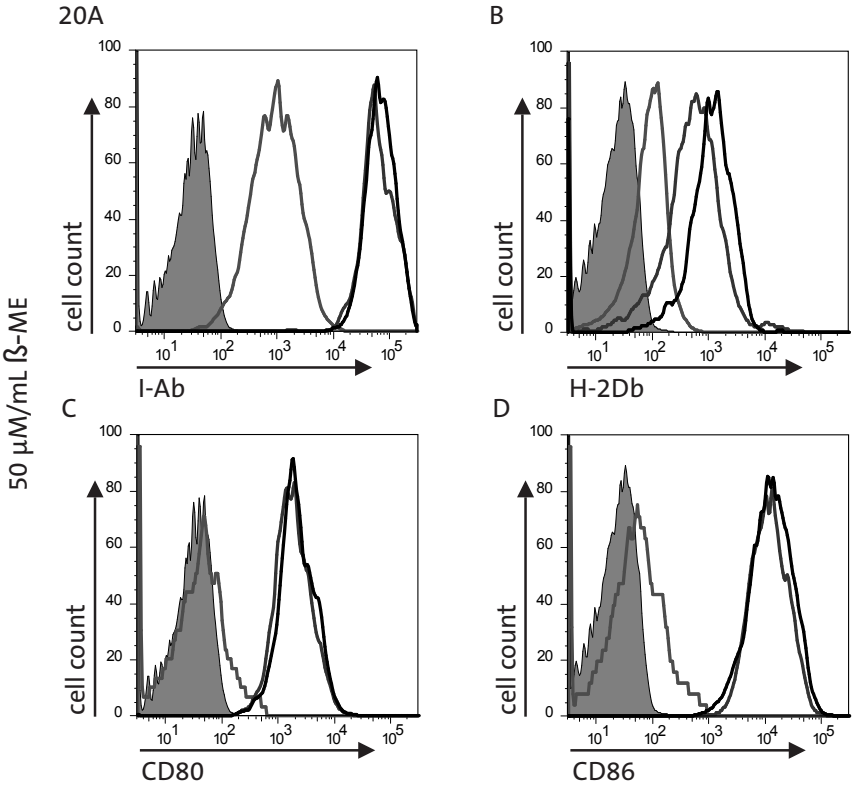


Figure 21: Activation status of mCD40B with 100 µM/mL ß-ME

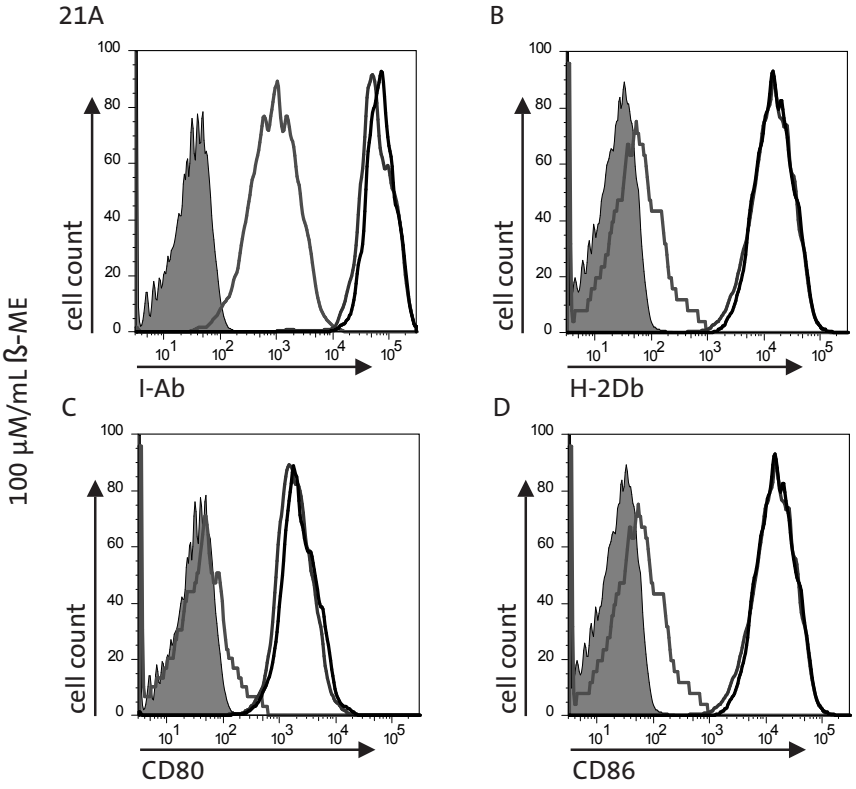
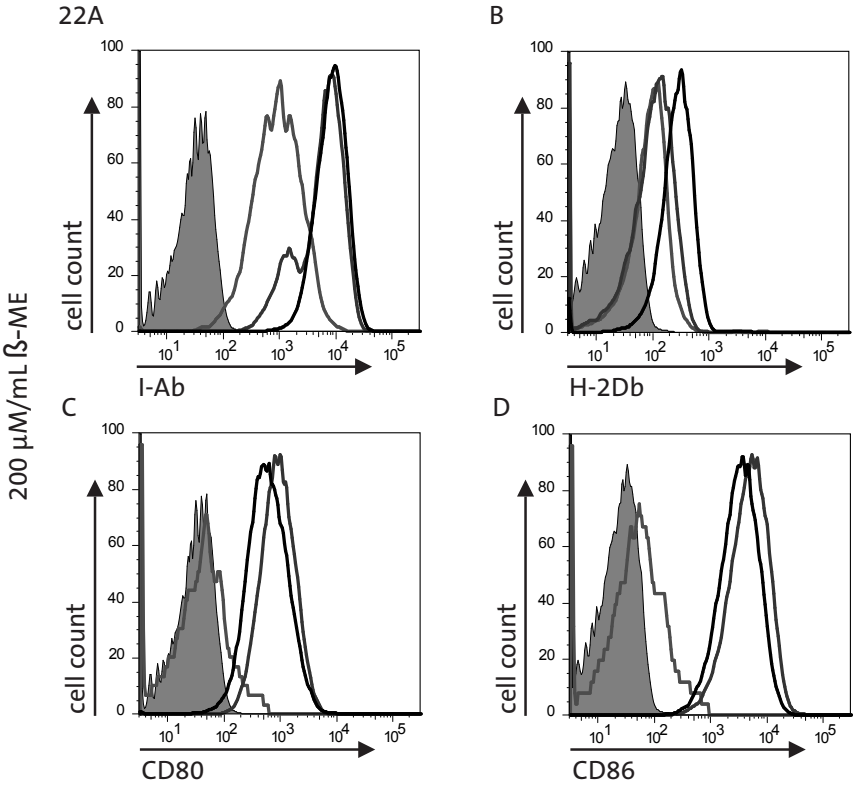


Figure 22: Activation status of mCD40B with 200 µM/mL ß-ME



Due to improved proliferation and consistent clustering 100 µM/mL ß-ME was applied in further mCD40B cultures.

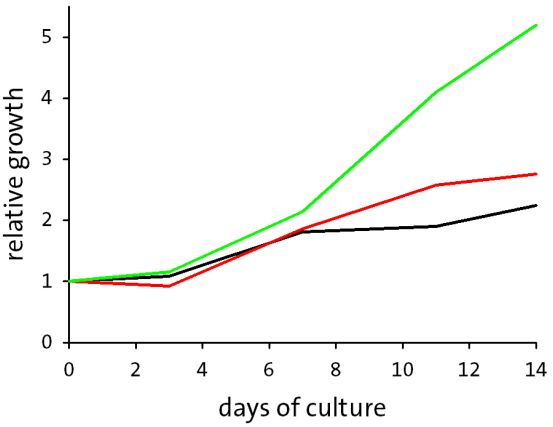
3.1.1.4. Cell culture media

To further optimize mCD40B culture conditions different media for optimal cell nutrition were tested. Dulbecco´s modified Eagle´s medium (DMEM) is the most frequently used classical basal medium suitable for a wide spectrum of mammalian cells. In particular primary and secondary murine cells and highly proliferating cell cultures are fed with this medium containing vitamins, amino acids and high glucose concentration.
Iscove´s Modified Dulbecco´s Medium (IMDM) is a completely defined variant of DMEM, including additional vitamins, amino acids, sodium pyruvate and HEPES buffer to especially support the growth of murine B-lymphocytes and rapidly proliferating high-density cell cultures.
DMEM-Ham´s F12 is a 1:1 mixture of DMEM and Ham´s F12 of which the latter was designed for cultivation of mammalian primary cells and murine L-cells containing increased concentrations of amino acids, vitamins and micronutricients as well as a additional buffering system for higher osmotic stability.
Murine splenocytes cultivated on tmuCD40L HeLa cells were grown in each of the three media supplemented with 1 U/mL IL-4 and 100 µM/mL ß-ME as defined before. Cultivation with IMDM and DMEM-Ham´s F12 media induced comparably low growth rates ranging from 1.0 to 2.8 and from 1.3 to 2.2, respectively (figure 23, red and black lines). In contrast performing mCD40B culture with DMEM medium resulted in constantly strong proliferation rates ranging from 2.4 to 5.2 until day 14 of culture (green line).

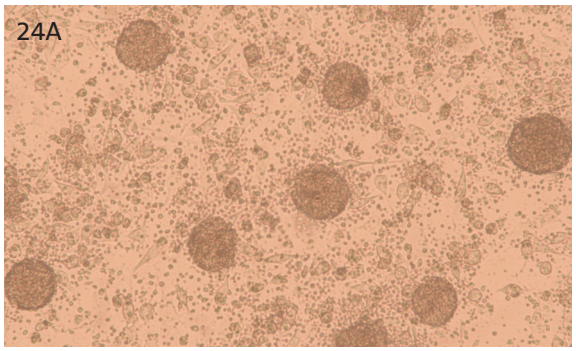
Figure 23: Proliferation of mCD40B is reliant on cell culture media

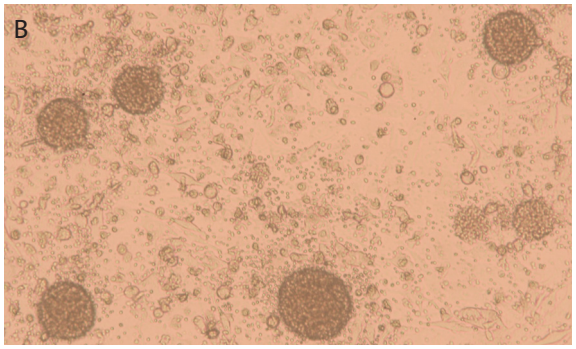
Proliferation of mCD40B cells is dependent on the selection of cell culture medium: cultivation in DMEM medium (green line), IMDM medium (red line) or DMEM-Ham´sF12 medium (black line).

(One representative experiment of at least 3 is shown.)



Considering cluster formation of mCD40B grown in different culture media only slight differences were found. Treatment with DMEM medium resulted in the highest density of clusters, which is also supported by the highest proliferation rates (figure 24A). Aside from this the use of IMDM and DMEM-Ham´s F12 media induced formation of cell clusters which were to some extent frayed and dissociated (figures 24B and C, respectively).





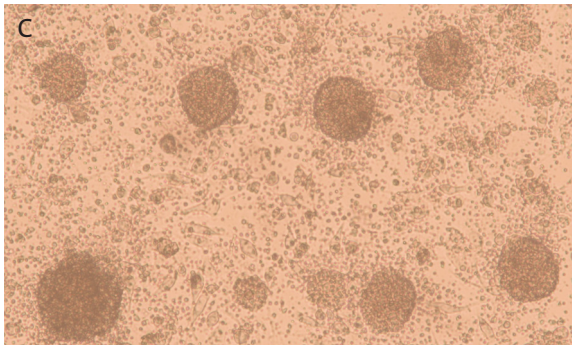


Figure 24: Cluster formation of mCD40B cells is dependent on selection of cell culture medium

Photographs were taken at a 5x magnification. B-cell cluster formation is dependent on cell culture medium: cultivation in DMEM medium (A), IMDM medium (B) and DMEM-Ham´s F12 medium (C).

(One representative picture of at least 3 independent cultures is shown.)

Comparing mCD40B cultures grown in different culture media no significant differences were found by flow cytometric analysis. After 14 days of cultivation on tmuCD40L HeLa cells with DMEM medium $CD19^+$ $CD3^-$ B cells with an average purity of 94.9 ± 3.6 % were generated. By treatment with IMDM or DMEM-Ham´s F12 media the B cell proportion was diminished to 93.2 ± 2.8 % and 92.6 ± 2.7 %, respectively. One representative test series of at least 3 is shown in figure 25 A-G.

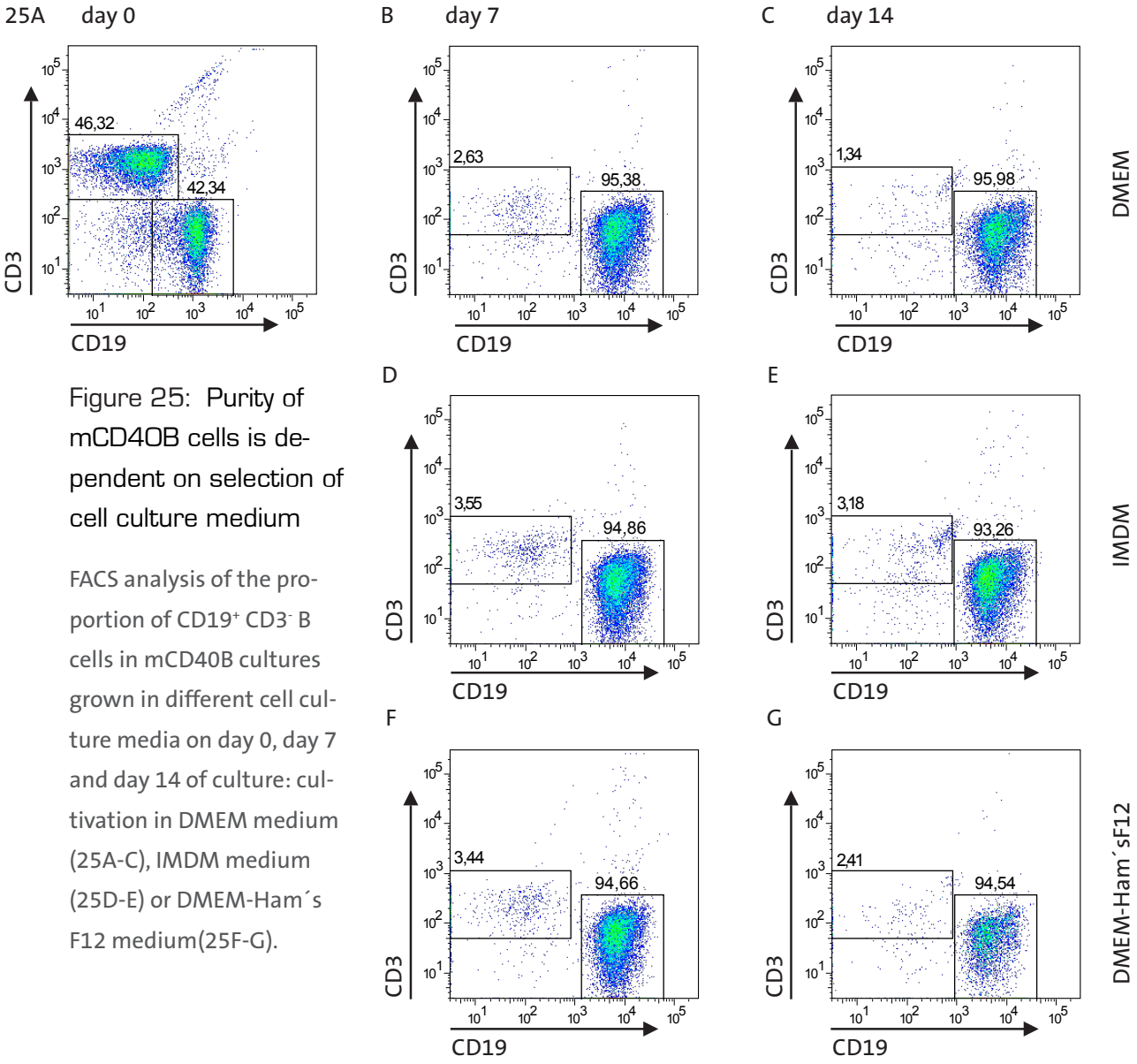


Figure 25: Purity of mCD40B cells is dependent on selection of cell culture medium

FACS analysis of the proportion of $CD19^+$ $CD3^-$ B cells in mCD40B cultures grown in different cell culture media on day 0, day 7 and day 14 of culture: cultivation in DMEM medium (25A-C), IMDM medium (25D-E) or DMEM-Ham´s F12 medium(25F-G).

(One representative experiment out of 3 is shown.)

Evaluation of the activation status showed that there were no significant differences in expression of MHC molecules $I\text{-}A^b$ and $H\text{-}2D^b$ as well as CD80 and CD86. Only cultivation in IMDM tended to result in lower expression levels. One representative test series of at least 3 is shown in figures 26 - 28.

Due to increased growth rate as well as slightly augmented purity and activation DMEM medium was chosen for further mCD40B cultures.

Figure 26: Activation status of mCD40B in DMEM medium

Phenotypical FACS analysis was performed assessing the expression of MHC molecules Class-I (H-2Db) and Class-II (I-Ab) and the expression of co-stimulatory molecules CD80, CD86 on $CD19^{+}$ $CD3^{-}$ mCD40B cells cultivated in different cell culture media on day 0, day 7 and day 14 of cell culture: DMEM - (26A-D) IMDM - (27A-D) DMEM-Ham´s F12 - (28A-D).

Figure 27: Activation status of mCD40B in IMDM medium

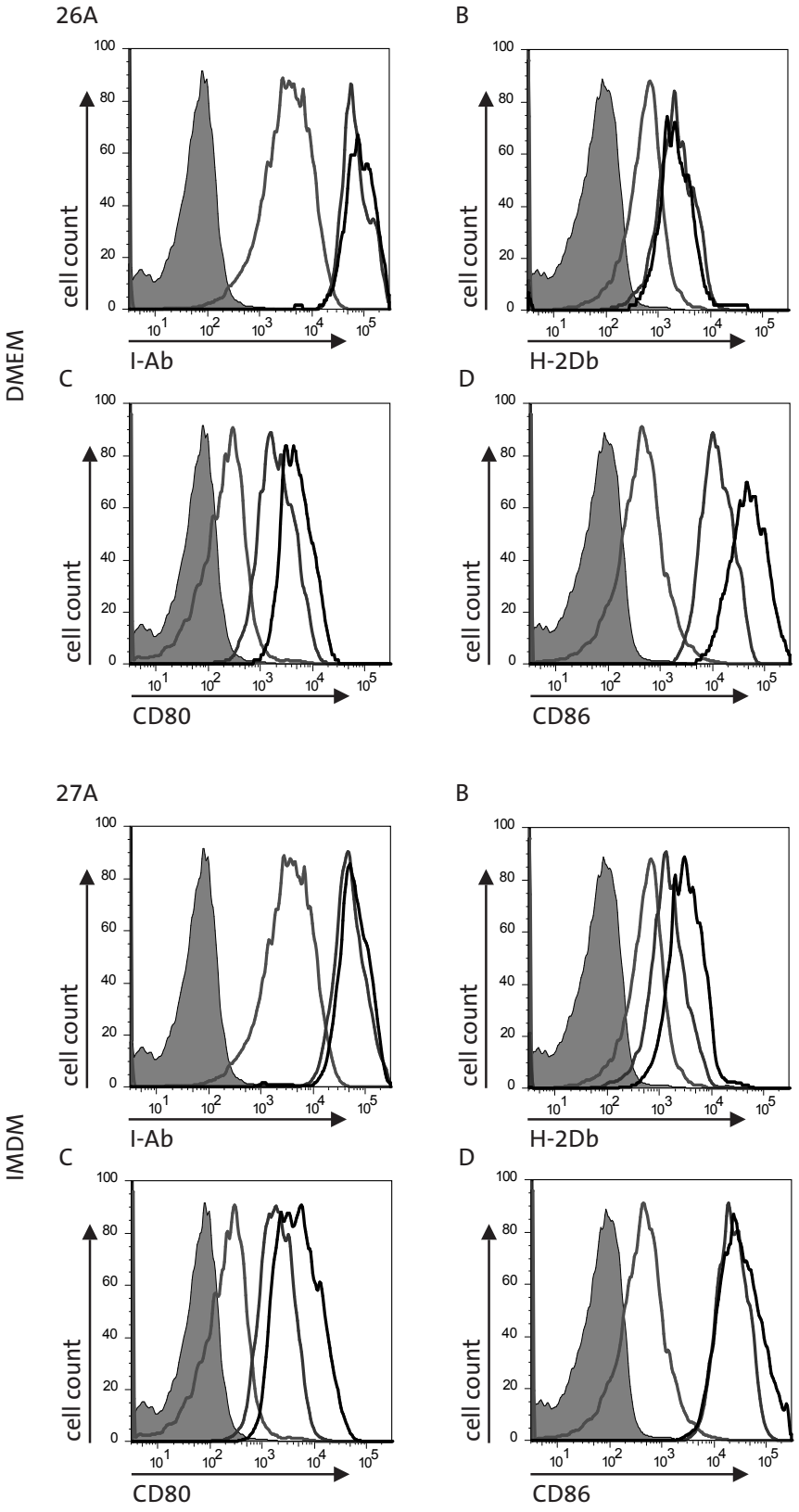
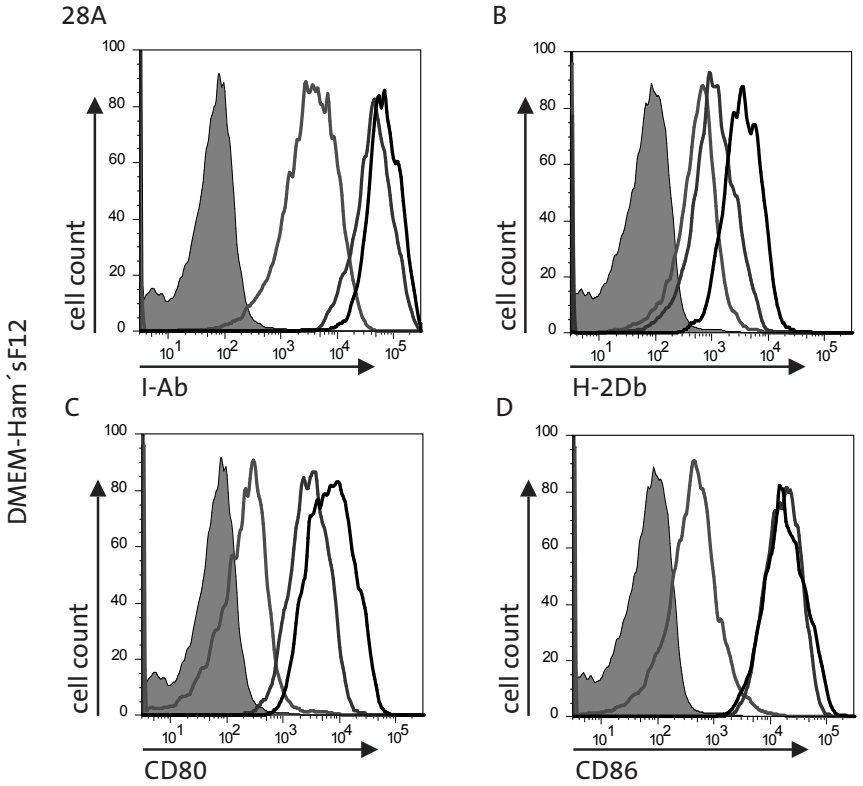


Figure 28: Activation status of mCD40B in DMEM-Ham´s F12 medium



Histograms shaded in dark gray represent the isotype controls; lines in light grey stand for specific antibody staining on day 0, line in dark grey on day 7 and black lines represent day 14.

(One representative experiment out of 3 is shown.)

3.1.2. Optimized system for generation of CD40-activated B cells from C57BL/6 mice

The assessment of the key components to generate effective mCD40B culture conditions resulted in following strategy (Liebig et al., 2010 218):

Splenocytes from C57BL/6 mice (8-12 weeks of age) are enriched by density gradient centrifugation with specific mouse Pancoll. Purified lymphocytes are co-cultivated with adherent irradiated murine CD40L expressing feeder cells (tmuCD40L HeLa) providing the CD40 stimulus. The murine CD40B culture is performed on 6-well plates in DMEM-Medium supplemented with fresh IL-4 [1 U/mL], ß-ME [100 µM/mL] and Cyclosporin A (CsA) [0.63 µg/mL]. Every 3 - 4 days the cell suspension is transferred onto fresh irradiated CD40L expressing feeder cells, and cell culture medium and additives are replenished meanwhile (see illustration 29). Until day 14 of culture cell cluster formation is induced resulting in typical round CD40B cell cluster due to homotypic aggregation (figure 30A, B). During time course $CD3^+$ T-cell population is diminished from an average 30 % to less than 5 % compared to the proliferating $CD19^+$ B-cell population that steadily increases to more than 95 % until day 14 of culture representing an average 3.7 fold increase (figure 30C). Under these conditions the so-termed “murine CD40-activated B cells” (mCD40B) continuously

up-regulate activation marker CD80, CD86 and MHC molecules I-A^b and H-2D^b compared to immature splenocytic B cells (figure 30D).

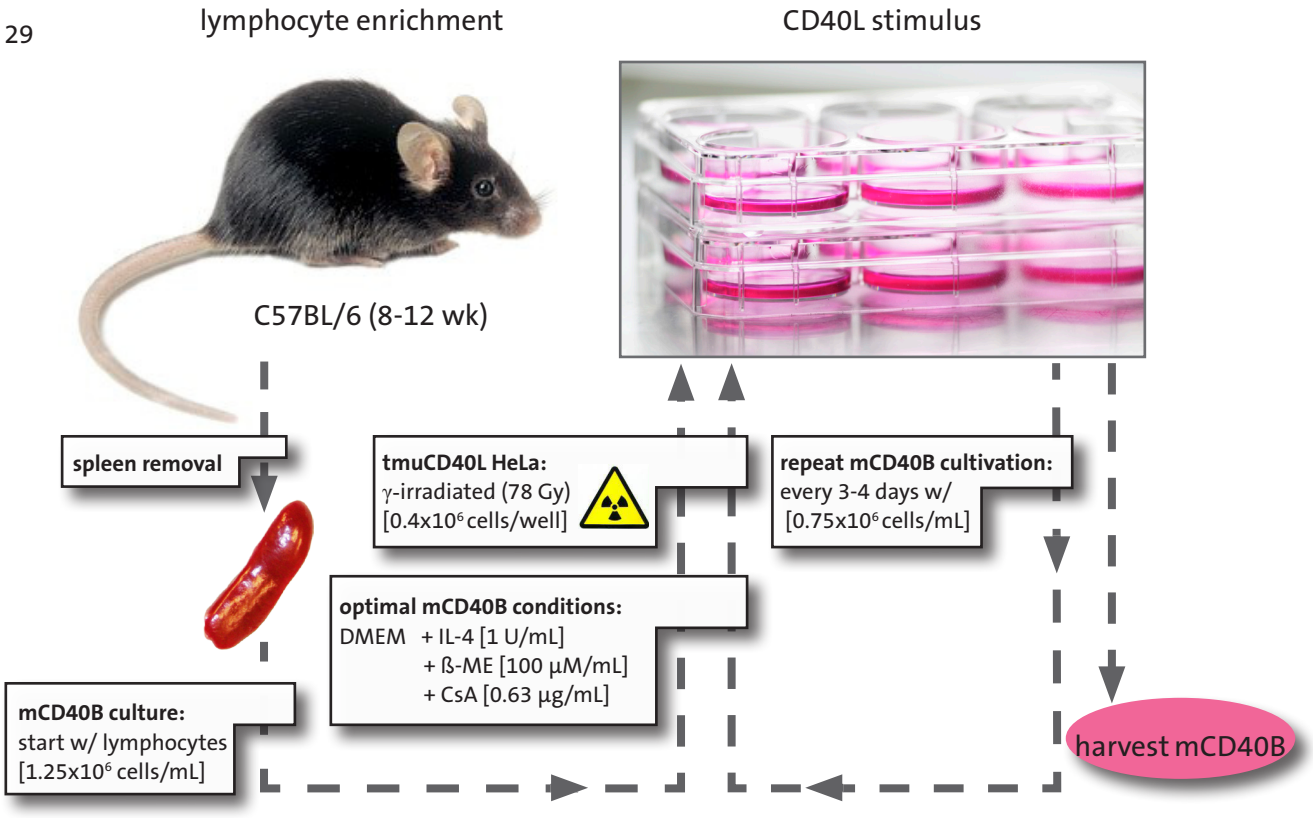


Figure 29: Illustration of the optimized cell culture system for generation of highly activated proliferating murine CD40-activated B cells

This optimized cell culture system was established to generate highly activated proliferating murine CD40B cells within 14 days. In brief, splenic lymphocytes from C57BL/6 mice are enriched and plated onto irradiated tmuCD40L HeLa in DMEM-Medium supplemented with fresh IL-4, ß-ME and CsA. After 3-4 days of co-cultivation feeder cells and growth stimuli are replenished leading to an accumulation of murine CD40B cells.

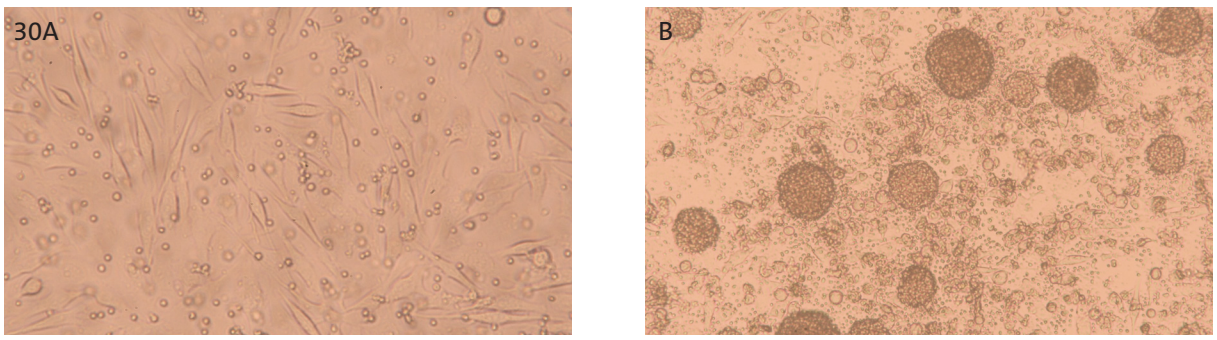
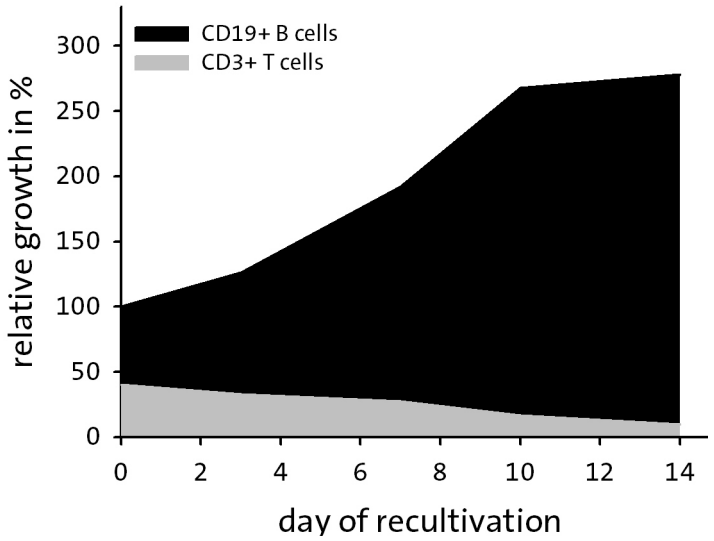


Figure 30: Characterisation of second generation activated murine CD40B cells

Photographs were taken at a 5x magnification using a light optical microscope fitted with a digital camera. (A) No B-cell clusters on day 0 when splenocytes are plated onto adherent muCD40L HeLa compared to (B) formation of typical round clusters after 14 days of culture.

(One representative photograph of at least 15 independent CD40B-cultures is shown.)

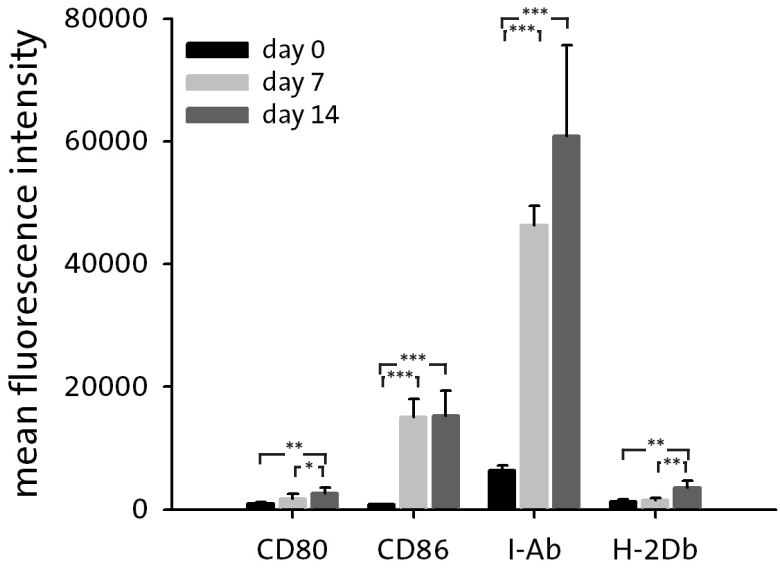
C



(C) Using the trypan blue exclusion test the number of viable mCD40B cells was determined every re-cultivation event and at each point of time the relative growth was assessed normalising the relative growth to the percentage of $CD3^+$ and $CD19^+$ cells in the culture determined by flow cytometric analysis.

(The growth curve represents the relative growth normalised to the fraction of B cells and T cells of at least 19 independently grown cultures.)

D



(D) Activation status of mCD40B grown in optimal culture condition was documented by analysing the expression of MHC molecules Class-I (H-2Db) and Class-II (I-Ab) and the expression of co-stimulatory molecules CD80 and CD86 on day 0, day 7 and day 14.

(Mean values of fluorescence intensity gated on viable $CD19^+$ cells ± SD of at least 9 experiments is shown.)

For all further experiments mCD40B were generated according to this optimized system, otherwise marked differently. Every mCD40B culture was routinely checked for proliferation, purity, activation status and cluster formation as well. Exclusively cultures comprising highly-activated, ≥ 90 % pure $CD19^+$ B cells were used.

3.2. Characterisation of the immune-stimulatory capacity of murine CD40-activated B cells “in vitro”

To study the antigen-presenting function of the above described murine CD40-activated B cells their ability to stimulate a proliferative response to allogeneic $CD3^+$ T cells *in vitro* was initially investigated. Here T cell activation is induced via signalling through binding of the T-cell receptor to allo-antigens in context of MHC on antigen-presenting cells. For complete activation of T cells additional co-stimulatory signals are required which are supplied by interaction of CD28 on T cells as well as CD80 (B7-1) and CD86 (B7-2) on APCs (Galvin et. al, 1992 219). This fact is utilized in the mixed lymphocyte reaction (MLR) assay (Steinman et al., 1978 220) where the *in vitro* proliferation of > 90 % pure negatively selected $CD3^+$ T cells (figure 31A) from BALB/c wild type mice (MHC haplotype d) in response to stimulation by mCD40B from C57BL/6 wild type mice (MHC haplotype b) was determined.

Since dendritic cells have long been viewed as the most potent APC due to their ability to induce primary immune responses *in vivo* (Inaba et al., 1990 221) bone marrow-derived DC served as alternative source of antigen-presenting cells and as positive control in the MLR. Therefore $CD34^+$ bone marrow cells from hind limbs of C57BL/6 wild type mice (MHC haplotype b) were isolated (figure 31B) and differentiated in endotoxin-tested medium containing GM-CSF growth factor [500 U/mL] (Lutz et al., 1999 222). Mature DCs were generated to assure activation of T cells in the MLR and 2 different maturation stimuli namely lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria or anti-CD40 antibody were tested to cover the heterogeneity of DC subsets (herein after referred to as DC LPS and DC aCD40Ab, respectively). Myeloid-derived DC phenotype was controlled by determination of the $CD11b^+$ $CD11c^+$ MHC-II^{high} (I-Ab) population (figure 31C and H, respectively). After 24h maturation with LPS [10 ng/mL] or anti-CD40 antibody [1 µg/mL] the expression of co-stimulatory molecules CD80, CD86 and CD83 on DC LPS and DC aCD40Ab was monitored by flow cytometry (figures 31 D-G and I-L, respectively).

31A

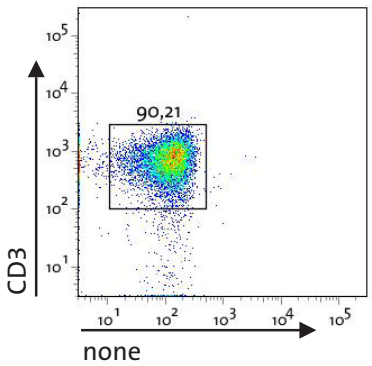
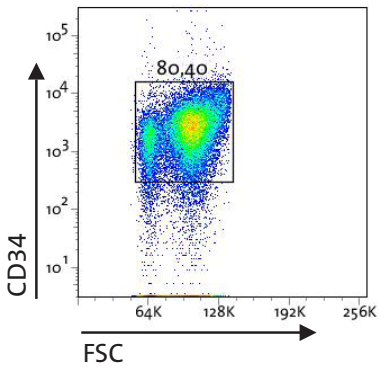


Figure 31: Phenotypical analysis of T cells and DC subsets induced in allogeneic MLR

(A) T cells for MLR were purified by negative selection for $CD3^+$ T cells from BALB/c mice and used when FACS analysis resulted in purity > 90 %.

(One representative T-cell enrichment of at least 12 is shown.)

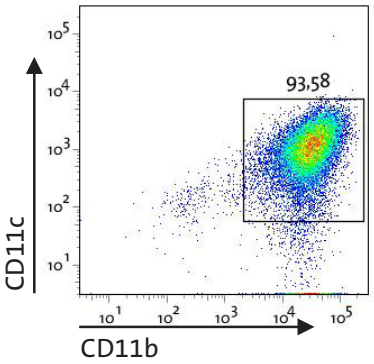
B



(B) Myeloid DCs were generated from 75-80 % pure, positively selected CD34⁺ bone marrow progenitor cells through stimulation with GM-CSF [500 U/mL] for 7 days.

(One representative CD34⁺ cell-enrichment of at least 26 is shown.)

C



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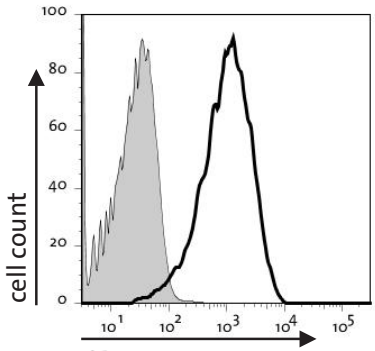

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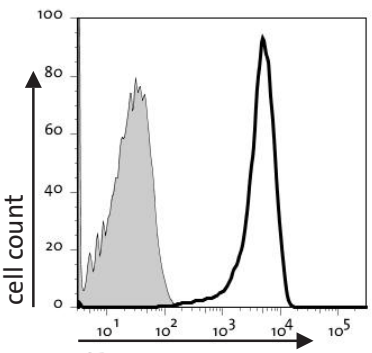

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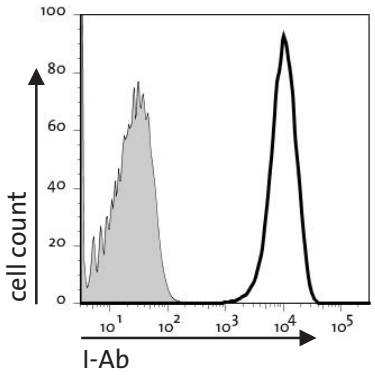

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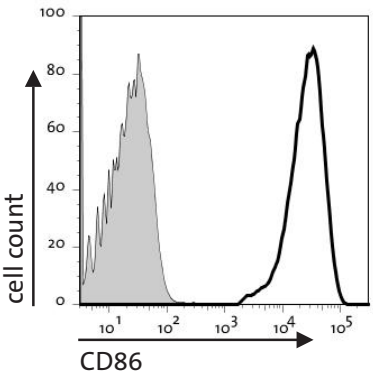
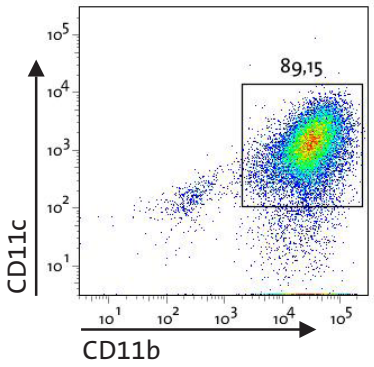
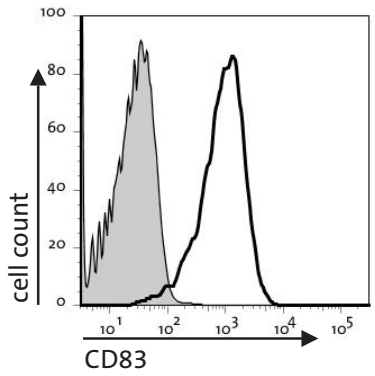


H



I



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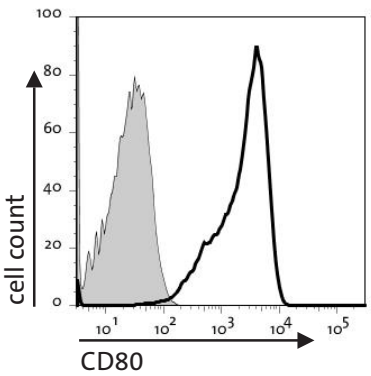
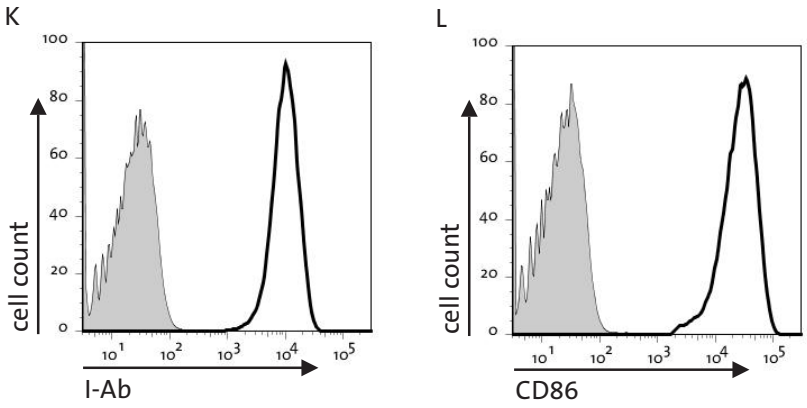




(C-G and H-L) mDC LPS and mDC aCD40Ab were finally matured for 24h using LPS [10 ng/mL] or aCD40 antibody [1 µg/mL], respectively. Maturation status was controlled by FACS analysis of expression of co-stimulatory molecules CD80, CD86, and CD83 on > 90 % pure $CD11c^+$ $CD11b^+$ $MHC\text{-}II^{high}$ (I-Ab) cell population.

(One representative DC maturation of at least 26 is shown.)

The phenotype of mCD40B cultivated for 14 days or for 7 days (herein after referred to as mCD40B d14 or mCD40B d7, respectively) was routinely checked as well (data not shown).

To measure the dynamics of the T-cell response T cells were labelled with the fluorescent proliferation marker 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and cultivated together with DC LPS, DC aCD40Ab, mCD40B d14 or mCD40B d7 at various APC-to-T cell ratios (0:1, 3:1, 1:1, 1:10, 1:20, 1:50, 1:100) in triplicates in 96 well plates for 5 days. CFSE segregates equally between daughter cells upon cell division, resulting in the sequential halving of cellular fluorescence intensity with each successive generation. When analyzed by flow cytometry, this sequential halving of fluorescence is visualized as distinct peaks in the histogram (figure 32A-J). Furthermore, the expression of the activation marker CD25 on divided T cells as a function of mitotic number was monitored as well (figure 33A-J).

Neither $CD3^+CD4^+$ nor $CD3^+CD8^+$ T cell population proliferated or showed up-regulation of CD25 expression when T cells were cultivated without APC (negative control, figures 32 and 33 A and B, respectively). Notably, T cell proliferation occurred not only when DC LPS (figure 32C, D) or DC aCD40Ab (32E, F) were used as APC, but also when mCD40B d14 (32G, H) or mCD40B d7 (32I, J) were used at a 1-to-1 ratio to stimulate allogeneic T cells. Additionally, stimulation of T cell division by either source of APC was accompanied by an up-regulation of CD25 expression (figure 33C-J).

Figure 32: Proliferation of allogeneic T cells in MLR at APC-to-T cell ratio 1-to-1

Typical sequential halving of CFSE fluorescence intensity with each generation is detected by flow cytometric analysis.

(A, B) Negative control: $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells were cultivated without stimulating APC.

(C and D, E and F, G and H, I and J) Cultivation of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T-cells with DC LPS, DC aCD40Ab, mCD40B d14 or mCD40B d7 as stimulating APC in MLR, respectively.

(One representative experiment of 8 is shown.)

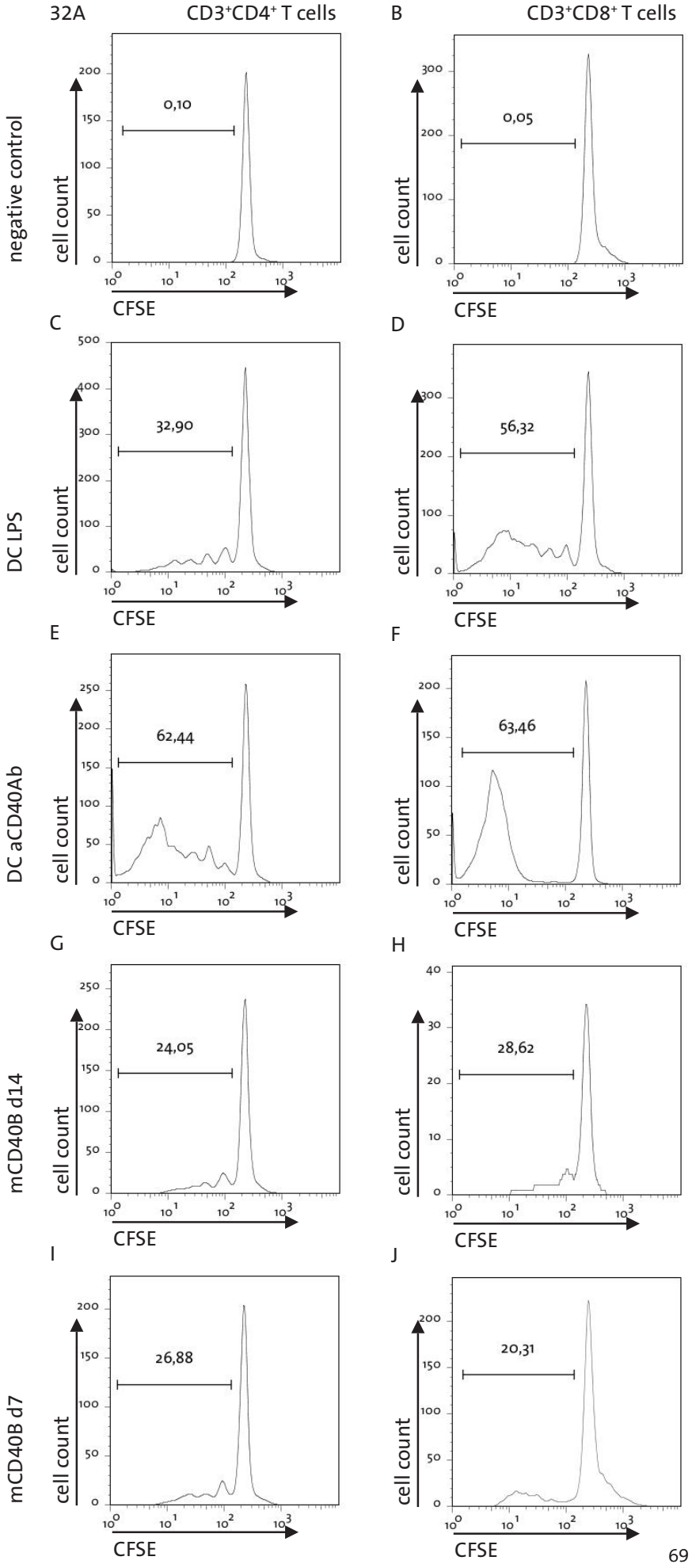


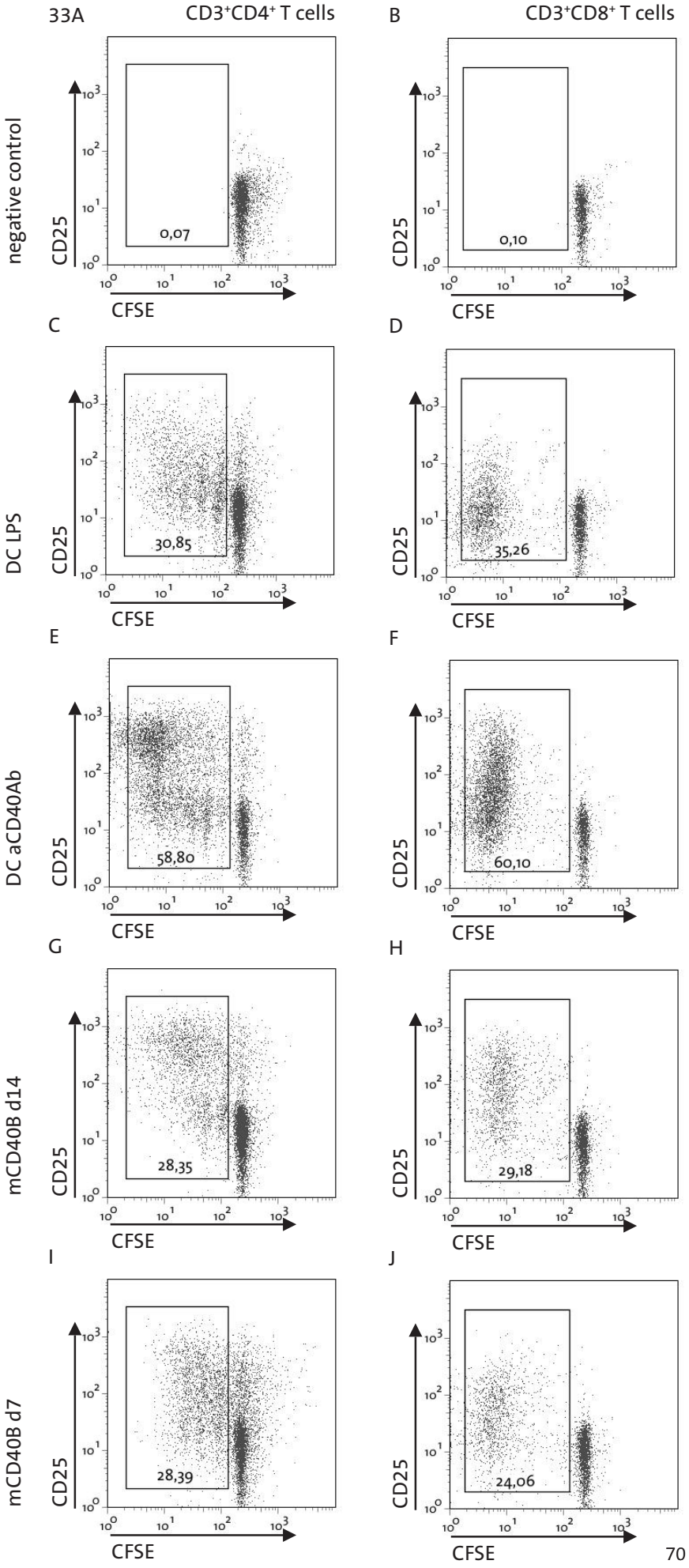
Figure 33: CD25 expression of proliferated allogeneic T cells in MLR at ratio 1-to-1

Sequential halving of CFSE fluorescence intensity which is accompanied by up-regulation of CD25 expression on $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cell populations is documented by FACS analysis.

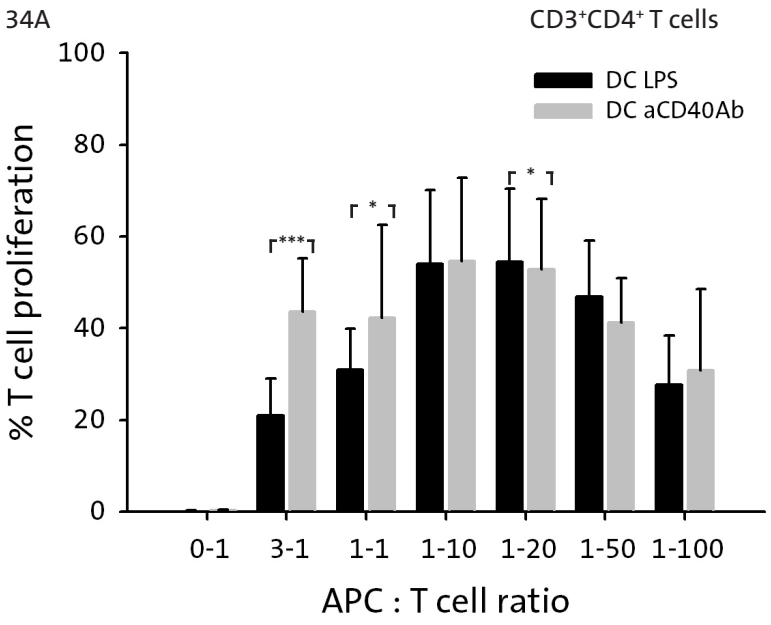
(A, B) Negative control: T cells were cultivated without stimulating APC.

(C and D, E and F, G and H, I and J) Cultivation of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T-cells with DC LPS, DC aCD40Ab, mCD40B d14 or mCD40B d7 as stimulating APC in MLR, respectively.

(One representative experiment of 8 is shown.)



Furthermore, by performing 8 independent allogeneic MLRs it was observed that mCD40B were able to stimulate $CD3^+$ T cells to proliferate as measured by decrease in CFSE-fluorescence intensity (figure 34A-D). Comparing different mCD40B and DC stimulator subtypes at various ratios it was noticed that mCD40B subtypes performed best using bigger APC-to-T cell ratios (1:1 to 1:10 for $CD3^+CD4^+$ T cells and 3:1 to 1:1 for $CD3^+CD8^+$ T cells) compared with DC subtypes performing best at lower ratios (1:10 to 1:20 for $CD3^+CD4^+$ and 1:1 to 1:10 for $CD3^+CD8^+$ T cells). As there were significant differences in the percentage of proliferated T cells at different ratios in favour of both DC subtypes DC LPS as well as DC aCD40Ab were used as reference APCs in further *in vivo* experiments. Comparing mCD40B d14 and mCD40B d7 significant differences were seen in stimulating proliferation of $CD3^+CD4^+$ T cells at ratios 3-to-1 and 1-to-1 when mCD40B d7 were used as APC. In contrast $CD3^+CD8^+$ T cell proliferation was induced significantly stronger when mCD40B d14 were used as APC at ratios 3-to-1 and 1-to-1.
For the induction of a sufficient and long-lasting immune response by antigen-presenting cells the action of $CD4^+$ helper T cells as well as $CD8^+$ cytotoxic T cells is needed. Since there was no clear result in favour of one mCD40B subtype mCD40B d14 and mCD40B d7 were used in the following experiments to characterize their antigen-presentation capacity *in vivo*.

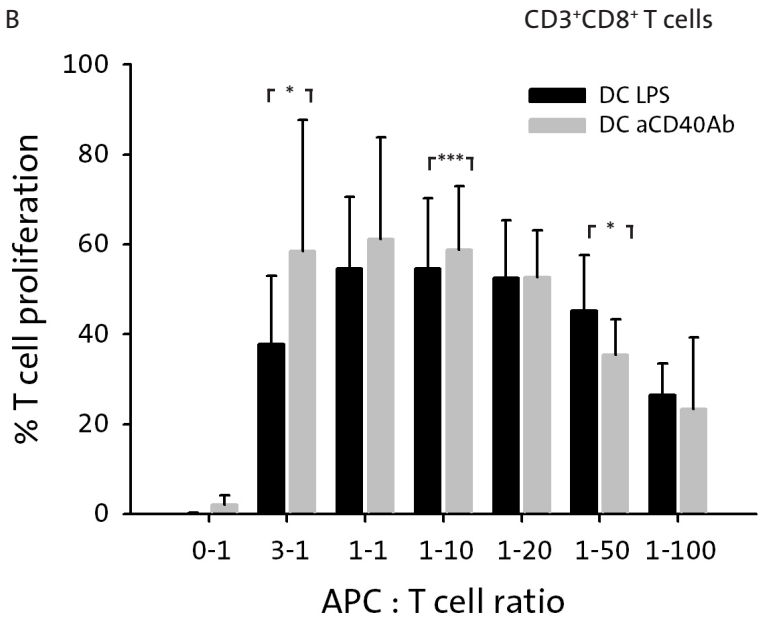


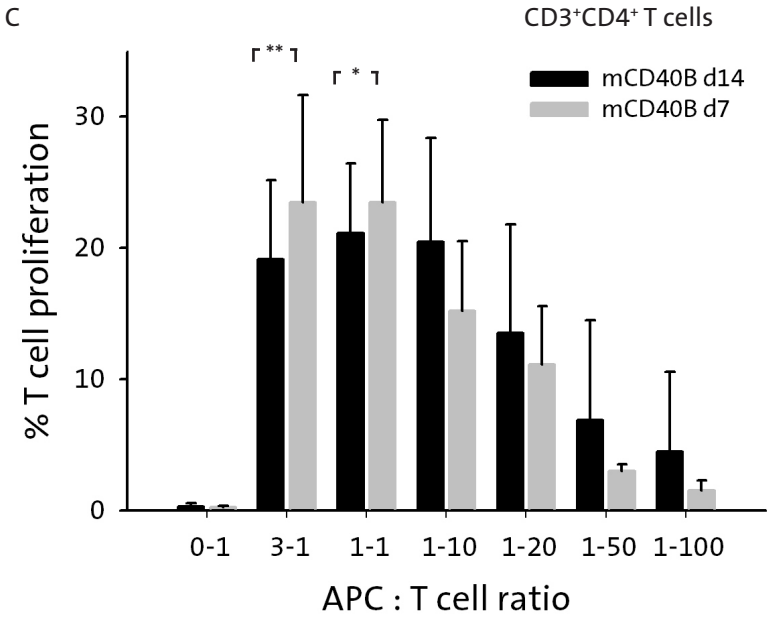
(Mean values of the percentage of proliferated T-cells ± SD of 8 independent MLR experiments is shown. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

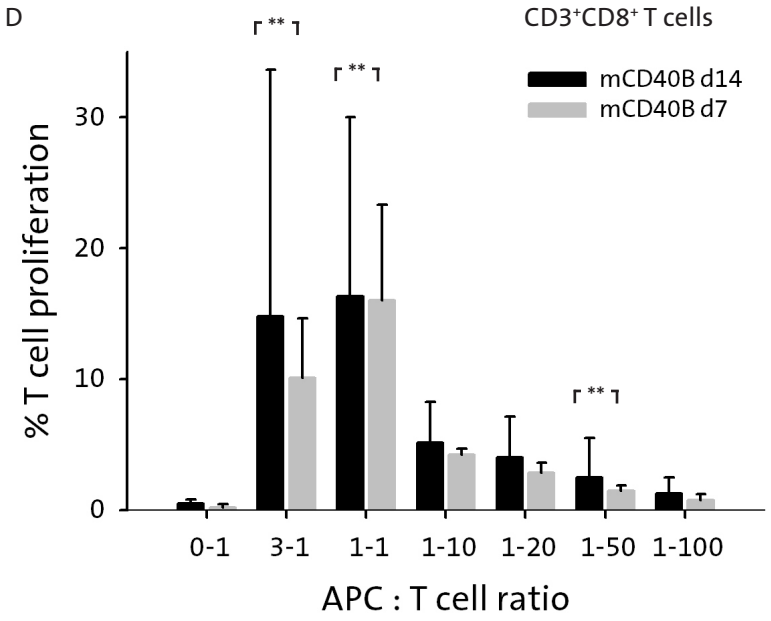
Figure 34: Statistical analysis of T-cell proliferation of eight independent allogeneic MLRs

$CD3^+CD4^+$ T-cell proliferation at various ratios in allogeneic MLR with either DC LPS and DC aCD-40Ab (A) or with mCD40B d14 and mCD40B d7 (C) as APCs.

$CD3^+CD8^+$ T-cell proliferation at various ratios in allogeneic MLR with either DC LPS and DC aCD-40Ab (B) or with mCD40B d14 and mCD40B d7 (D) as APCs.


B
CD3+CD8+ T cells
DC LPS
DC aCD40Ab
% T cell proliferation
100
80
60
40
20
0
*
***
*
0-1
3-1
1-1
1-10
1-20
1-50
1-100
APC : T cell ratio


C
CD3+CD4+ T cells
mCD40B d14
mCD40B d7
% T cell proliferation
30
20
10
0
**
*
0-1
3-1
1-1
1-10
1-20
1-50
1-100
APC : T cell ratio


D
CD3+CD8+ T cells
mCD40B d14
mCD40B d7
% T cell proliferation
30
20
10
0
**
**
**
0-1
3-1
1-1
1-10
1-20
1-50
1-100
APC : T cell ratio

3.3. Characterisation of the immunologic activity of mCD40B "in vivo" - Establishment of a murine vaccination model

In order to analyze whether highly activated, > 90 % pure mCD40B are potent antigen-presenting cells *in vivo* their systemic effects concerning safety were addressed in the first part - in the second part the efficacy regarding the elicited immune response was investigated for the first time.

3.3.1. Analysis of secondary immunological effects of adoptive mCD40B cell transfer

In cancer immunotherapy the adoptive transfer of tumour-reactive T cells directed against self-derived tumour-antigen can induce severe adverse effects like auto-immunity. In fact rarely, but also in vaccinations with dendritic cells toxicities like immunologic injection site reactions or autoimmune disorders arising from humoral or cellular immune responses of the individual to his own tissue constituents have been observed. To assess whether the administration of mCD40B *in vivo* would be safe or would lead to any toxic side effects C57BL/6 mice were challenged with pure mCD40B suspended in PBS buffer in three different routes (intraperitoneal, intravenous and subcutaneous) to allow the distribution into all systemic lymphoid organs and tissues. In various murine tumour models mice are repeatedly vaccinated with 2 x 10e4 to 2 x 10e6 dendritic cells for up to four weeks to induce sufficient anti-tumour immunity (Balkow et al., 2009 223; Galea-Lauri et al., 2004 224; Eggert et al., 2002 225). Therefore to address long-term toxicity of mCD40B administration high doses [1 x 10e6 cells/injection] of mCD40B were injected two times a week for five weeks. Furthermore acute toxicity was assessed by injecting mice once with very high mCD40B doses [1 x 10e7 cells/injection]. Matched groups of mice received PBS buffer only in three different routes serving as negative control. After five weeks of further observation mice were sacrificed and no changes in behaviour, weight and survival of mCD40B treated mice compared to PBS injected control mice were detected (figure 35A and B).

35A

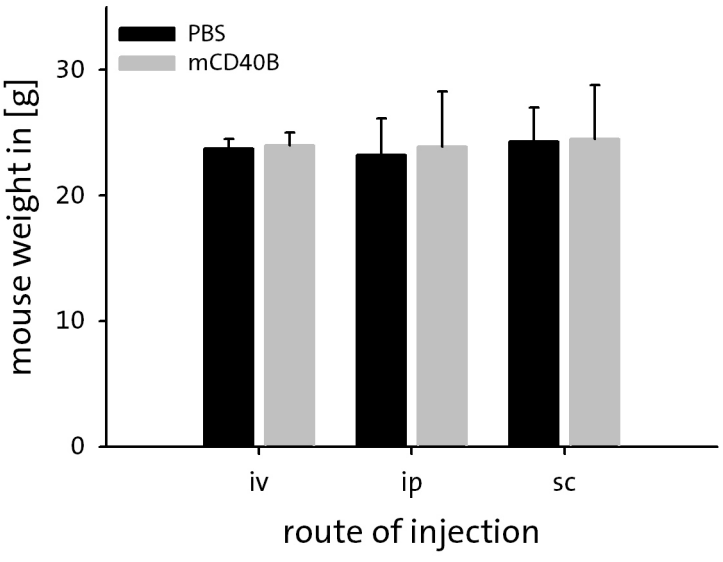
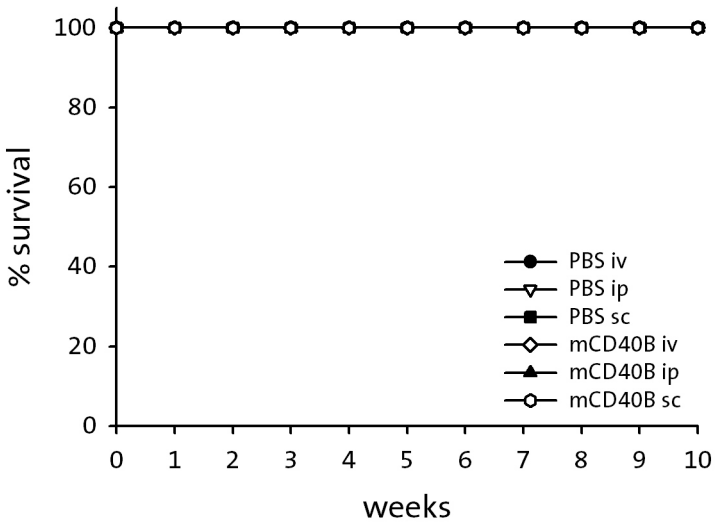


Figure 35: No difference in weight and survival of mice treated with > 90 % pure mCD40B

Per group five C57BL/6 mice were challenged with either mCD40B or PBS alone as negative control in three different routes (intraperitoneal - ip, intravenous - iv or subcutaneous - sc) and weight (A) and survival (B) were documented.

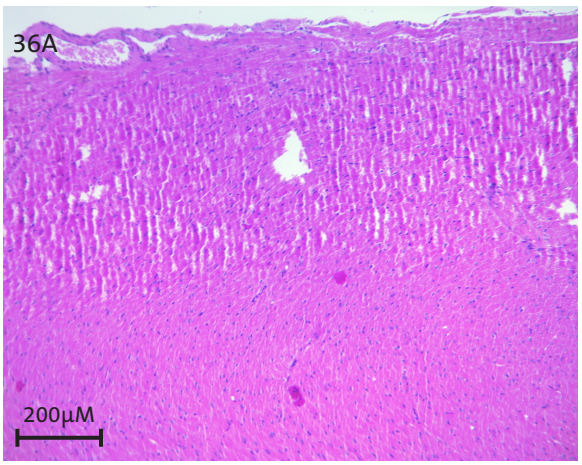
(Mean values ± SD of 4 independent experiments with five C57BL/6 mice per group are shown.)

B



For histo-pathological analysis clinical relevant organs (heart, lung, liver, kidney and spleen) were removed and paraffin-embedded sections prepared by hematoxylin and eosin (H&E) staining (figure 36A-E). Classification of histological sections was done by an experienced pathologist looking for lymphocytic infiltrations, structural tissue injury and other indications for inflammation. Therefore three non-consecutive sections of each organ were completely screened, lymphocytic infiltrates were counted and a semi-quantitative analysis was performed (figure 36F) revealing normal histology in all organs analyzed. Comparing mCD40B treated mice with PBS injected control mice there were no significant differences. However there were lymphocytic infiltrations found in lung sections accompanied by discrete chronic bronchitis and in some liver sections in mCD40B treated animals and the PBS injected control group as well. Therefore these results were not attributed to administration of mCD40B cells but were the result of suboptimal animal housing caused by dusty air - probably due to non-filtered, non-ventilated caging systems and fine granulation beddings.

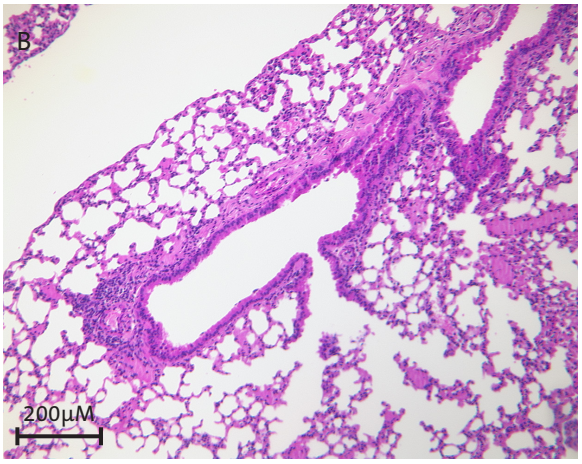
Figure 36: Normal histology of organs after mCD40B administration



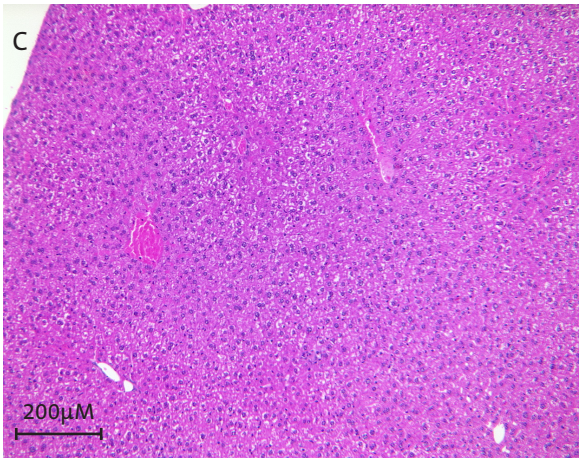
Histo-pathological analysis of H&E stained sections of heart (A), lung (B), liver (C), spleen (D) and kidney (E) exhibited normal histology.

(Organ sections of five mCD40B treated and PBS injected C57BL/6 mice per group (injection routes iv, ip or sc) were completely screened and 4 independent experiments performed.)

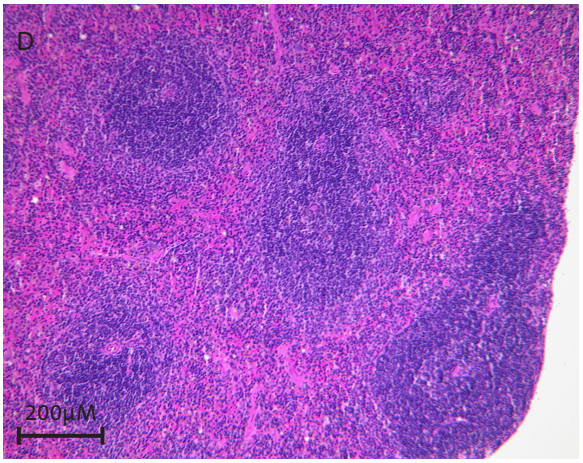
(A) Heart: Heart muscle overlayed by thin epicardial tissue.



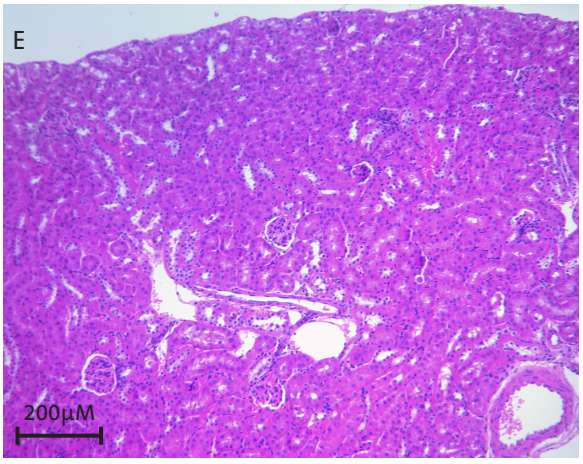
(B) Lung: Well developed lung tissue. In the centre two bronchioli lined with regular respiratory epithelium. Discrete chronic bronchitis.



(C) Liver: Liver tissue with fine central veins and regular portal fields.



(D) Spleen: Regular structured splenic tissue with prominent secundar follicle and a fine capsule. White pulp (WP) and red pulp (RP).



(E) Kidney: Renal cortex tissue. Regular glomerular and tubular structures.

(Representative photographs of mice treated with 1 x 10e7 mCD40B cells injected intravenously taken at 100x magnification are shown.)

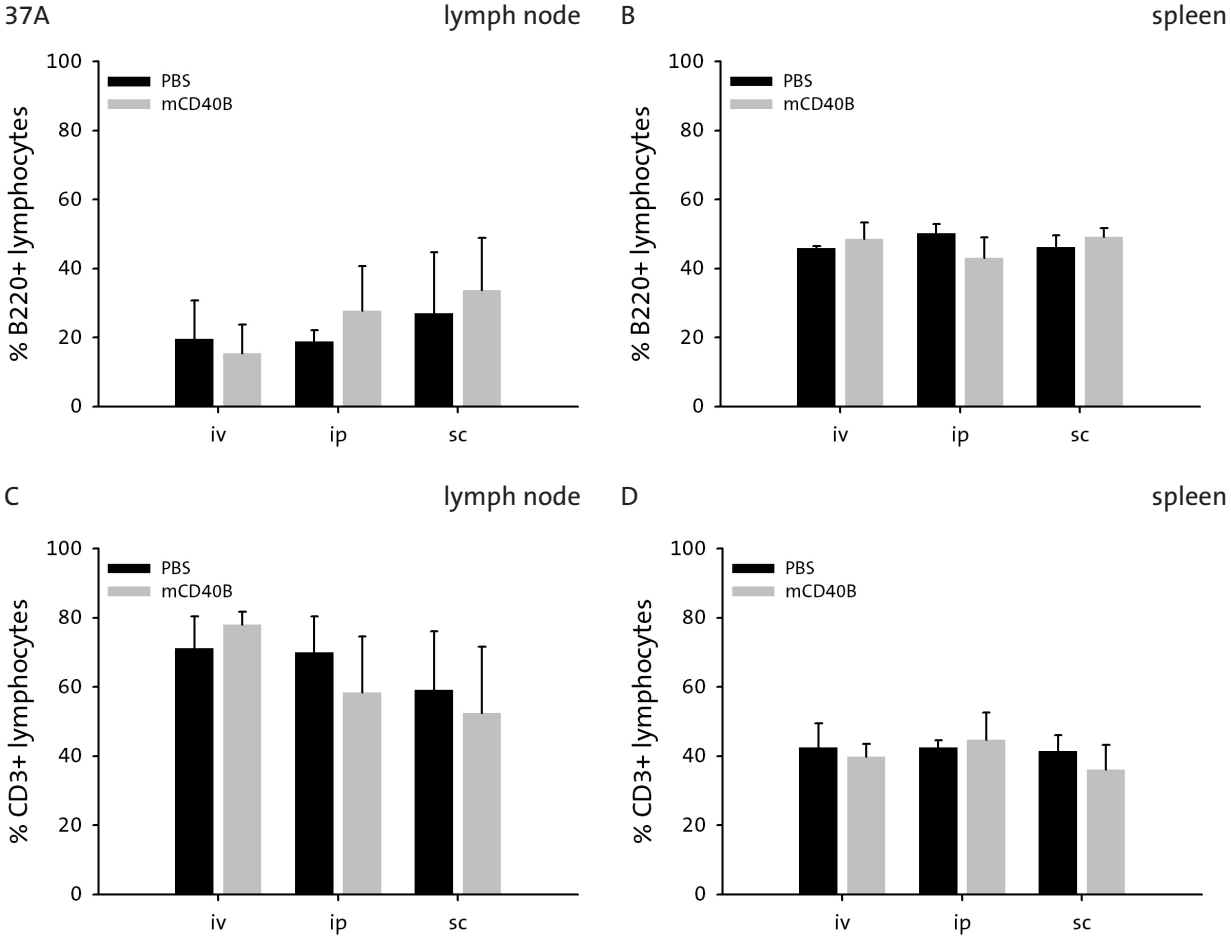
F

			heart	lung	liver	kidney	spleen
lymphocytic infiltrates	PBS	iv	o	+	+	o	o
	PBS	ip	o	++	o	o	o
	PBS	sc	o	+	+	o	o
	mCD40B	iv	o	+	o	o	o
	mCD40B	ip	o	+	o	o	o
	mCD40B	sc	o	+	+	o	o

(F) Semi-quantitative analysis was performed by screening three non-consecutive histological sections of each organ for lymphocytic infiltrations and counting them. Mean values of 4 independent experiments were calculated and assigned to one of three categories:

o no infiltrate, + few lymphocytic infiltrates ($1 \leq x \leq 3$) ++ several lymphocytic infiltrates ($4 \leq x \leq 8$).

Furthermore secondary lymphoid organs were screened for rejection reactions by assessment of the lymphocyte subsets involved in adaptive immune reactions. Therefore mesenteric and inguinal lymph nodes as well as spleen were dissected, single cell suspensions prepared and composition of $B220^+$ B cells, $CD3^+$ T cells as well as $CD3^+CD4^+$ helper T cell and $CD3^+CD8^+$ cytotoxic T cell populations determined by FACS analysis (for figure 37A-H see also next page). Comparing mCD40B treated mice and PBS injected control group (injection routes iv, ip or sc) no significant differences in the composition of the four cell populations were detected, neither in the spleen, nor in the lymph nodes in four experiments performed independently.



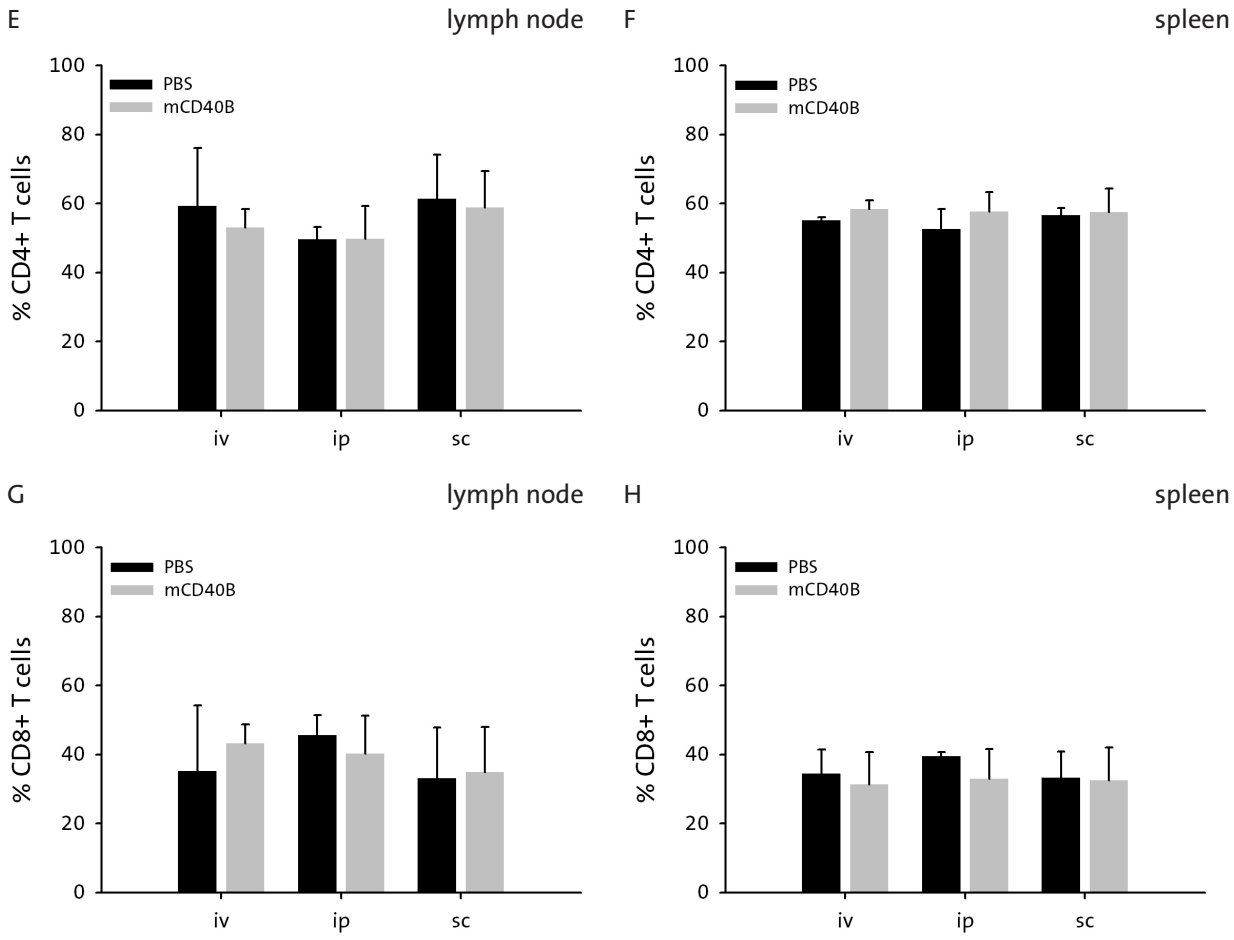


Figure 37: No secondary immunological effects after mCD40B administration

Assessment of lymphocyte composition of B220⁺ B cells (A and B), CD3⁺ T cells (C and D), CD4⁺ helper T cells (E and F) and CD8⁺ cytotoxic T cells (G and H) in mesenteric and inguinal lymph nodes and spleen of mCD40B treated mice and PBS injected control mice (injection routes iv, ip or sc).

(Mean values ± SD of 4 independent experiments with five C57BL/6 mice per group are shown.)

To summarize, histo-pathological assessment of behaviour, weight, survival, clinical relevant organs and secondary lymphoid organs after administration of mCD40B revealed no differences compared to PBS injected control group. After showing the safety and lack of toxicity of the administration of high doses of mCD40B in C57BL/6 mice we next developed the mCD40B vaccination schedule.

3.3.2. Characterization of mCD40B as cellular adjuvant - Induction of immunity

As professional APCs dendritic cells are able to take up antigen, thus become activated and migrate to secondary lymphoid organs were they present antigens to T cells (Inaba et al., 1990 221). By using mCD40B cells as cellular adjuvant it was tested whether those cells are basically able to efficiently present antigen to T cells in order to induce specific immune responses in adaptive im-

munotherapy. Therefore mCD40B cells were loaded with peptide by peptide-pulsing, which is a common technique to deliver antigens to APCs (Inaba et al., 1990 221; Zitvogel et al., 1996 120).
Several parameters were systematically analysed to set up an efficient vaccination schedule with mCD40B, namely route of administration (iv, ip or sc), cell dose [1 x 10e5, 1 x 10e6, 1 x 10e7 cells/injection], vaccination intervals (day 0, day 7, day 14) and formulation (mCD40B d7 or mCD40B d14 and mCD40B γ-irradiated with 26 Gy). Peptide-pulsed DC which were injected subcutaneously served as a gold standard to which mCD40Bs were compared (Balkow et al., 2009 223; Galea et al., 2004 224; Eggert et al., 2002 225). As positive control specific peptide was emulsified in incomplete Freund's adjuvant (IFA), because IFA is considered to be one of the most effective adjuvants and administered subcutaneously into the right flank once. Herein adjuvant activity is a result of sustained release of antigen from the oily deposit (depot effect), and stimulation of a local innate immune response leading to enhanced adaptive immunity. An essential component of this response is an intense inflammatory reaction at the site of antigen deposition resulting from an influx of leukocytes and their interaction with antigen (Hossain et al., 2001 226; Shibaki et al., 2002 227).
The efficacy of different mCD40B cell-based peptide vaccine formulations in inducing specific immune responses was tested by the functional analysis of antigen-specific T cells: 1) the *in vivo* cytolytic activity of antigen-specific $CD8^+$ cytotoxic T cells and 2) the magnitude of the $CD8^+$ T cell response by production of intracellular IFN-γ. Therefore two assays were established: 1) the *in vivo* cytotoxicity assay and 2) the intracellular cytokine staining for IFN-γ in $CD8^+$ cytotoxic T cells.

To basically test peptide-antigen presentation by mCD40B *in vivo* for the first time the H-$2D^b$ restricted (MHC-I) synthetic peptide [amino acid sequence KAVYNFATM] derived from murine pathogenic lymphocytic choriomeningitis virus (LCMV) glycoprotein 33-41 (GP33) was used to load mCD40B with (Gairin et al., 1995 228; Stemmer et al., 1999 229). For vaccination mCD40B d14 were either pulsed with 10 µM LCMV peptide (mCD40B d14+) or left untreated (mCD40B d14-) and 1 x 10e6 cells were administered intraperitoneally into C57BL/6 mice twice at a 7 day interval in the style of DC vaccinations. To assess the functionality of LCMV-specific $CD8^+$ T cells the *in vivo* cytotoxicity assay was performed (figure 38A, next page). To this end immunized mice were injected with CFSE-stained splenocytes from naïve mice loaded with LCMV peptide. Unloaded splenocytes stained with a lower CFSE concentration served as reference. Specific killing of peptide-loaded $CFSE^{high}$ target cell fraction was determined by flow cytometric analysis calculating the ratio of the frequencies of CFSE-labelled splenocytes loaded with LCMV peptide and unloaded splenocytes (One representative gating for CFSE-positive target cells is displayed in figure 38B). With induction of LCMV-specific immune response cytotoxic potential is demonstrated by decrease of percentage of the $CFSE^{high}$ fraction (Representative data for identification of $CFSE^{high}$ (right gate) and $CFSE^{low}$ (left gate) target cell fraction is shown in figures 38C-G).
No specific lysis was detected when mice were vaccinated with PBS alone (NC). While only low levels of specific lysis were detected in mice vaccinated with peptide alone (pep only) or mCD40B d14-, the LCMV-specific lysis was significantly increased to 33 % in mice vaccinated with mCD40B d14+. Similarly in mice vaccinated with IFA plus peptide (PC) specific lysis rose to 30 %. (Combined data of 7 independent experiments with 2 mice per group is shown in figure 38H.)

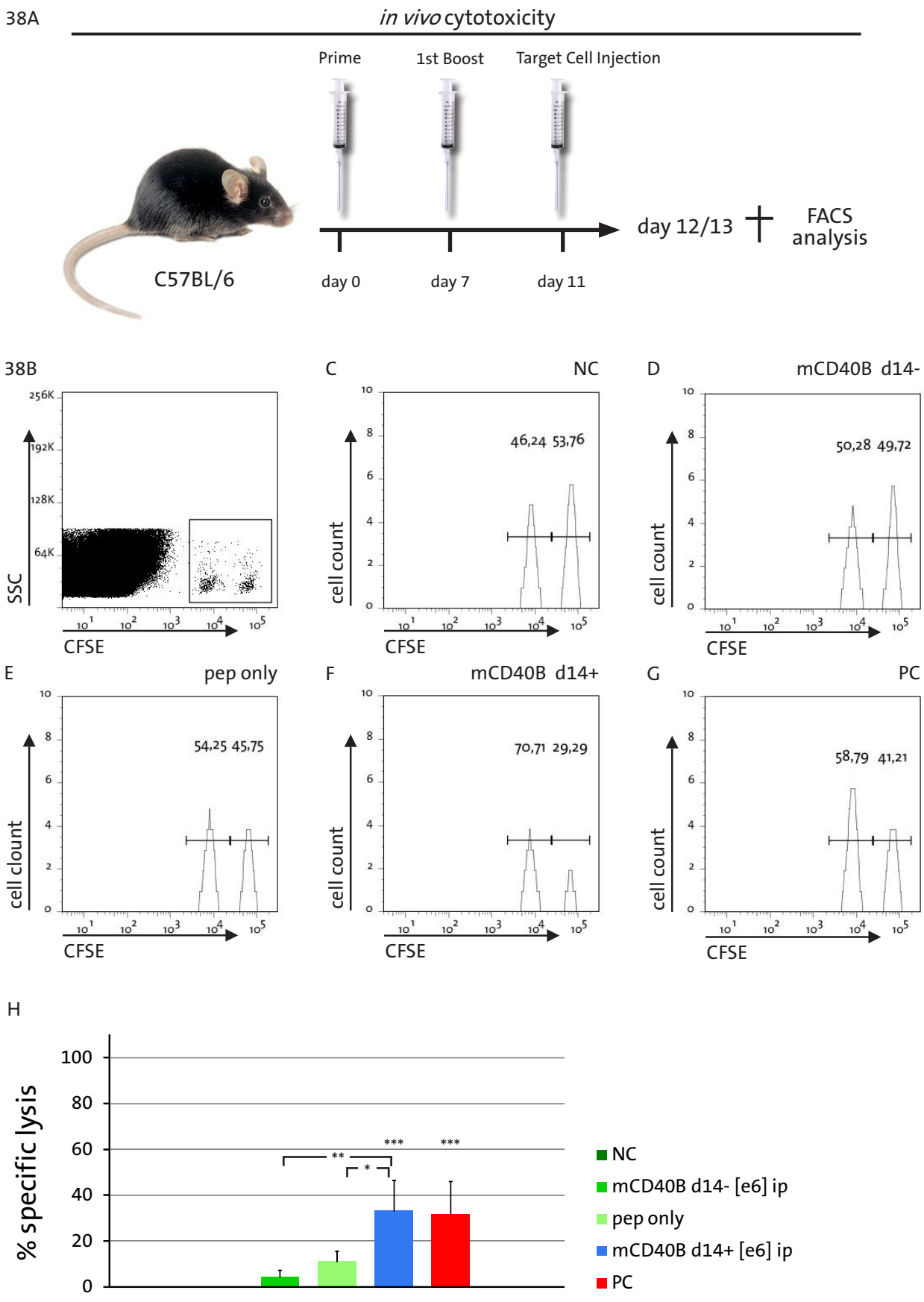


Figure 38: Vaccination with mCD40B LCMV induces antigen-specific cytotoxicity of CD8+ T cells

(A) C57BL/6 mice were vaccinated with 1 x 10e6 mCD40B d14 pulsed with LCMV peptide (mCD40B d14+) or left unpulsed (mCD40B d14-) at a 7 day interval. Vaccination with IFA plus peptide served as positive control, administration of PBS alone (NC) or peptide alone (pep only) served as negative controls. 4 days after the second immunization 1 x 10e7 target cells labelled with different amounts of CFSE were administered intraperitoneally into vaccinated mice. Specific lysis by $CD8^+$ cytotoxic T cells *in vivo* was determined by flow cytometry calculating the ratio of the frequencies of CFSE-labelled splenocytes loaded with LCMV peptide and unloaded splenocytes.

In (B-G) representative FACS data from one of 7 vaccination experiments is shown. (B) Typical gating on CFSE-stained target cell fraction. Further gating of target cells on peptide-pulsed $CFSE^{high}$ (right gate) and unpulsed $CFSE^{low}$ fraction (left gate) of mice vaccinated with (C) PBS alone (NC), (D) mCD40B d14-, (E) peptide alone (pep only), (F) mCD40B d14+ or (G) IFA (PC).

(H) Bar chart of mean values ± SD of specific lysis combining data of 7 independent vaccination experiments with two C57BL/6 mice per group are shown. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

For establishment of the intracellular cytokine staining for IFN-γ in $CD8^+$ cytotoxic T cells identical vaccinations as described above were performed in C57BL/6 mice using the same LCMV-peptide. To determine the frequencies of $CD8^+$ $IFN\text{-}\gamma^+$ T cells vaccinated mice were sacrificed, spleens removed and purified lymphocytes re-stimulated with 10 µM LCMV-peptide to induce production of intracellular IFN-γ by specific antigen-experienced $CD8^+$ cytotoxic T cells. During re-stimulation lymphocytes were treated with BD Golgi-Stop™ containing monensin to block cytokine transport outside the cell. As a consequence IFN-γ accumulates in the Golgi-complex and detection by flow cytometry is facilitated (figure 39).

39

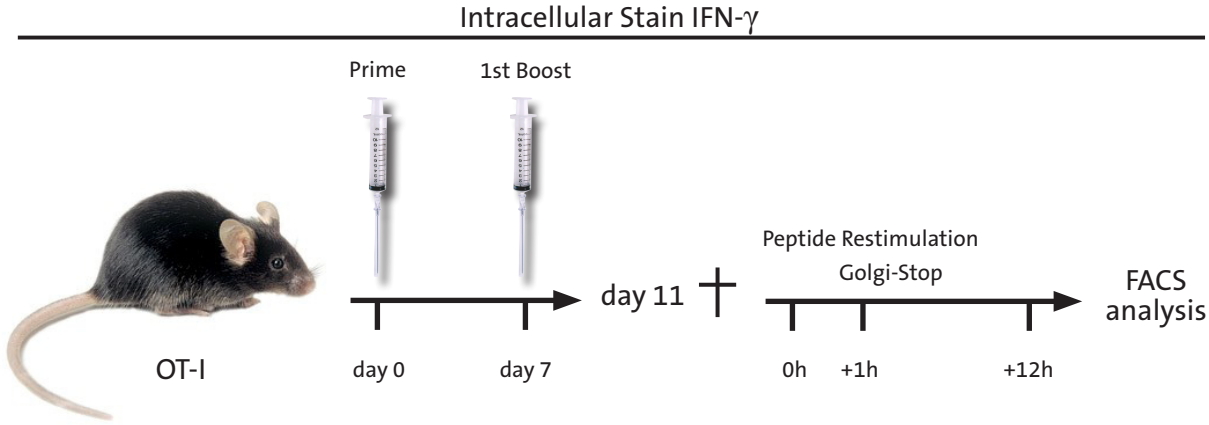


Figure 39: Illustration of vaccination schedule and intracellular cytokine staining procedure

When vaccinating C57BL/6 mice with LCMV-peptide levels of intracellular accumulated IFN-γ were generally low (< 2 %) and production of IFN-γ was independent from treatment of mice, likely due to intra-individual differences (data not shown) in this wild-type line. Therefore the TCR-transgenic OT-I line was used in further vaccination experiments to detect intracellular IFN-γ. In OT-I mice T-cell levels are strongly skewed towards $CD8^+$ cytotoxic T cells so it is facilitated to follow their fate during immune responses. Furthermore these T cells express a single T-cell receptor

specific for H-2K^b restricted (MHC-I) synthetic peptide OVA$_{257-264}$ [SIINFEKL] derived from hen eggs ovalbumin (Hogquist et al., 1994 230) representing a well studied foreign model-antigen.
For that reasons in order to find the optimal mCD40B formulation inducing specific immune responses *in vivo* vaccination parameters were systematically analyzed by performing the cytotoxicity assay in C57BL/6 mice and the intracellular cytokine staining for IFN-γ produced by CD8$^+$ cytotoxic T cells in OT-I mice both treated with foreign peptide OVA vaccine formulations.
Before using in vaccination experiments OT-I mice were routinely monitored for expression of TCR-chain V$_\beta$5 on CD3$^+$CD8$^+$ T cells (figure 40). All mice checked expressed TCR-chain V$_\beta$5 in more than 90 % of all CD3$^+$CD8$^+$ T cells.

40

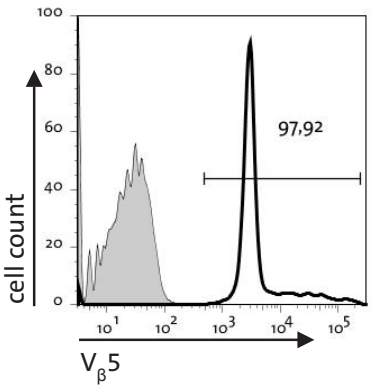
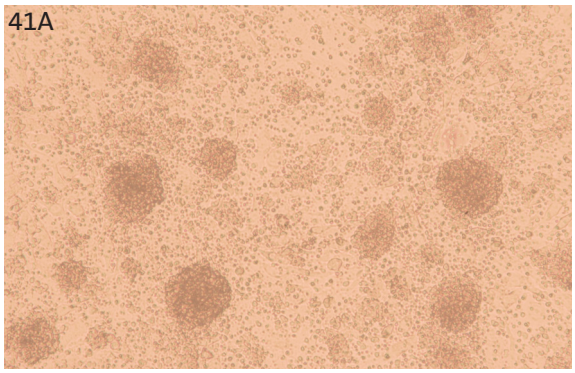


Figure 40: Vβ5 expression by CD3+CD8+ T cells in OT-I mice

Splenocytes from OT-I mice were monitored for expression of transgenic TCR-chain V$_\beta$5 by flow cytometric analysis using V$_\beta$5-PE antibody.

(One representative FACS analysis is shown.)

For vaccination experiments in OT-I mice generation of mCD40B and DC originated from OT-I line (herein after referred to as mCD40B OT-I and DC OT-I, respectively) was performed according C57BL/6 protocol without any changes. Thereby growth, phenotype and purity of mCD40B OT-I were comparable to that of mCD40B C57/BL6. Formation of typical round cell clusters was observed and an average 4.1 fold increase in growth of 10 independently performed mCD40B cultures was documented when B cells originated from OT-I mice (figure 41A,B). Cultivation according optimal mCD40B C57BL/6 culture protocol led to a similar purity (A) and activation profile (B-E) as determined by expression of MHC molecules Class-I (H-2Db) and Class-II (I-Ab) and the expression of co-stimulatory molecules CD80, CD86 on CD19$^+$ CD3$^-$ mCD40B OT-I compared to mCD40B C57BL/6 (figure 42 A-E).



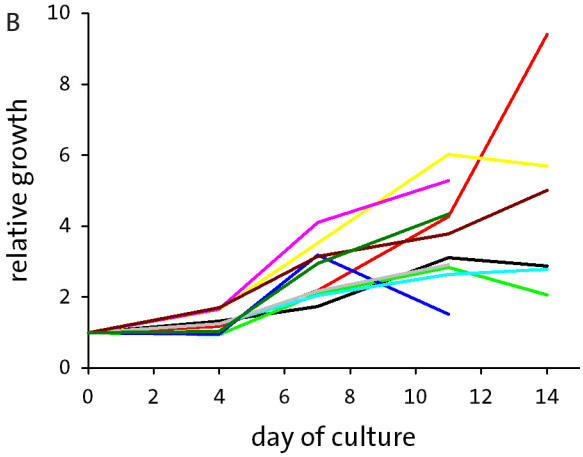


Figure 41: Cluster formation (A) and proliferation (B) of mCD40B cells originated from OT-I splenocytes

(A) Photographs were taken at a 5x magnification using a light optical microscope fitted with a digital camera.

(One representative picture of at least 10 independent mCD40B OT-I cultures is shown.)

(B) The number of viable mCD40B cells was determined every 3-4 days using the trypan blue exclusion test. Until day 14 of culture the relative growth was assessed by calculating the relative increase between two passages.

(Representative data of 10 independently performed mCD40B OT-I cultures is shown.)

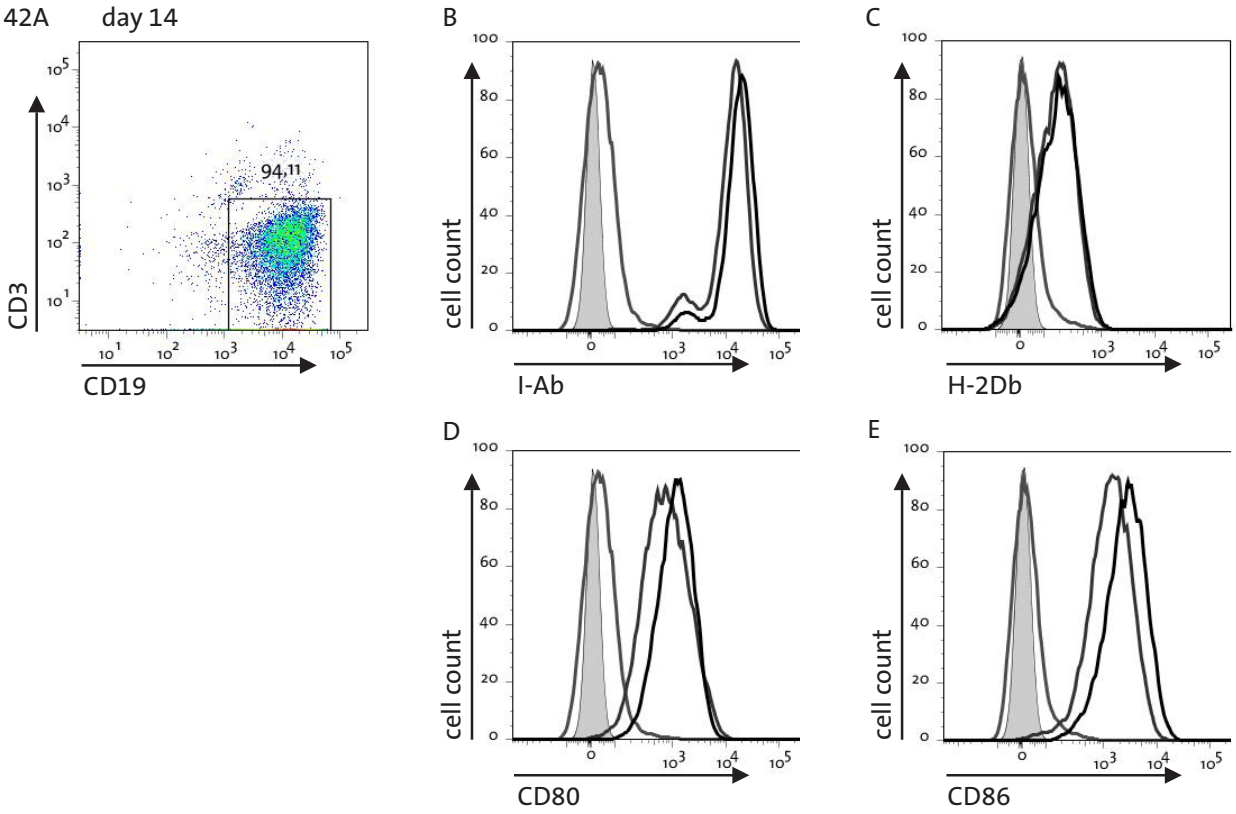


Figure 42: Purity (A) and activation status (B-E) of mCD40B cells originated from OT-I splenocytes

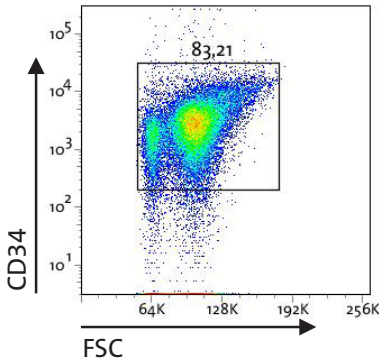
FACS analysis of the proportion of $CD19^+$ $CD3^-$ B cells in mCD40B cultures grown from OT-I splenocytes according optimal mCD40B C57BL/6 culture protocol displaying purity (A) and activation profile (B-E) as determined by expression of MHC molecules Class-I (H-2Db) and Class-II (I-Ab) and the expression of co-stimulatory molecules CD80, CD86 on $CD19^+$ $CD3^-$ mCD40B OT-I.

(One representative FACS analysis of at least 10 is shown.)

Likewise phenotype of DC generated from ≥ 80 % pure $CD34^+$ bone marrow progenitor cells of OT-I mice (figure 43A) was similar to that of DC C57BL/6 monitored by determination of the $CD11b^+$ $CD11c^+$ MHC-II^{high} (I-A^b) population. After 24h maturation with LPS [10 ng / mL] or anti-CD40 antibody [1 µg/mL] the expression of co-stimulatory molecules CD80, CD86 and CD83 on DC LPS and DC aCD40Ab was up-regulated as monitored by flow cytometry. (One representative analysis of

at least 10 DC LPS and DC aCD40Ab cultures originated from OT-I mice is shown in figure 43 B-F and G-K, respectively.)

43A



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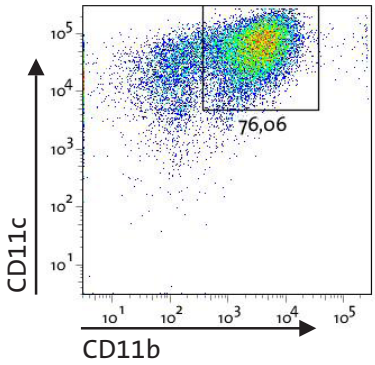

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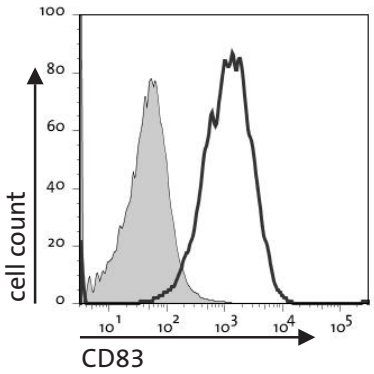

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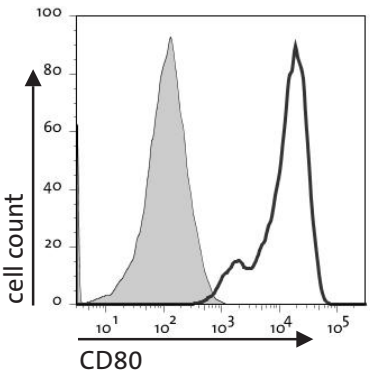

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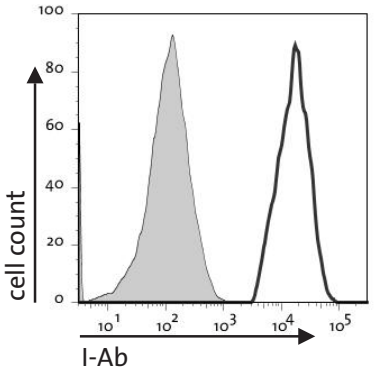

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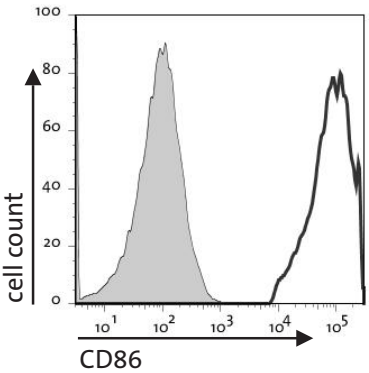

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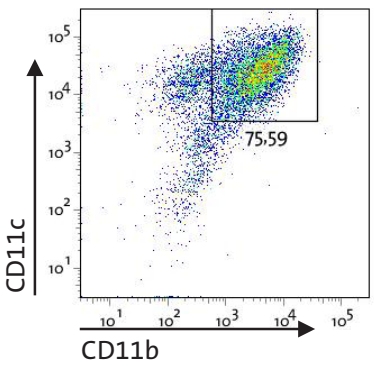

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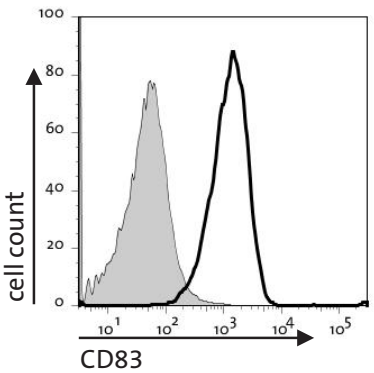

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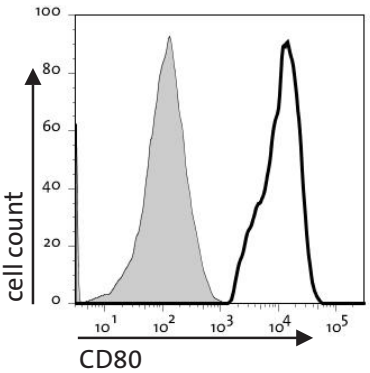


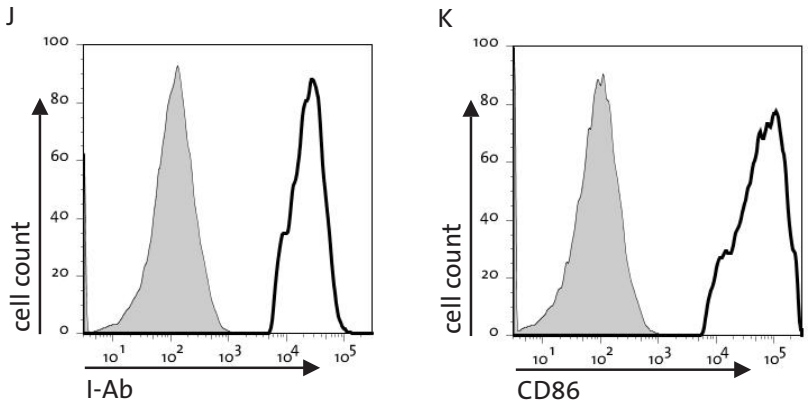


Figure 43: Phenotypical analysis of DC subsets originated from OT-I CD34+ bone marrow

(A) Myeloid DCs were generated from 80-85 % pure, positively selected $CD34^+$ bone marrow progenitor cells from OT-I mice through stimulation with GM-CSF [500 U/mL] for 7 days.

(B-F and G-K) mDC LPS and mDC aCD40Ab from OT-I mice were finally matured for 24 hours using LPS [10 ng/mL] or aCD40 antibody [1 µg/mL], respectively. Maturation status was controlled by FACS analysis of expression of co-stimulatory molecules CD80, CD86, and CD83 on > 90 % pure $CD11c^+CD11b^+MHC\text{-}II^{high}$ ($I\text{-}A^b$) cell population.

(One representative FACS analysis of DC OT-I from at least 10 is shown.)

Starting with identification of the optimal mCD40B formulation vaccinations with OVA peptide were performed comparing mCD40B d14 and mCD40B d7. Furthermore sub-lethally irradiated (26Gy) mCD40B were tested to thereby see whether their antigen-presenting function would be diminished.

Similar to LCMV vaccinations no specific lysis was detected when mice were vaccinated with PBS alone (NC). While only low levels of specific lysis were detected in mice vaccinated with peptide alone (pep only) or mCD40B d14- (green bars), the OVA-specific lysis was significantly elevated from 71 ± 28 % when 1 x 10e6 mCD40B d7+ (dark blue) were used to 84 ± 13 % using 1 x 10e6 mCD40B d14+ (blue) while irradiation of mCD40B d14+ diminished specific lysis rate to 76 ± 22 % (grey bar), all injected ip. Similarly in mice vaccinated with IFA plus peptide (PC) specific lysis rose to 74 ± 24 %. (Mean values of 7 independent experiments with 2 mice per group ± SD. Bar graph shows combined data in figure 44A.) Furthermore specific lysis rates in successful vaccinated C57BL/6 mice were increased compared to LCMV vaccinations revealing strong immunogenicity of the foreign OVA peptide.

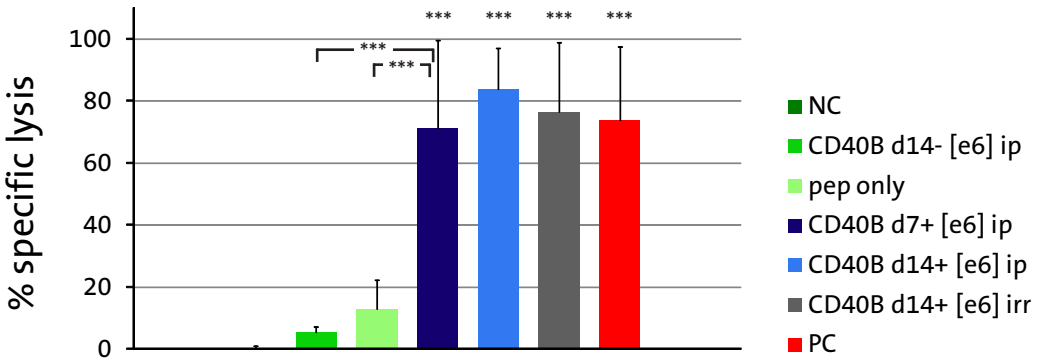


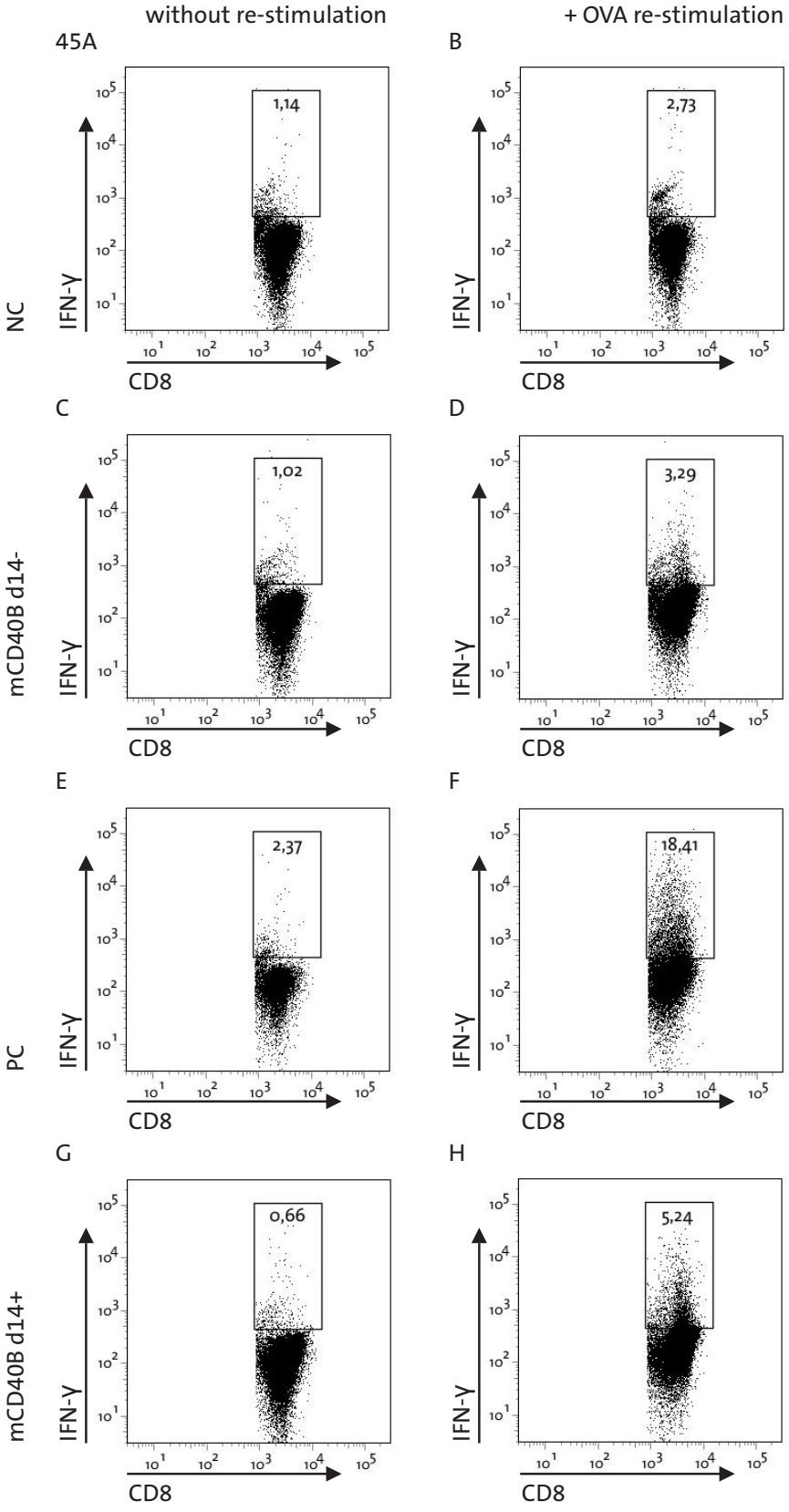
Figure 44: Vaccination with mCD40B OVA induces antigen-specific cytotoxicity dependent from adjuvant formulation

C57BL/6 mice were vaccinated with 1x10e6 mCD40B d7, mCD40B d14 or sub-lethally irradiated mCD40B pulsed with 10 µM OVA peptide (mCD40B d14+) or left unpulsed (mCD40B d14-) all injected ip at a 7 day interval. Vaccination with IFA plus peptide served as positive control, administration of PBS alone (NC) or peptide alone (pep only) display further negative controls. 4 days after the second immunization 1 x 10e7 target cells labelled with different amounts of CFSE were administered intraperitoneally into vaccinated mice. Specific lysis by $CD8^+$ cytotoxic T cells *in vivo* was determined by flow cytometry calculating the ratio of the frequencies of CFSE-labelled splenocytes loaded with OVA peptide and unloaded splenocytes.

Bar chart of mean values ± SD of specific lysis combining data of 7 independent vaccination experiments with two C57BL/6 mice per group are shown. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Performing vaccinations in OT-I mice elevated levels of intracellular IFN-γ^+ $CD8^+$ cytotoxic T cells were detected dependent on treatment of mice as determined by flow cytometric analysis. In OT-I mice vaccinated with PBS alone (NC) or 1 x 10e6 mCD40B d14 left unpulsed (mCD40B d14-) basal IFN-γ levels were detected in $CD8^+$ cytotoxic T cells. In contrast, when mice were vaccinated with IFA plus peptide (PC) or mCD40B d14 pulsed with peptide (mCD40B d14+) IFN-γ levels of antigen-specific $CD8^+$ cytotoxic T cells were increased from basal levels of 1.1 ± 0.7 % or 0.6 ± 0.5 % to 16.2 ± 3.2 % or 5.0 ± 1.8 %, respectively, but only when purified lymphocytes were re-stimulated with OVA peptide. (Mean values of 7 independent experiments with 2 mice per group ± SD.) Therefore IFN-γ production was specifically attributed to response by antigen-experienced $CD8^+$ cytotoxic T cells showing that those T cells were functionally competent. (One representative gating for IFN-γ^+ $CD8^+$ cytotoxic T cells is displayed in figure 45A-H.)

Figure 45: Vaccination with mCD40B OVA induces intracellular IFN-γ production by specific antigen-experienced CD8+ T cells



OT-I mice were vaccinated with 1 x 10e6 mCD40B d14 pulsed with OVA peptide (mCD40B d14+) or left unpulsed (mCD40B d14-) at a 7 day interval. Vaccination with IFA plus peptide represents the positive control, administration of PBS alone (NC) displays the negative control. Production of intracellular IFN-γ by specific antigen-experienced $CD8^+$ cytotoxic T cells was detected by flow cytometry after re-stimulating purified lymphocytes of vaccinated animals with the same peptide.

(A-H) IFN-γ levels of un-stimulated $CD8^+$ T cells (left column) vs. IFN-γ levels of $CD8^+$ T cells from vaccinated mice re-stimulated with 10 µM OVA peptide *in vitro* (right column).

(One representative gating for IFN-γ^+CD8^+ cytotoxic T cells out of at least 17 independently performed experiments is displayed.)

In order to identify the optimal mCD40B formulation by the second approach vaccinations with mCD40B d14, mCD40B d7 and sub-lethally irradiated (26 Gy) mCD40B d14, all injected ip were performed in TCR-transgenic OT-I mice with OVA peptide comparing specifically induced IFN-γ production of antigen-experienced $CD8^+$ cytotoxic T cells. Levels of IFN-γ were significantly elevated from basal levels of < 2 % (green bars) to 6.5 ± 2.1 % (dark blue) or 5.5 ± 3.8 % (blue) when vaccinations were performed with mCD40B d7+ or mCD40B d14+, respectively. In contrast, irradiated mCD40B d14 pulsed with OVA peptide (grey bar) did not lead to elevated levels of $CD8^+$ IFN-γ^+ T cells. (Mean values of 7 independent experiments with 2 mice per group ± SD. Bar graph shows combined data in figure 46).

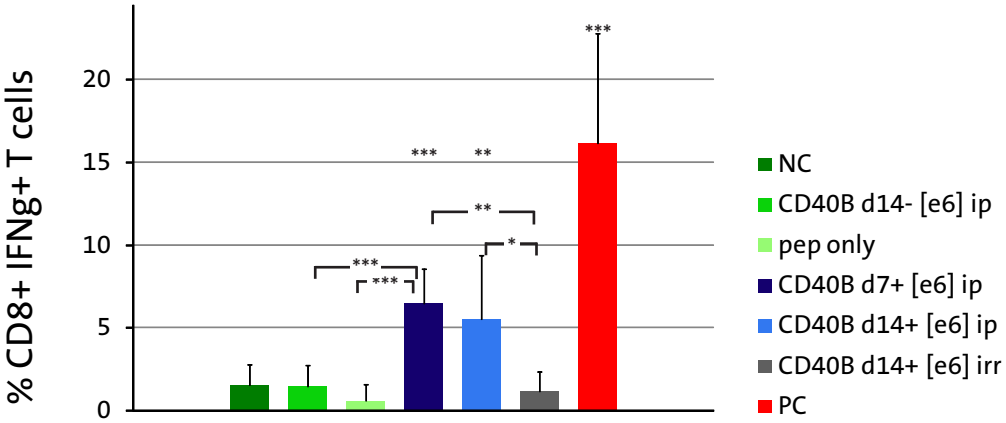


Figure 46: Vaccination with mCD40B OVA induces intracellular IFN-γ production by specific antigen-experienced CD8+ T cells dependent from adjuvant formulation

OT-I mice were vaccinated with 1x10e6 mCD40B d7, mCD40B d14 or sub-lethally irradiated mCD40B pulsed with OVA peptide (mCD40B d14+) or left unpulsed (mCD40B d14-) all injected ip at a 7 day interval. Vaccination with IFA plus peptide served as positive control, administration of PBS alone (NC) or peptide alone (pep only) display further negative controls.

Mean values of frequencies of IFN-γ^+ $CD8^+$ T cells re-stimulated with OVA peptide were normalized to corresponding un-stimulated sample of the same animal. Bar chart of mean values ± SD combining data of 7 independent vaccination experiments with two OT-I mice per group are shown. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

A comparison of OVA-specific T cell reactions in response to the different mCD40B formulations as assessed by *in vivo* cytotoxicity assay and production of intracellular IFN-γ did not clearly favour one particular formulation. Irrespective of the duration of CD40-activation the T cell response induced with mCD40B d7 and mCD40B d14 was significantly increased compared to NC, pep only or mCD40B left unpulsed. Whereas irradiated mCD40B d14 significantly raised levels of specific lysis there was no effect measured by IFN-γ production. Comparing mCD40B d7 and mCD40B d14 there was no significant difference detected by both methods. As cultivation of mCD40B for 14 days resulted in higher amounts of cells generated this formulation was used in further experiments.
Second, the impact of injection route of mCD40B introduced in vaccination experiments to induce specific immune response was examined by both methods (figure 47A and B). Therefore 1 x 10e6 mCD40B d14+ were injected ip (blue), sc (purple) or iv (magenta) and specific lysis or production of intracellular IFN-γ were measured as described before. Whereas vaccination in all three routes were able to significantly increase levels of specific lysis of target cells there was no significant effect on IFN-γ production detectable when mCD40B were administered iv. Given that intraperitoneal mCD40B injection had a significant benefit over other routes in specific induction of immunity as determined by specific lysis of target cell testing of further vaccination parameters was compared to mCD40B d14+ injected ip.

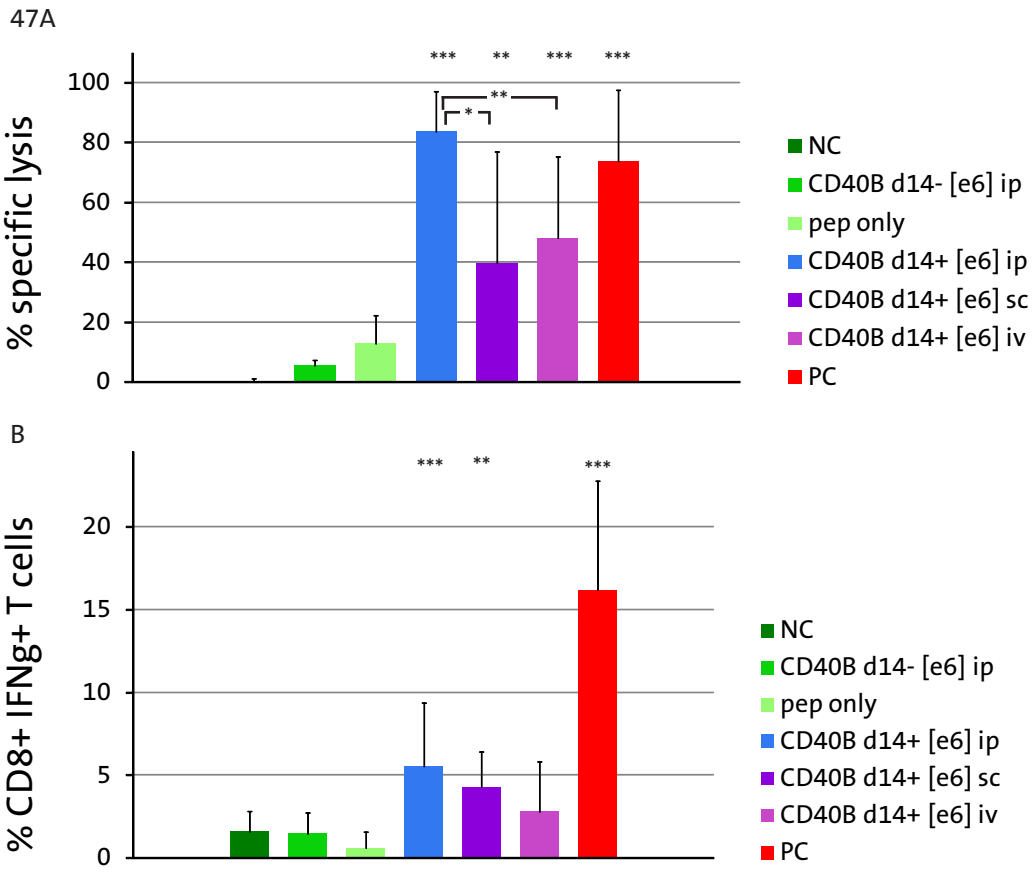


Figure 47: Vaccinations with mCD40B OVA d14 induces antigen-specific T cell responses dependent from injection route

C57BL/6 mice (A) or OT-I mice (B) were vaccinated with 1x10e6 mCD40B d14 pulsed with OVA peptide (mCD40B d14+) injected ip, sc or iv at a 7 day interval (blue, purple or magenta bar, respectively). Vaccina-

tion with IFA plus peptide served as positive control (red bar), administration of PBS alone (NC), mCD40B left unpulsed (mCD40B d14-) or peptide alone (pep only) display further negative controls (green bars).

(A) 4 days after the second immunization 1 x 10e7 target cells labelled with different amounts of CFSE were administered intraperitoneally into vaccinated mice. Specific lysis by $CD8^+$ cytotoxic T cells *in vivo* was determined by flow cytometry calculating the ratio of the frequencies of CFSE-labelled splenocytes loaded with OVA peptide and unloaded splenocytes.

(B) Production of intracellular IFN-γ by specific antigen-experienced $CD8^+$ cytotoxic T cells was detected by flow cytometry after re-stimulating purified lymphocytes of vaccinated animals with the same peptide. Mean values of frequencies of IFN-γ^+CD8^+ T cells re-stimulated with OVA peptide were normalized to corresponding un-stimulated sample of the same animal.

Bar charts of mean values ± SD of specific lysis (A) and frequency of $CD8^+$ IFN-γ^+ T cells (B) combining data of 4 independent vaccination experiments with three mice per group are shown. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Third, the effect of varying cell doses of mCD40B inserted in vaccinations was studied with both assays. Hence 1 x 10e5, 1 x 10e6 and 1 x 10e7 mCD40B d14+ were administered ip at a 7 day interval and read out assays performed as described before (figure 48A and B). Thereby a dose dependent effect of vaccinations with mCD40B was identified as increasing cell doses from 1 x 10e5 to 1 x 10e6 or 1 x 10e7 significantly raised levels of specific lysis of target cells from 26.4 ± 8.7 % (light blue) to 83.7 ± 13.4 % (blue) or 96.0 ± 3.4 % (dark blue), respectively. (Mean values of 3 independent experiments with 3 mice per group ± SD).

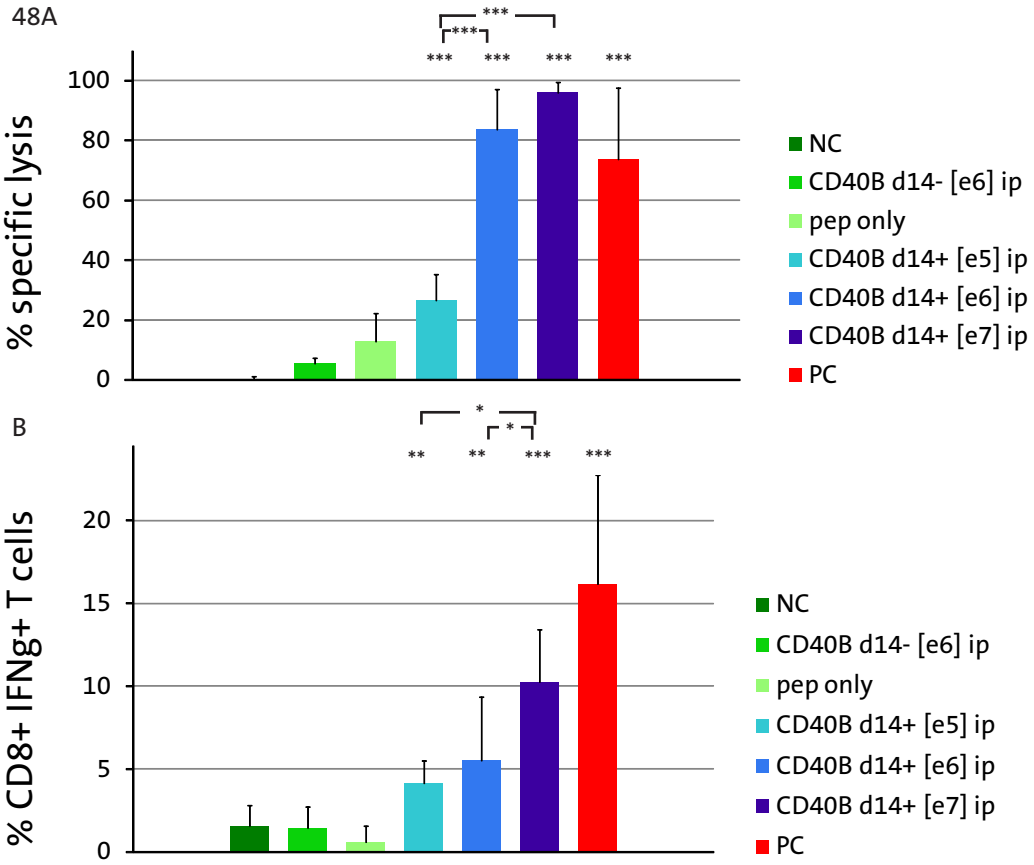


Figure 48: Vaccinations with mCD40B OVA d14 induce antigen-specific T cell responses dose dependently

C57BL/6 mice (A) or OT-I mice (B) were vaccinated with 1 x 10e5, 1 x 10e6 or 1 x 10e7 mCD40B d14 pulsed with OVA peptide (mCD40B d14+) injected ip at a 7 day interval (light blue, blue or dark blue bar, respectively). Vaccination with IFA plus peptide served as positive control (red bar), administration of PBS alone (NC), mCD40B left unpulsed (mCD40B d14-) or peptide alone (pep only) display further negative controls (green bars).

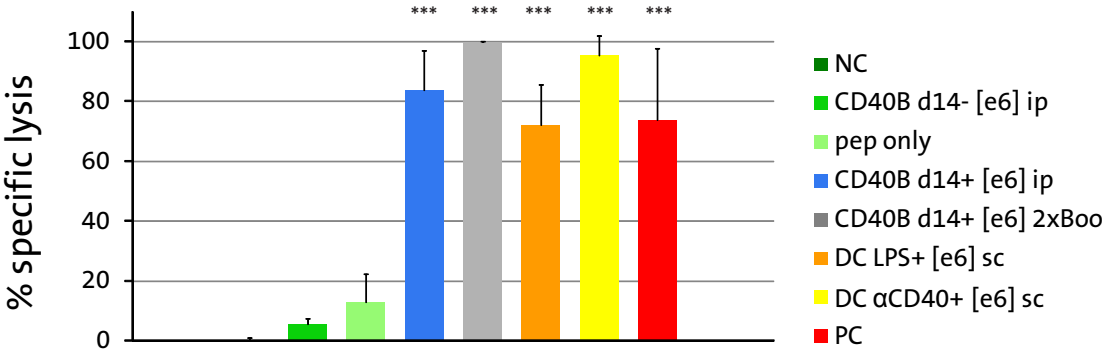
(A) 4 days after the second immunization 1 x 10e7 target cells labelled with different amounts of CFSE were administered intraperitoneally into vaccinated mice. Specific lysis by $CD8^+$ cytotoxic T cells *in vivo* was determined by flow cytometry calculating the ratio of the frequencies of CFSE-labelled splenocytes loaded with OVA peptide and unloaded splenocytes.

(B) Production of intracellular IFN-γ by specific antigen-experienced $CD8^+$ cytotoxic T cells was detected by flow cytometry after re-stimulating purified lymphocytes of vaccinated animals with the same peptide. Mean values of frequencies of IFN-γ^+CD8^+ T cells re-stimulated with OVA peptide were normalized to corresponding un-stimulated sample of the same animal.

Bar charts of mean values ± SD of specific lysis (A) and frequency of $CD8^+$IFN-γ^+ T cells (B) combining data of 3 independent vaccination experiments with three mice per group are shown. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Fourth, the effect of varying mCD40B vaccination schedules was analysed with both assays. Furthermore DC LPS or DC aCD40Ab were pulsed with OVA peptide and included into vaccination experiments to see whether mCD40B used under optimal conditions would be able to keep up with the widely recognised antigen-presentation function of DC. And so mCD40B d14+ were administered ip at a 7 day interval with one or two booster vaccinations and read out assays performed as described before. According to literature 1 x 10e6 DC^{OVA} were administered sc in additional experiments (figure 49A and B). In doing so the levels of specific lysis were increased from 83.7 ± 13.4 % (blue) to 99.8 ± 0.3 % (light grey) when the booster vaccination was repeated. However this effect could not be confirmed by detection of IFN-γ production. Vaccination with DC LPS or DC aCD40Ab raised levels of specific lysis to 72.2 ± 13.5 % (orange) or 95.3 ± 6.8 % (yellow), respectively. Again this result could not be verified by analysis of IFN-γ production. (Mean values of 3 independent experiments with 3 mice per group ± SD).

49A



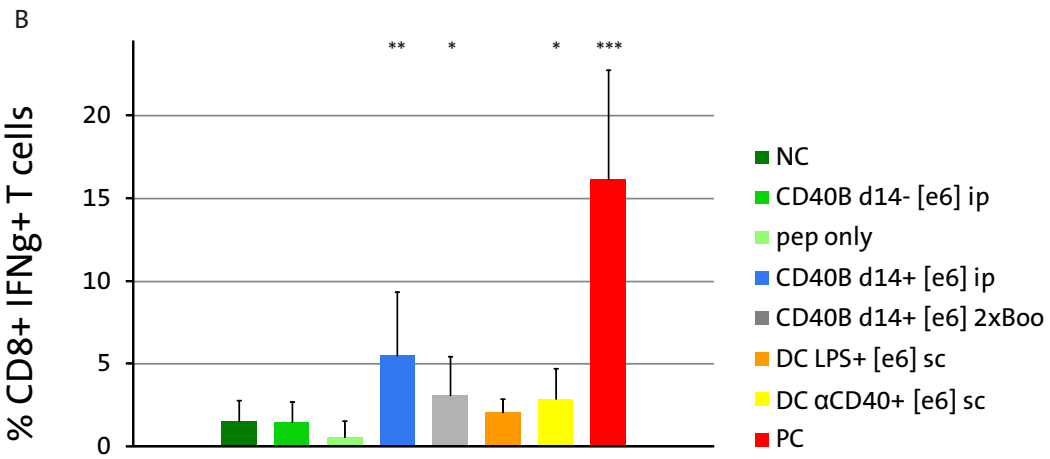


Figure 49: Repetitive vaccinations with mCD40B OVA d14 induce strong antigen-specific T cell responses

C57BL/6 mice (A) or OT-I mice (B) were treated with 1 x 10e6 mCD40B d14 pulsed with OVA peptide (mCD40B d14+) injected ip at a 7 day interval in a primary and booster vaccination or in one primary and two booster vaccinations (blue or light grey bar, respectively). Vaccinations with 1 x 10e6 DC LPS or DC aCD40Ab pulsed with peptide were injected sc. Vaccination with IFA plus peptide served as positive control (red bar), administration of PBS alone (NC), mCD40B left unpulsed (mCD40B d14-) or peptide alone (pep only) display further negative controls (green bars).

(A) 4 days after the second immunization 1 x 10e7 target cells labelled with different amounts of CFSE were administered intraperitoneally into vaccinated mice. Specific lysis by $CD8^+$ cytotoxic T cells *in vivo* was determined by flow cytometry calculating the ratio of the frequencies of CFSE-labelled splenocytes loaded with OVA peptide and unloaded splenocytes.

(B) Production of intracellular IFN-γ by specific antigen-experienced $CD8^+$ cytotoxic T cells was detected by flow cytometry after re-stimulating purified lymphocytes of vaccinated animals with the same peptide. Mean values of frequencies of IFN-γ^+CD8^+ T cells re-stimulated with OVA peptide were normalized to corresponding un-stimulated sample of the same animal.

Bar charts show mean values ± SD of specific lysis (A) and frequency of $CD8^+$IFN-γ^+ T cells (B). Combined data of 3 independent vaccination experiments with three mice per group is shown. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

By analysing mCD40B formulation, injection route, cell dose and vaccination schedule with specific lysis of target cells and production of intracellular IFN-γ through antigen-specific $CD8^+$ cytotoxic T cells the following optimal vaccination algorithm for induction of a specific immune response against foreign model antigen OVA was developed. Formulation of mCD40B was set at mCD40B+ d14 given that specific immune responses detected by elevated lysis of target cells and increased IFN-γ production by $CD8^+$ cytotoxic T cells were induced and high amounts of cellular vaccine could be generated. Furthermore intraperitoneal injection of $1x10^7$ mCD40B cells yielded in clear, significant benefit over other routes and doses. Finally repeated booster vaccinations elevated levels of specific lysis of target cells and were chosen for application in the most effective mCD40B

vaccination schedule. In figure 50 the chosen one schedule is clearly represented. This schedule was finally applied in first mCD40B vaccination experiments to study their ability to induce anti-tumour immunity.

50

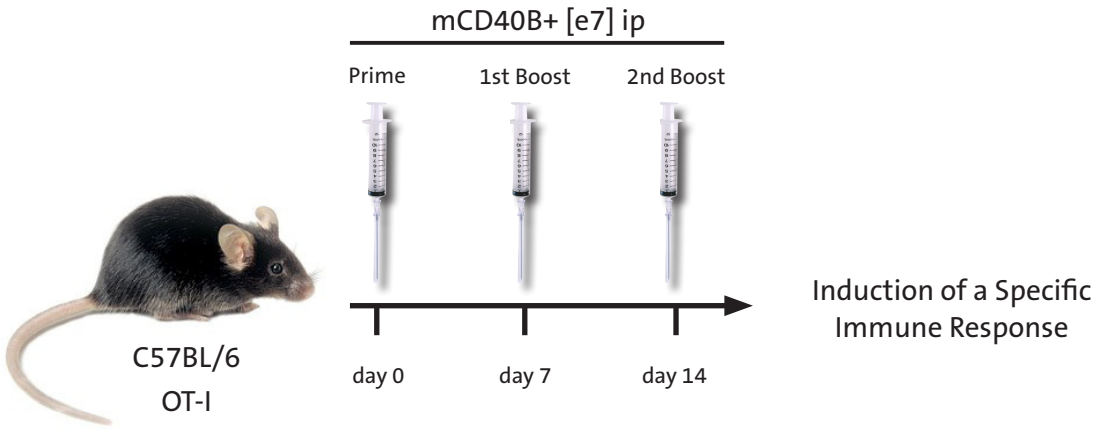


Figure 50: Illustration of an effective mCD40B vaccination schedule

Induction of specific immune responses is optimally gained by vaccinating C57BL/6 mice or OT-I mice with 1 x 10e7 mCD40B d14 pulsed with peptide (mCD40B+) injected ip at a 7 day interval in one primary and two booster vaccinations.

3.4. mCD40B as cellular adjuvant against B16 melanoma

In order to evaluate the protective effect of mCD40B anti-tumour vaccination the B16 melanoma a well established and widely used mouse tumour model was chosen. In the 80s the B16 cell line was established originating in spontaneously arising melanoma in C57BL/6 mice by Dr. I.J. Fidler and colleagues. B16 melanoma cells can be cultivated *in vitro*. After subcutaneous injection of B16 melanoma cells into a syngeneic animal (H-D^b) again a solid tumour can develop. Fidler et al. established several sub-lines with different metastasizing potential. Thus the parental cell line B16.F1 is able to form lung metastases upon intravenous tail vein injection. Furthermore the number of metastases was increased upon isolation of those tumour cells from lung-metastases and re-injection into syngeneic mice, a technique which is called passaging. By repeating passaging for several times the variant tumour line B16.F10 with higher survival and growth potential was established (Fidler et al., 1973 231; Fidler et al., 1976 232).

Human melanomas express different tumour-associated antigens which are potential targets for novel designs for therapeutic cancer vaccines. Melanoma-associated antigens include melanosomal proteins (tyrosinase, tyrosinase-related proteins 1 and 2 (TRP-1/2) and gp100) and tumour-specific antigens comprising the MAGE, BAGE and GAGE class of antigens. These antigens are non-mutated self-peptides, thus not representing strong immunogens or tumour-rejections antigens. Therefore to efficiently induce T cells specific for self-epitopes it is important ensure high expression of these epitopes on the cell surface of antigen-presenting cells. Pulsing professional APC with exogenously peptides is aiming at this issue (Kawakami et al., 1998 233). B16 melanoma cells express some non-mutated, melanoma-associated antigens (TRP-2 and gp100) analogue to antigens in human melanoma (Schreurs et al., 1997 234; Schreurs et al., 2000 235). So we chose the TRP-2 peptide [amino acid sequence SVYDFFVWL] to pulse mCD40B cells with representing a model of tumour immunotherapy closely mimicking cancer therapy in melanoma patients (Bloom et al., 1997 236).

To examine the effect of peptide-loaded mCD40B in a preventive vaccination approach the treatment regimen started with a first vaccination (Prime) followed by two further vaccinations (1^{st} and 2^{nd} Boost) of C57BL/6 mice in 7 day interval (figure 51A). One week after the last injection B16 tumour was inoculated by sc injection of 1 x 10e6 B16.F10 melanoma cells into the right flank and tumour volume was determined daily until day 13 after inoculation by measuring tumour diameter [mm^3] using a vernier calliper.

In groups of mice receiving 1 x 10e6 mCD40B d14 tumour growth was significantly decelerated compared to PBS-injected control mice only when mCD40B d14 had been pulsed with TRP-2 peptide [10 µM]. This growth slow-down was observed from onset of tumour growth on at day seven after tumour challenge. Raising mCD40B dose from 1 x 10e6 to 1 x 10e7 cells per injection did not show an enhancement of vaccine efficacy but growth rate was also significantly decreased compared to PBS control group. In groups of mice receiving preventive treatment with 1 x 10e6 dendritic cells loaded with the same peptide matured with LPS or anti-CD40 antibody a decelerating effect on tumour growth was even stronger with two mice treated with DC LPS remaining tumour free until termination on day 13 post tumour inoculation (figure 51B).

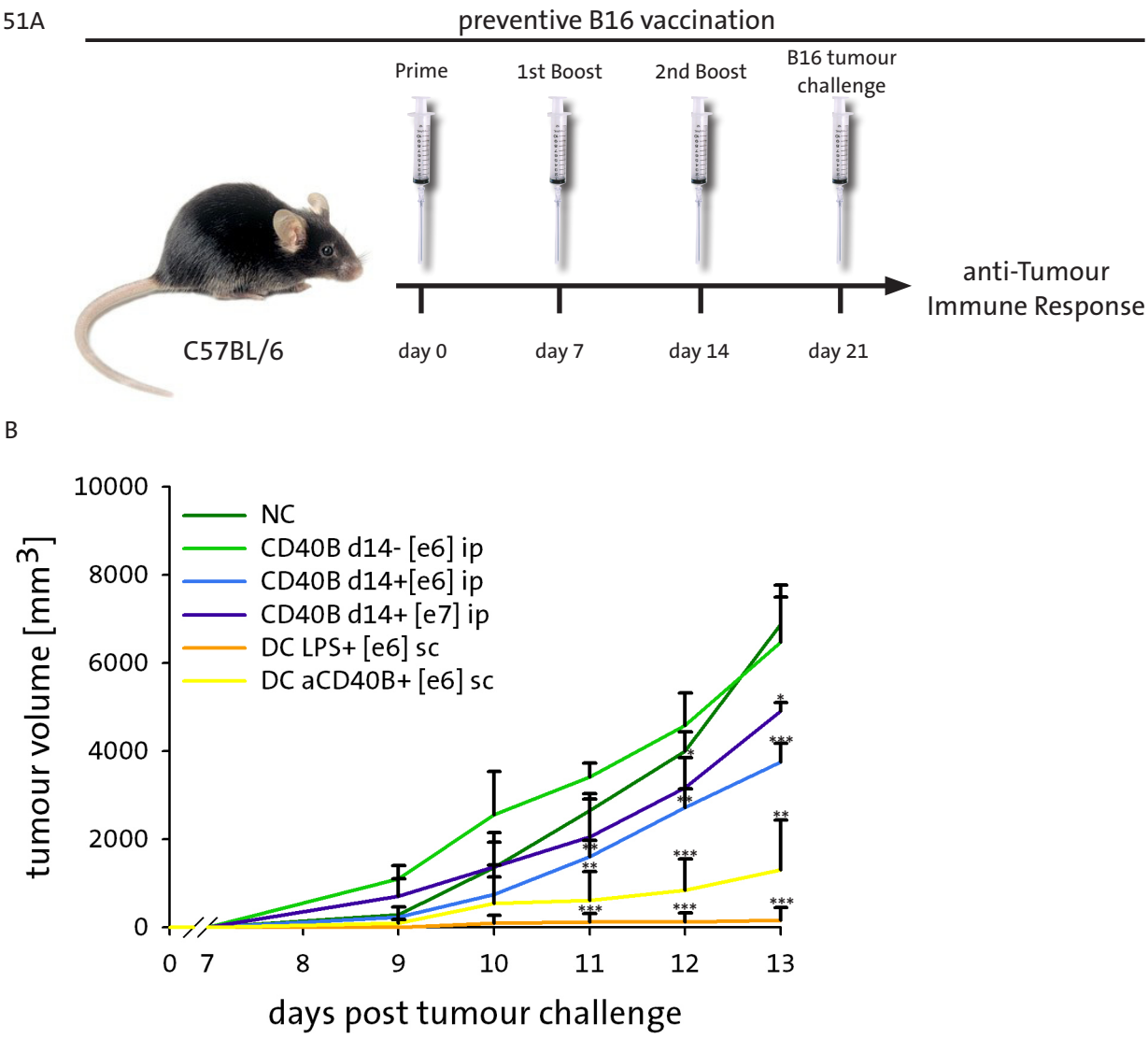


Figure 51: Preventive vaccination using mCD40B TRP-2 d14 induces growth deceleration of B16 melanoma

(A) Preventive vaccination schedule performing three subsequent vaccinations administered in a seven day interval followed by B16.F10 tumour inoculation (1 x 10e6) sc into the right flank of C57BL/6 mice one week after the last injection.

(B) Volume of subcutaneous B16 melanoma in groups of C57BL/6 mice treated by ip injection of 1 x 10e6 or 1 x 10e7 mCD40B d14 pulsed with TRP-2 peptide (mCD40B d14+) (blue and dark blue line, respectively). Alternatively mice were vaccinated with 1 x 10e6 DC LPS or DC aCD40Ab pulsed with peptide injected sc into the left flank (orange and yellow line, respectively). Administration of PBS alone (NC) or 1 x 10e6 mCD40B left unpulsed (mCD40B d14-) injected ip served as negative controls (green and light green line, respectively).

Tumour growth was assessed daily by multiplying the largest tumour diameters of three dimensions [mm^3]. Line plot shows mean values ± SD of tumour volume of indicated treatment groups from two independent vaccination experiments with 3 mice per group. Significant differences calculated with paired Student´s t-test are marked by an asterisk. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

In conclusion first anti-tumour vaccination experiments with TRP-2 peptide-pulsed mCD40B show that specific immune response is induced thereby leading to a significant reduction in tumour growth. Whether this noticeable but weak effect can be increased to a level of complete tumour remission needs to be further evaluated.

DISCUSSION

4. DISCUSSION

4.1. A murine system for the generation of immune-stimulatory B cells

Fundamental for this work was the development of effective cultivation conditions in order to generate murine CD40-activated B cells that fulfil the prerequisites to operate as antigen-presenting cells.

CD40 stimulation in the presence of IL-4 has shown to efficiently stimulate human B lymphocytes to proliferate and differentiate. However, varying effects on responses of B lymphocytes have shown to be induced by different sources of CD40 stimulus, namely soluble agonists or membrane-associated cellular forms of the CD40L differing in the degree of cross-linking and density (Kehry M.R. and Castle B.E., 1994 237; Néron et al., 2011 205). A higher degree of cross-linking and multimerization of CD40L has been shown to be associated with increased B-cell proliferation reflecting different modes of B and T-cell interaction. To provide strongly stimulating conditions in our model naïve B cells were activated with membrane-bound CD40L expressed by adherent feeder cells additionally facilitating removal of residual feeder cells from B-cell culture. Induction of proliferation and up-regulation of co-stimulatory as well as MHC-molecules on mCD40B cell cultures confirmed sufficient CD40 triggering in our model (see figures 6B, 7D-G).

The effect of CD40 stimulation on B cell proliferation and differentiation has been shown to be synergistically enhanced by IL-4 addition due to up-regulation of CD40 expression upon IL-4 treatment (Vallé et al., 1989 238; Maliszewski et al., 1993 239). This effect was in line with our results comparing proliferation rates of mCD40B cultures in the presence or absence of IL-4 reaching a plateau between 1 and 2 U/mL in a dose-dependent-manner (see figures 6B and 8). However, a transfection of IL-4 into feeder cells resulting in membrane-bound IL-4 has been suggested for further improvement of expansion of human CD40-activated B cells by others and an analogous approach would be worthwhile to be tested in the murine expansion system (Park et al., 2007 240). Notably, we were not able to expand murine CD40-activated B cells for longer than three weeks (data not shown). This stands in great contrast to reports of expansion durations for human CD40-activated B-cell cultures from several months to a few years as reported by several groups (Jung et al., 2001 241; O´Nions J. and Allday M.J., 2004 242; Wiesner et al., 2008 243). Furthermore, Ahmadi T. mentioned expansion of murine CD40-activated B cells by activation with human CD40L-transfected L cells for several weeks, but did not show them in his paper. However, the proliferation rates he depicted were equal to the ones we found (Ahmadi et al., 2008 208). After all, sufficient cell numbers were produced in our system with 30-50 x 10e6 primary B cells being obtained from a single murine spleen. Moreover, the duration of the expansion period was long enough in order to evaluate up-regulation of co-stimulatory molecules and immune-stimulatory capacity of mCD40B cells.

To date several *in vitro* models mimicking CD40 activation have been established using soluble and membrane-bound forms of CD40 ligand. Besides qualitative and quantitative optimization of

the adjuvant applied there is no CD40-activating reagent which is suitable for application in human clinical trials at present. Therefore, omission of allogeneic and xenogeneic components from cell culture represents an open gap to be filled through the development of a human GMP-grade soluble recombinant forms of CD40L and serum-free culture conditions (Neron et al., 2011 244). Nevertheless, murine CD40Bs expanded in our system did not induce any overt side effects after systemic application to mice, therefore their administrations was considered as safe (see figures 35, 36, 37). Per se this model significantly facilitates the immunological characterisation of the murine CD40-activated B cells.

4.2. Antigen-presentation and T-cell activation by CD40-activated B cells

There is solid evidence that B cells act as professional antigen-presenting cells given that they are able to internalize antigens, process and present them via MHC-II to T cells, which upon cognate antigen-recognition build up an "immunological synapse" (Cassell D.J. and Schwartz R.H., 1994 49; Grakoui et al., 1999 39). Proper activation of T cells according the "two-signal model" of lymphocyte activation drives the generation of potent effector T cells culminating in efficient immune responses (Bretscher P.A., 1999 6). Furthermore CD40-CD40L interaction between APCs and $CD4^+$ T cells has been shown to be required for sufficient induction of anti-tumour immunity (Mackey et al., 1998 187; Diehl et al., 1999 188). Therefore triggering CD40 on APCs has been investigated as possibility to bypass $CD4^+$ T cell function. Several studies have addressed different aspects of CD40-activated B cell´s antigen-presenting function mostly *in vitro*, and there is controversial data showing induction of tolerogenic or immunogenic T-cell responses with certain B-cell activation stimuli (Parekh et al., 2003 245; Tu et al., 2008 209; Dumont et al., 2008 246; Zheng et al., 2009 210; Zheng et al., 2010 211).

In vitro induction of T-cell responses in allogeneic MLRs require TCR signalling through binding of alloantigen-MHC complex and T-cell activation through co-stimulation by APCs. In our model murine CD40-activated B cells rapidly up-regulated and maintained expression of CD80 and CD86 and MHC-molecules needed for antigen presentation (see figure 30D). As this immune-phenotype was regarded potent in co-stimulating T cells, it was not astonishing that murine CD40-activated B cells of either subset induced significant proliferative responses to both $CD4^+$ and $CD8^+$ T cells in allogeneic MLR. Moreover, both mCD40B subsets were superior in inducing $CD4^+$ over $CD8^+$ T cell proliferation, possibly being a result of high peak expression of MHC-II molecule $I\text{-}A^b$. Finally, at low APC-to-T cell ratios mCD40B day 7 and mCD40B day 14 were significantly better in inducing $CD4^+$ and $CD8^+$ T cell proliferation, respectively. Whether this observation can be translated into a meaning of biological relevance e.g. that the duration of CD40-activation has a significant impact on T cell tropism needs to be further examined.

Little is known about the antigen-presenting capacity of CD40-activated B cells *in vivo*, however their utilization as cellular vaccine for the immunotherapy of cancer has been proposed. For the

purpose of assessing the applicability of mCD40B *in vivo* we studied whether antigen-loaded CD40-activated B cells are able to induce antigen-specific T-cell responses *in vivo*.

Previous studies have suggested a variety of methods for loading of human CD40-activated B cells with viral or tumour-derived antigens, such as direct antigen-pulsing with peptides or tumour-lysates, gene delivery by retroviral transduction and DNA/RNA transfection, or fusions of CD40Bs and tumour cells (Schultze et al., 1997 113; Kondo et al., 2002 196; Lapointe et al., 2003 195; Coughlin 2004 200; Fujiwara et al., 2005 197; Klier et al., 2010 247). Additionally, few reports on human peptide-pulsed CD40Bs have already shown efficient induction of primary and secondary antigen-specific $CD4^+$ and $CD8^+$ T cells *in vitro* (Schultze et al., 1997 113; von Bergwelt-Baildon et al., 2002 199; Lapointe et al., 2003 195; Zentz et al., 2007 248). Still, little is known about the presentation of MHC-I restricted antigens by CD40-activated B cells so far. By performing peptide pulsing with either viral peptide LCMV or foreign model-antigen OVA antigen delivery to mCD40B was ensured as antigen is loaded directly onto MHC-I molecules and the antigen-processing machinery can be omitted.

In accordance with the study of Ritchie D.S. who used membrane fractions from insect cells containing muCD40L injected i.v., we could show that LCMV-pulsed mCD40B induce *in vivo* cytotoxicity after ip injection and that this killing of target cells was antigen specific (Ritchie et al., 2004 214 and figure 38H). Furthermore, using model peptide OVA for mCD40B-based induction of immunity we found that induction of CTL response *in vivo* is dependent on injection route (ip > iv > sc), cell dose (10e7 > 10e6 > 10e5) and number of vaccination repetitions (2 > 1 booster vaccinations). However, our results contradict Lee J.´s to some extent. He reported that OVA-transfected B cells activated via anti-CD40 antibody (HM40-3) and CpG induced only weak CTL responses *in vitro* and failed to prime $CD8^+$ T cells after i.p. injection *in vivo* (Lee et al., 2008 249). Furthermore Guo S. also published recently, that OVA-peptide pulsed B cells activated with the same HM40-3 antibody injected i.v. were weak inducers of $CD8^+$ T cell responses as determined by IFN-γ secretion and that they failed to induce *in vivo* cytotoxicity similar to OVA-nucleofected murine CD40B cells. On the other hand he demonstrated that in his model OVA-transgenic but not irradiated OVA-transgenic mCD40Bs were as efficient as OVA-transgenic DC in inducing T cell responses *in vivo* (Guo et al., 2010 212). Conversely, we could show that OVA-pulsed mCD40B cells were competent in inducing specific lysis of target cells when using our cultivation system.

How can those differing results be obtained? First, contrary to Lee J. and Guo S. we used CD40L feeder cells to stimulate B cells with, probably resulting in a stronger CD40 stimulus due to higher cross-linking capacity of membrane-bound CD40 (Fanslow et al., 1994 206; Néron et al., 2005 207). Comparing the immune-phenotype of both mCD40B subsets as follows, the expression of MHC and co-stimulatory molecules is largely up-regulated in our system whereas Lee J. and Guo S. do not achieve the same elevated expression levels with their anti-CD40 antibody treatment. Second, we could show that the *in vivo* induction of the CTL response is dependent on injection route and dose of antigen-loaded CD40-activated B cell. Whereas Lee J. immunizes his mice with a low dose of 4 x 10e5 cells i.p. Gou S. applies 1-2 x 10e6 cells injected iv, which is likely to additionally account for the poor results.

Furthermore, in his model using transgenic mice with mutant MHC-I molecule $H\text{-}2D^b$ Ritchie D.S.

could show that antigen-presentation by mCD40B was partly dependent on cross-presentation of antigen by other endogenous APCs (Ritchie et al., 2004 214). This would explain why using irradiated mCD40B resulted in specific killing of target cell in our hands similar to non-irradiated mCD40B. Upon sufficient CD40-stimulation and following stable expression of their immune-phenotype mCD40B might directly present antigen and deliver antigen to endogenous APC as well, with the latter mechanism being prevalent upon irradiation.
In order to clarify the mechanism the induced immune response was functionally characterised by the amount of IFN-γ secretion and the cytolytic activity of antigen-specific $CD8^+$ cytotoxic T cells. Another question that remains to be answered is why different results were obtained for equal parameters (e.g. injection route iv) applying both techniques as a read-out of APC function. First, the techniques used to assess vaccinations with OVA-pulsed mCD40B were applied to different mouse models. At the beginning of our studies we were not able to detect IFN-γ production of antigen-specific $CD8^+$ T cells from immunized wild-type mice upon peptide re-stimulation *in vitro*. Therefore we switched to use TCR-transgenic OT-I mice analogous to Gou S. and then detected up to 10.2 % (± 3.17) $CD8^+$ IFN^+ T cells upon mCD40B vaccinations whereas *in vivo* cytotoxicity still was performed in wild-type mice. Second, IFN-γ production is initiated very early upon cross-linking of the T cell receptor complex whereas cytolysis of target cells occurs later during immune response (Ullman et al., 1990 250). Therefore minimal IFN-γ secretion may not exclude functional T cell response, but rather show time progression and thus we set higher value to results on cytolysis of target cells.

4.3. Induction of anti-tumour immunity by vaccination with mCD40B

The particular focus of this thesis was to develop a murine model to study B cells as APCs *in vivo* and test their applicability in a realistic tumour model. Hence, this work provides the first murine model to study CD40 ligand activated B cells as APC *in vivo* which allowed us the induction of tumour regression of the strongly immunogenic B16.F10 melanoma by mCD40Bs pulsed with the naturally expressed tumour-antigen TRP-2.
The identification of TAAs, the inherent nature of the host´s immune system to respond to TAAs and the ability to *ex vivo* generate and manipulate APCs in order to present TAAs has stimulated the concept of anti-tumour vaccination. In order to elicit effective anti-tumour-immunity the vaccination approach must overcome inhibitory mechanisms such as tumour-mediated immune evasion by secretion of immunosuppressive cytokines and induction of tolerance through tolerization of T cells by insufficient costimulation. Thereby, recognition and destruction of the tumour by the immune system is prevented.
In a study on tumour-mediated immune suppression of CD40-activated B cells we demonstrated that phenotype, migratory potential and antigen presenting function of human CD40-activated B cells is resistant to PGE-2, IL-10, TGF-β and VEGF (Shimabukuro-Vornhagen et al., PLoS One *in revision*) whereas DCs are thereby inhibited in differentiation and antigen presenting function (Steinbrink et al, 1999 251; Gabrilovich et al., 2004 252). This resistance of activated B cells against

immune-suppressive cytokines might reflect situations in autoimmune disease where B cells function as APCs *in vivo* - on the one hand their treatment being difficult, on the other hand B-cell depletion by monoclonal antibodies being that efficient.
Furthermore, both CD40-activated B cells and immature DCs have been shown to induce Treg in mice and human (Reichardt et al., 2007 253; Zheng et al., 2010 211; Steinman et al., 2003 136). But whether or not B cells act as stimulatory or tolerogenic cells has been suggested to depend on the strength of B cell activation (Shimabukuro-Vornhagen et al., 2009 254).
When using mCD40B stimulated with murine CD40L-expressing feeder cells, pulsed with TAA Trp-2 according the optimal vaccination schedule to induce protective immunity against B16 melanoma we found that tumour growth was significantly reduced (figure 51). However unexpectedly, when increasing the cell dose from 1 x 10e6 to 1 x 10e7 mCD40B per vaccination no further reduction of tumour growth was observed. Therefore the window of immunogenicity might be met with a certain cell dose underlining the need for studying the APC functions of CD40B *in vivo*.
Ritchie D.S. demonstrated that using mCD40B pulsed with viral peptide in a preventive setting against model-tumour cells expressing the corresponding epitope, reduction of tumour growth is only provided upon stimulation with CD40L-membrane fractions, but not upon activation of B cells with IL-4 or LPS (Ritchie et al., 2004 214).
On the contrary, despite significant CTL induction *in vivo* Lee J. showed that therapeutic vaccination with 4 x 10e5 mCD40B injected ip, activated with anti-CD40 antibody and transfected with RNA encoding for TAA TRP-2 and co-stimulatory molecules, did not prevent from B16 melanoma growth (Lee et al., 2008 249). Furthermore, using B cells activated with anti-CD40 antibody and/or CpG, Guo S. reported on the failure of 1 x 10e5 OVA-peptide pulsed mCD40B injected iv to reduce EG-7 tumour growth in a therapeutic setting as well, whereas vaccination with OVA-transfected mCD40B protected similar to OVA-transgenic DCs against tumour growth (Guo et al., 2010 212).
Even though our data and Ritchie´s study appear to be contradictory to Lee´s and Guo´s work at first sight, one can draw several conclusions from those pieces of data. First, there is evidence that murine CD40-activated B cells are capable of efficiently transporting antigens from the periphery to lymphoid organs and interact with T cells *in vitro* and *in vivo*, thus eliciting specific T cell responses regulated by the nature and strength of stimulus inserted. Whereas “innate” stimulation of B cells with LPS will lead to induction of Treg, insufficient T cell co-stimulation will lead to T cell anergy or apoptosis. Upon cognate interaction of activated antigen-loaded B cells and T cells specific T cells are expanded and activated via a combination of direct T-cell stimulation and cross-presentation of antigen by local APCs. Due to the migratory potential of mCD40Bs this happens upon i.v. and i.p. injection in lymph nodes and spleen, and to a lesser extent upon sc injection as well. Dependent on the stimulus used to activate B cells different ways of antigen-loading may lead to functional antigen-presentation. Whereas weak B cell activation would require internal antigen-processing for efficient and durable antigen-presentation to T cells, it would be sufficient for properly activated B cells to present exogenous peptide upon external loading. Furthermore, mCD40B vaccination might induce both cellular and humoral immune responses thereby improving immunity against tumour antigens.

4.4. Comparison of mCD40B to mDC as APC

In order to investigate whether mCD40B are comparable to DCs in eliciting T cell responses the APC capacity of two subsets of each group with highly activated phenotype were introduced to MLR. Herein, the protein expression levels of co-stimulatory molecules of mCD40B subsets were comparable to that of matured DCs (figures 30D and 31C-L). However, the comparison of mDC and mCD40B subsets revealing that mDC were superior to mCD40B in increasing the number of T cells did not meet our expectation (figures 34A-D). Hence, closer examination differences between mCD40B and mDC contributing to T-cell activation in allogeneic MLR is required.

Morphological factors that might be relevant are cell size as well as number and size of dendrites. To this end, APC cell sizes were assessed by flow cytometric analysis of their forward scatter area, which is approximately proportional to size. We found that DCs introduced in MLR were about 1.5 times bigger than mCD40Bs at least partly accounting for their differential capacity to stimulate T-cell proliferation *in vitro* (data not shown).

Biochemical differences of mCD40B and mDC affecting MLR might be turnover, expression levels and density of MHC, co-stimulatory and co-inhibitory molecules as well as up-take, processing and presentation of self-proteins as allo-antigens. Furthermore a differential impact of irradiation on mCD40B and mDC might explain for this discrepancy as sub-lethal irradiation would enable further cell proliferation leading to antigen loss due to rapid turnover. Also Guo´s study demonstrating that OVA-transfected mCD40B or mDC but not OVA-peptide pulsed mCD40B were able to induce reduction of EG-7 tumour growth confirms that prevention from antigen loss by stable transfection may achieve a beneficial result.

There is only very limited data available on the ability of activated B cells to induce anti-tumour immunity *in vivo*. In particular, there are only few studies that directly compared B cells with DCs in the ability to induce an anti-tumour immune response.

Due to their efficient APC capacity DCs have been most frequently used in cellular immunotherapy for the generation of tumour-specific immune responses. In a systemic review of clinical trials using DC vaccination a proof of this concept was provided showing an association between induced cellular immune response and clinical benefit rate (Draube et al., 2011 144). Essential parameters accounting for outcome of DC-based cancer vaccination have been revealed, namely adequate cell number, appropriate DC subset at proper stage of differentiation and the injection route supporting migration of DCs to secondary lymphoid organs. Thus, despite proven safety and feasibility DC-based cancer vaccination is limited. In order to evaluate CD40-activated B cells as alternative source of APC we systemically investigated several vaccination parameters and compared them to DCs. Performing mCD40B vaccinations we also found a positive association between elevation of mCD40B cell numbers as well as vaccination repetitions and functional T cell response (figures 48A and 48A). In contrast to DCs CD40B cells stimulated *in vitro* show enormous proliferating capacity and large amounts can be obtained from few mL of peripheral blood (Wiesner et al.,2008 243; Schultze et al., 1997 113). Therefore cell number would not represent a major challenge in CD40B-based immunotherapy - not even in immune-compromised patients.

Antigen-presentation to T cells and following induction of an immune response requires physically interaction of lymphocytes in secondary lymphoid organs. Previously, we could show that

CD40-activated B cells do not only express certain chemokines and receptors allowing them to transmigrate the walls of high endothelial venules (HEV) and attract T cells, but also that they localize in spleen and lymph nodes upon i.v. and s.c. injection (von Bergwelt-Baildon et al., 2006 213 and Klein-Gonzáles, unpublished data). On the contrary DCs lack CD62L (L-selectin) required for entering the draining lymph node across the HEV and only few DC administered i.v., s.c. or intradermally reach the draining lymph nodes (Ridolf et al., 2004 145; Quillien et al., 2005 146). Furthermore, when injected iv DCs accumulate in the spleen and non-lymphoid tissues, but not in the lymph node (Robert et al., 2003 255). In order to meet the migration requirements of mCD40B for *in vivo* CTL induction different injection routes were performed and analyzed. We were able to identify several key parameters which influence the induction of T-cell responses by mCD40B. Vaccine formulation, cell dose, the injection route, as well as the vaccination schedule all had a significant impact on the strength of the elicited T-cell response.
In line with Park´s data on LPS-activated B cells loaded with antigen by RNA-transfection we found that s.c. administration of peptide-pulsed mCD40B had significant lower effect on CTL response induction in the spleen than DCs (Park et al., 2008 256; figures 47A and 49A). Furthermore upon iv injection mCD40B also induced significant CTL responses in the spleen as expected from *in vivo* migration experiments performed by us and others (Klein-Gonzáles, unpublished data and Ahmadi et al., 2007 208). This was not surprising as B lymphocytes travel through peripheral blood and lymphoid tissues finally entering the follicles to form germinal centres. On the other side DCs reside in peripheral tissues and organs induced to migrate through the afferent lymphatic vessels to the regional lymph node upon antigen up-take. To our knowledge there is only one report on i.p. vaccination of mice with OVA-transfected B cells activated with CpG and anti-CD40 antibody showing absent induction of CTL response by *in vitro* europium release (Lee et al., 2008 249). However, insufficient activation stimulus may again account for this negative result. In our model ip injections of peptide-pulsed mCD40B were significantly better over other routes and rates of target cell lysis indeed comparable to DC vaccinations (figures 47A and 49A). Interestingly, the level of induced anti-tumour immunity on day 13 post tumour inoculation was significantly higher when using mDC compared to mCD40B, although either treatment regimen was found to be similar in inducing CTL responses (figure 49A). Differential regulation of cellular and humoral immune responses improving immunity against tumour antigens by both cell types might lead to these varying results.

4.5. Concluding remarks

When we compare our data to other studies using CD40-activated B cells we learn that in order to generate B cells with effective APC function adequate CD40 signalling has to be provided. To date membrane-bound forms stimulating via CD40 exceed over soluble forms probably due to differential cross-linking and signal transduction. Several soluble human CD40 ligands in trimeric and hexameric composition and agonistic antibodies have been tested by us and others and to date there is no GMP-grade construct with equal potential to induce activation and proliferation. This step indispensable for testing CD40-activated B cells in Phase I trial is worked on in cooperation with Miltenyi Biotech Company.

FUTURE PROSPECTS

5. FUTURE PROSPECTS

At the moment this model for the generation of murine CD40-activated B cells capable of inducing anti-tumour immunity is used to further evaluate the mechanisms by which antigen-presenting B cells confer or inhibit effective anti-tumour responses. In order to determine the window of immunogenicity the quality and the quantity of the elicited anti-tumour response will be analyzed in more detail. Therefore the capacity of mCD40B to regulate T-cell differentiation will be characterised phenotypically for composition of B cell subsets and for their cytokine profiles driving either T_h1 (IFN-γ) or T_h2 (IL-4) skewed $CD4^+$ or $CD8^+$ T cell responses *in vivo*. Furthermore we will clarify whether sub-optimal B cell activation leads to induction of inhibitory B cell subsets, such as B cells producing neutralizing antibody or regulatory B cells with $CD19^{hi}$ $CD1d^{hi}$ $CD5^+$ phenotype secreting immune-suppressive cytokines (IL-10). We will test whether by adaption of the B cell subset therapeutic and preventive immunity against tumour antigens can be improved and whether a tumour antigen-specific T-cell memory can be generated to meet the requirements for full anti-tumour immunity.

Second, CD40-activated B cells have been used to present antigens to $CD4^+$ and $CD8^+$ T cells. However, antigen processing mechanisms in CD40B cells are still unclear. By use of antigen processing inhibitors of endocytosis (NaN_3 and 2-deoxy-d-glucose), endosomal acidification (Chloroquine), proteases (E-64d) and synthesis of new MHC II molecules (BrefeldinA), we want to clarify how CD40Bs can take-up, process and present antigen by the MHC-class II pathway and to what extent other mechanisms like trogocytosis and cross-presentation on MHC class I molecules are relevant.

Third, specific B cells act as antigen-presenting cells due to their ability of BCR-mediated endocytosis of whole protein antigen. Utilizing the B cell receptor-mediated enhanced antigen-uptake and processing the interaction with T cells and their activation may be improved. Therefore the CD40B approach will be applied to clonal B cell populations with a tumour-antigen-specific B-cell receptor to test whether those B cells represent a more effective alternative to previous CD40B vaccines.

ATTACHMENTS

6. ATTACHMENTS

6.1. References

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6.2. List of Figures

6.3. List of Tables

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Erklärung §4 Abs. 1 Nr. 9

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von den angegebenen Teilpublikationen - noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.
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Teilpublikationen

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Lebenslauf

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Kongressbeiträge

10/2011 Poster	*In vivo* Evaluation von CD40-aktivierten B Zellen als zelluläres Adjuvans für die Krebsimmuntherapie, DGHO, Basel
09/2010 Vortrag	*In vivo* Evaluation einer CD40-B Zell-basierten Tumor-Vakzine zur Verwendung in der Immuntherapie, DGHO, Mannheim
02/2010 Poster	Developing a murine model to study B cells as antigen presenting cells *in vivo*, ASBMT, Orlando
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Fernsehbeiträge

04/2011	„Kampf gegen Krebs“ WDR - Planet Wissen, Studiointerview
04/2010	„Krebs - Wie können wir ihn besiegen?“ WDR - Quarks und Co, Filmbeitrag
02/2010	„Nur ein erster Schritt - Jede Krebsimpfung muss auf den Tumor passen“ 3Sat - Nano, Filmbeitrag