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Characterization of human MHC II-restricted T cell receptors with reactivity against B cells and tumor cells for therapeutic application in the context of adoptive T cell transfer of transgenic CD4 T cells

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ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
α-h	Anti-human
ALL	Acute lymphoblastic leukemia
AML	acute myleiod leukemia
APC	Allophycocyanin
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CLL	Chronic lymphatic leukemia
CML	Chronic myleiod leukemia
CMV	Cytomegalovirus
CpG	CpG deoxynucleotides
CspA	Cyclosporine A
CTLA-4	Cytotoxic T-lymphocyte antigen 4 (CD152)
DC	Dendritic cells
DEPC water	Diethyl pyrocarbonate water
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBV	Eppstein-Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
E:T	Effector : Target ratio
FACS	Fluorescence-activated cell sorting
Δ FCS	heat inactivated fetal calf serum
FITC	Fluoresceinisothiocyanat
FMNL1	Formin-like protein 1

FoxP3	Forkhead box P3
GFP	Green fluorescent protein
GvL	Graft versus leukaemia
GvHD	Graft versus host disease
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HS	Human serum
IFN-γ	Interferone-γ
IL-x	Interleukin-x
kDa	Kilodalton
KOD	Thermococcus kodakaraensis
LB	Lysogeny Broth
LCL	Lymphoblastoid cell line
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption/ionization
MHC	Major histocompatibility complex
mu	Mouse
NK	Nuclear killer cells
PBMC	Peripheral blood mononuclear cells
PE	Phycoerythrin
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGE_2	Prostaglandin E_2
PP124	Peptide 124
RNA	Ribonucleic acid
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TCR	T cell receptor
TCRmu	Anti-mouse αβ-TCR antibody

TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
Th	T helper cell
TIL	Tumor infiltrating lymphocytes
Treg	Regulatory T cells
VEGF	Vascular endothelial growth factor

1 ZUSAMMENFASSUNG

Adoptive T-Zelltherapie ist ein neuer vielversprechender Ansatz zur Tumortherapie. Da $CD8^+$ T-Zellen in der Lage sind Tumorzellen direkt anzugreifen werden derzeit die meisten Studien, bei denen tumorspezifische T-Zellen adoptiv transferiert werden, mit $CD8^+$ T-Zellen durchgeführt. Die Verwendung von $CD4^+$ T-Zellen könnte die Effektivität der adoptiven T-Zelltherapie möglicherweise steigern, da $CD4^+$ T-Zellen die Durchlässigkeit von Turmorgewebe verbessern und das $CD8^+$ T-Zellgedächtnis unterstützten.

Ziel dieses Projektes war es tumorreaktive $CD4^+$ T-Zellen für eine potentielle spätere klinische Anwendung zu generieren. Zu diesem Zweck haben wir zwei unterschiedliche Ansätze im autologen System gewählt. Zum Einen haben wir eine Peptidbibliothek des tumorassoziierten Antigens Formin-like protein 1 (FMNL1) verwendet. Mit diesem Ansatz konnten wir T-Zellklone generieren, die ein spezifisches FMNL1 Peptid erkannt haben. Allerdings wird dieses Peptid höchstwahrscheinlich nicht durch zelluläre Proteasome hergestellt. Zum Anderen haben wir unterschiedlich gereifte FMNL1 Protein-gepulste dendritische Zellen zum Aktivieren spezifischer T-Zellen verwendet. Wir konnten so zwei unterschiedliche selbst-reaktive T-Zellklone aus Zellen eines gesunden Spenders generieren. Diese T-Zellklone wurden von zwei unterschiedlichen Ausgangspopulationen generiert. Klon Aa2.2 wurde aus mononukleäre Blutleukozyten gewonnen, während Klon Bb5.14 aus der naiven T-Zellpopulation isoliert wurde.

Wir konnten die T-Zellrezeptoren (TCR) von beiden Klonen (Aa2.2 und Bb5.14) nach erfolgreichem Transfer in unterschiedliche autologe und allogene T-Zellpopulationen eingehend untersuchen. TCR Aa2.2 (Aa2.2) erkannte ausschließlich B-Zellen, während TCR Bb5.14 (Bb5.14) außerdem dendritische Zellen und Makrophagen erkannt hat. Es kam weder bei Aa2.2- noch bei Bb5.14-transduzierten T-Zellen zu Brudermord. Wir konnten zeigen, daß Aa2.2 HLA-DR1-restringiert ist, während Bb5.14 HLA-DR11 restringiert ist. Beide TCR erkannten HLA-DR kompatible lymphoblastoide Zellinien und chronische lymphatische Leukämiezellen. Aufgrund der gezeigten Tumorreaktivität könnten beide TCR von Interesse für eine spätere klinische Anwendung sein.

Toll-like Rezeptor (TLR) Agonisten werden als potentielle Adjuvanzien für immun-therapeutische Therapien untersucht. Wir haben überraschender Weise unterschiedliche Effekte von TLR Liganden auf Aa2.2- und Bb5.14-transduzierte T-Zellen festgestellt. Aa2.2, der in den Versuchen funktionell deutlich schwächer war als Bb5.14, zeigte eine deutlich bessere Reaktivität gegen TLR Ligand behandelte Zielzellen. Im Gegensatz dazu war die Reaktivität von T-Zellen, die mit Bb5.14 transduziert waren deutlich verringert, wenn Zielzellen zuvor mit TLR Agonisten behandelt wurden. Dieses Ergebnis wurde bei einer Multi-

Zytokinanalyse und einen Chrom-Freisetzungsversuch bestätigt. Allerdings wurde die Proliferation gleichermaßen durch B-Zellen verstärkt, wenn diese mit Lipopolysaccharid (LPS) behandelt waren. Diese Ergebnisse zeigen, daß bei der Anwendung von TLR Liganden als Adjuvanzien die Eigenschaften der TCR möglicherweise berücksichtigt werden sollten.

Verschiedene T-Zellpopulationen die mit beiden TCR transduziert wurden konnten Zielzellen töten, zeigten Antigen-abhängiges Wachstum und verschiedene Zytokinmuster. Diese Multifunktionalität der TCR-transduzierten T-Zellen ist von großer Bedeutung für eine potentielle klinische Anwendung.

2 ABSTRACT

Adoptive T cell therapy is a promising approach for the treatment of malignancies. Presently, most studies focus on the use of tumor-specific cluster of differentiation $CD8^+$ T cells, since these are able to directly eliminate cancer cells. Application of tumor-specific $CD4^+$ T cells may improve permeability of the tumor tissue and enhance $CD8^+$ T cell memory formation. Therefore, $CD4^+$ T cells may highly improve this therapeutic approach.

We aimed to generate tumor reactive $CD4^+$ T cells for potential future treatment of hematologic malignancies. For this purpose, we chose two approaches, both applied in the autologous system. On the one hand we used a peptide library of the tumor-associated antigen Formin-like protein 1 (FMNL1). Using this approach, we were able to generate T cell clones with specificity for a defined FMNL1 epitope. However, this epitope is most likely not endogenously processed by cellular proteasomes. On the other hand, we used differentially matured dendritic cells (DC) pulsed with recombinant FMNL1 for T cell priming. In this way, we were able to recover two self reactive distinct T cell clones from a healthy donor. Both T cell clones were isolated from different initial populations. Clone Aa2.2 was isolated from untouched peripheral blood mononuclear cells (PBMC), whereas clone Bb5.14 was generated from the naïve T cell population.

After successful transfer of both T cell receptors (TCR) in different autologous and allogeneic effector populations, we were able to characterize TCR Aa2.2 (Aa2.2) and TCR Bb5.14 (Bb5.14) in detail. Aa2.2 recognized exclusively B cells, whereas Bb5.14 additionally recognized DC and macrophages. Both TCR did not commit fratricide and recognize distinct restriction elements. Aa2.2 is Human leukocyte antigen (HLA)-DR1-restricted, whereas Bb5.14 exhibits HLA-DR11 restriction. Both TCR were reactive in response to HLA-DR matched lymphoblastoid cell lines (LCL) and chronic lymphocytic leukemia (CLL) samples. Tumor reactivity renders both TCR interesting for potential clinical application.

Toll-like receptor (TLR) agonists are investigated as potential adjuvants for immune-therapeutic approaches. We unexpectedly found differential effects of TLR ligands on the functionality of both TCR. Aa2.2, generally functionally weaker than Bb5.14, was significantly more reactive in response to TLR ligand pulsed target cells. In contrast, Bb5.14-transduced T cells were notably inhibited when they were incubated with targets previously treated with TLR agonists. This finding was confirmed by the results of multi-cytokine analysis and chromium release assay. However, proliferation of both TCR-transduced populations was similarly enhanced when these were incubated with B cells treated with Lipopolysaccharide (LPS) compared to untreated B cells. This oppositional regulation of distinct TCR by innate

immune stimulators shows, that for application of TLR agonists as adjuvants in adoptive T cell therapy, characteristics of the transferred TCR may need to be taken in account.

Effector cell populations transduced with both TCR were able to kill target cells, demonstrated antigen-induced proliferation and showed a mixed cytokine pattern. This multifunctionality of TCR-transduced T cells is of high importance for potential clinical application.

3 INTRODUCTION

3.1 Hematologic malignancies and treatments

3.1.1 Leukemia and lymphoma

Leukemias and lymphomas are hematologic malignancies originating from diverse hematopoietic cells. Lymphomas derive from diverse mature and precursor lymphocyte populations and develop mostly in lymph nodes. Malignancies of the lymphatic system are divided into two groups: Hodgkin and Non-Hodgkin lymphomas. Hodgkin lymphomas are characterized by Hodgkin cells and formation of multinucleated Sternberg-Reed cells (Begemann, 1999). Non-Hodgkin lymphomas are a vast group of highly diverse malignancies (WHO-classification, 2007). The most prevalent Non-Hodgkin Lymphoma is chronic lymphocytic leukemia (CLL). This disease regularly develops slower than most other lymphomas. CLL generally originates from B cells, in rare cases it may however also originate from T lymphocytes (Begemann, 1999; Berger, 2010). In contrast to CLL, Burkitt lymphoma is a fast growing entity. Burkitt lymphoma appears in three types: the endemic variant prevalent in Africa, which is associated to the Eppstein-Barr Virus (EBV), the sporadic variant originating from genetic disorders, and the immunodeficiency associated variant affecting human immunodeficiency virus (HIV) -infected patients suffering from acquired immune deficiency syndrome (AIDS) (Ferry, 2006). Acute lymphoblastic leukemia (ALL) is a high malignant neoplasia, developing from unmature B or T cells in the bone marrow (Berger, 2010). In contrast, chronic myleiod leukemia (CML) and acute myeloid leukemia (AML) originate from granulocytes and monocytes. In CML, the malignant cells are primarily able to differentiate to mature myeloid cells, whereas in AML cancer cells retain their immature characteristics (Begemann, 1999; Berger, 2010).

3.1.2 Present therapies and new approaches

Depending on the type of the hematologic malignancy, different therapeutical approaches can be applied or combined, such as chemotherapy, antibody therapy and radiotherapy potentially followed by stem cell transplantation. Hematopoietic stem cell transplantation may

be applied especially in patients with bad prognosis. This treatment has a curative potential by the Graft-versus-Leukemia (GvL) effect induced by the donors' T cells (Bottcher *et al.*, 2011; Horowitz *et al.*, 1990). T cells, which recognize a specific self major histocompatibility complex (MHC) molecule in combination with an immunogenic peptide in the autologous system, may additionally recognize allogeneic MHC molecules (Colf *et al.*, 2007). The recognition of allogeneic MHC molecules is currently seen to be peptide-dependent (Felix and Allen, 2007; Morris *et al.*, 2011). In the allogeneic transplant setting, minor histocompatibility antigens may be the source of peptides harboring distinct differences between donor and recipient resulting in the recognition of leukemic cells, but potentially also the recognition of healthy tissue. As a result of this therapy malignant cells may be eliminated but also the development of the life-threatening Graft-versus-Host disease (GvHD) may be induced (Horowitz *et al.*, 1990). Patients who do not suffer from a strong GvHD are at high risk to relapse. These patients may receive additional donor lymphocyte infusions to enhance the GvL effects (Kolb *et al.*, 2004; Roddie and Peggs, 2011).

Since present therapies for patients with bad prognosis demonstrate still low survival rates among patients, new approaches to treat leukemia, lymphoma and other cancers are required. Immunotherapies build a broad field of innovative therapies. Systemically active immunotherapies such as cytokine application, blocking of Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) or depletion of cluster of differentiation (CD) 25^+ T cells may sustain a potentially inhibited anti-tumor response (Dougan and Dranoff, 2009). These agents can be applied to any patient, independently on the antigenic composition of the tumor and the human leukocyte antigen (HLA)-type of the patient. However, these agents harbor the risk of side effects due to their systemic function (Weber, 2009). Specific immunotherapies, such as cancer vaccines or adoptive transfer of tumor-specific T cells may directly and exclusively target tumor cells, if the antigen is well chosen. Both approaches are presented more in detail in chapter 3.3. To date, some of these multiple new therapeutic approaches were able to elicit complete or partial remissions in individual cases, further encouraging the ongoing development of novel immunotherapies. Moreover, the combination of novel therapies including different immunotherapeutic and immune modulating approaches may be more effective in inducing an effective tumor-specific immune response.

3.2 “Tumor escape” mechanisms

3.2.1 Various mechanisms promote tumor immune evasion

“Tumor escape” mechanisms allow tumor cells to evade elimination by the immune system. Immune evasion is provided by the tumor microenvironment and modulation of the tumor cells themselves. Burnet claimed that the adaptive immune system would prevent tumor development in immunocompetent hosts. This over decades controversially discussed hypothesis was named the immunosurveillance hypothesis (Burnet, 1957). Dunn and colleagues published in 2002 the “cancer immunoediting” hypothesis building on the former principle of immunosurveillance (Dunn *et al.*, 2002). Cancer immunoediting is defined by three stages. The first stage, the tumor “elimination”, corresponds to the former immunosurveillance hypothesis. The second phase is “equilibrium” between tumor cells exhibiting effective immune evasion strategies and the immune system. In this phase, immune cells promote the growth of tumor cells able to evade immune destruction. This mechanism finally leads to the third stage, the tumor “escape”. Tumor cells are then able to escape the immune system of an immunocompetent host (Dunn *et al.*, 2002).

Various factors are responsible for the induction of tumor escape. During tumor growth, tumor tissue is hardly supplied with enough nutrients, due to low vascularisation. Cellular stress is then leading to the expression of Heat shock proteins, to the enhancement of reactive oxygen species and the deregulation of the pH balance in the tissue (Khong and Restifo, 2002). To enhance vascularisation, tumor cells express vascular endothelial growth factor (VEGF) (Khong and Restifo, 2002). However, VEGF may hinder dendritic cell (DC) differentiation and maturation, impeding a possible immune response (Oyama *et al.*, 1998). Other immunosuppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) may be secreted by tumor cells (Gorsch *et al.*, 1992). Moreover, prostaglandin E_2 (PGE_2), a proinflammatory cytokine, can induce IL-10 production by lymphocytes, macrophages and DC, which in turn impedes the immune response (Huang *et al.*, 1998; Kalinski *et al.*, 1997).

Tumors also evade the immune system by modulating surface expression of molecules crucial for immune intervention. Tumor cells may loose MHC I and MHC II expression through various mechanisms such as deregulations in the antigen processing pathway, MHC-loading or mutations of MHC-molecules preventing expression reviewed by Campoli and Ferrone (Campoli and Ferrone, 2008). In a healthy immunological context, these cells would be eliminated by natural killer (NK) cells. However, the function of NK cells is dependent on

costimulatory molecules and the cytokine milieu. Tumor cells escaping immune elimination may fail to express costimulatory molecules such as CD80, CD86 or CD40. Lack of costimulation not only impedes NK cell activity, but also obviates an effective T cell response by induction of T cell anergy (Schwartz, 1990). The cytokine milieu induces activity of regulatory T cells (Treg) as described in the following paragraph.

3.2.2 A major role of Treg in tumor escape

Treg are classically defined by CD4, CD25 and forkhead box P3 (FoxP3) coexpression and play a major role in peripheral tolerance induction. As tumor-associated antigens are primarily self antigens, $CD4^+$ $CD25^+$ $FoxP3^+$ T cells may suppress tumor-reactive T cells, impeding an effective anti-tumor immune response (Sakaguchi, 2005). In patients with different tumors, elevated Treg levels were detected (Zou, 2006). Increased presence of Treg in the tumor microenvironment is induced by several mechanisms. Treg can be attracted to the tumor tissue by CC chemokine ligand (CCL) 22, detected by CC chemokine receptor (CCR) 4 expressed on Treg surface. Moreover, disregulation of DC and cytokine milieu described above may lead to induction of Treg, to conversion of activated T cells to Treg and to heavy expansion of Treg (Zou, 2006).

Treg may suppress effective anti-tumor responses by direct cell contact through CTLA-4 and additionally through cytokine secretion. Treg may induce cell cycle arrest of activated T cells, or even T cell anergy or apoptosis. Moreover, IL-10 and TGF-β secreted by Treg may result in dysfunction of antigen presenting cells and NK cells (Zou, 2006).

In patients treated with immunotherapy, this accumulation of Treg may also impede effective tumor elimination. It has been described that lymphodepletion may enhance efficacy of adoptive transfer by depletion of inhibitory Treg (Muranski *et al.*, 2006). Currently, other strategies are developed specifically targeting Treg to enhance efficacy of immunotherapies. One strategy is application of the CTLA-4 antibodies ipilimumab and tremelimumab to block the inhibitory molecule CTLA-4 (Hodi *et al.*, 2003; Phan *et al.*, 2003). However blocking of CTLA-4 may induce severe side effects by inducing autoimmunity (Weber, 2009). Another strategy using anti-CD25 antibody or denileukin diftitox (diphteria toxin linked to IL-2) to delete $CD25^+$ T cells was partially successful (Barnett *et al.*, 2005; Dannull *et al.*, 2005). These approaches may be helpful in combination with adoptive T cell therapy or vaccination.

3.3 Immunotherapeutic approaches

3.3.1 Vaccination therapies

One immunotherapeutic approach is vaccination of cancer patients. Various antibodies targeting tumor cells equipped with multiple functions have been developed and are currently partially included in evidence-based treatment schedules (Dougan and Dranoff, 2009; Seimetz *et al.*, 2010). In addition, multiple therapeutic strategies are currently under investigation including active vaccination strategies with various agents such as recombinant proteins, peptides, viral vectors, deoxyribonucleic acid (DNA) and DC. As mentioned before, selection of the antigen is one crucial aspect for the development of specific immunotherapy. Generally, either self antigens exclusively expressed in the cancerous tissue or mutated antigens of the tumor tissue are targeted (Finn, 2008). Targeting self antigens is a challenge in tumor vaccination strategies, since T cells recognizing such self antigens with high avidity are likely deleted during negative selection in the thymus. If self antigen specific T cells have escaped negative selection, these might not be reactive due to peripheral tolerance mechanisms (Van Parijs and Abbas, 1998). Vaccination studies with recombinant protein or peptide have been applied with limited success even though some patients showed tumor specific T cell responses (Connerotte *et al.*, 2008; Kyte *et al.*, 2009; Ochsenreither *et al.*, 2011). Vaccination studies using DC may be advantageous as they provide functional DC presenting the designated peptides. There are a great number of vaccination strategies using DC. DC may be transfected with ribonucleic acid (RNA), loaded with tumor lysate, protein or peptides (Hsu *et al.*, 1996; Lesterhuis *et al.*, 2010; Nestle *et al.*, 1998; Van Tendeloo *et al.*, 2007). Moreover, DC can be matured with different cytokine cocktails playing a crucial role in the final function of the primed T cells (O'Neill *et al.*, 2004). Another approach involves application of immature DC. This method allows to minimize the costs of the therapy, since no cytokines are needed for maturation and *in vitro* culture is short (Nair *et al.*, 2003). However, superiority of any approach has not been demonstrated yet. Success of vaccination therapies might be enhanced by similar application of immune adjuvants (see 3.3.3).

3.3.2 Adoptive T cell therapy

Adoptive T cell therapy is an immunotherapeutic treatment currently investigated in clinical studies. For this treatment, autologous or allogeneic T cells may be expanded and selected

ex vivo before being transferred to cancer patients. One promising approach in adoptive T cell therapy is the isolation, stimulation and reinfusion of tumor infiltrating lymphocytes (TIL) in the autologous system (Dudley *et al.*, 2002a). In clinical studies, 50 to 75 % of patients transferred autologous TIL induced remissions (Dudley *et al.*, 2002a; Dudley *et al.*, 2005; Yee *et al.*, 2002). However, an unfavorable tumor microenvironment, intermediate T cell avidity or the absence of $CD4^+$ T helper (Th) cells may impede clinical responses.

In the allogeneic system, donor lymphocyte infusions are currently a powerful therapeutic approach for the treatment of patients with recurrent hematologic malignancies by inducing a GvL effect as described in 3.1.2 (Kolb *et al.*, 2004; Roddie and Peggs, 2011). However, this approach involves a high risk of development of life-threatening GvHD provided by alloreactive T cells affecting healthy host tissue (Horowitz *et al.*, 1990). The separation of both effects represents a longterm goal for cancer immunotherapists and may be achieved by transfer of high avidity T lymphocytes recognizing allorestriced MHC molecules in combination with a self peptide restrictedly presented on the tumor cell (Sadovnikova and Stauss, 1996). Sadovnikova and Stauss showed that transfer of allogeneic T cells recognizing the recipients' MHC molecule presenting a self peptide on the tumor cells was able to effectively eliminate the tumor. To date, a number of T cell receptors (TCR) of such allorestricted tumor specific T cells have been isolated and characterized in the human system (Amrolia *et al.*, 2003; Gao *et al.*, 2000; Liang *et al.*, 2010; Sadovnikova *et al.*, 1998; Schuster *et al.*, 2007). Genetic engineering of the patients' T cells allows transfer of such allorestricted TCR. Adoptive transfer of redirected $CD8^+$ T cells has been evaluated in several clinical studies (Burns *et al.*, 2009; Johnson *et al.*, 2009; Morgan *et al.*, 2006). In these studies clinical responses have been observed, however, genetically engineered highly reactive T cells may cause serious side effects when antigen expression is not restricted to the tumor tissue (Johnson *et al.*, 2009). Moreover, transferred TCR chains may pair with endogenous TCR chains building new unknown specificities (van Loenen *et al.*, 2010) or may be quickly down regulated failing to eliminate the tumor (Burns *et al.*, 2009).

Dudley et al. discussed that the clonal populations of autologous tumor reactive T cells transferred to melanoma patients possibly failed to lead to tumor elimination, because of the absence of specific $CD4^+$ T cells sustaining the transferred $CD8^+$ T cell populations (Dudley *et al.*, 2002b). Therefore, adoptive transfer of tumor reactive $CD4^+$ T cells might be an important tool in cancer therapy (Muranski and Restifo, 2009). One successful clinical case report using tumor specific $CD4^+$ T cells has been published in 2008 (Hunder *et al.*, 2008). Hunder et al. reported successful transfer of autologous cancer-testes antigen NY-ESO specific $CD4^+$ T cells to a patient with metastatic melanoma leading to durable clinical remission (Hunder *et al.*, 2008). Tumor specific $CD4^+$ T cells may provide anti-tumor immunity through diverse effects. Linda Sherman and colleagues showed that successful tumor elimination requires

tumor specific $CD4^+$ T cells rendering the tumor tissue permeable and enhancing cytotoxic activity of tumor reactive $CD8^+$ T cells (Bos and Sherman, 2010; Wong *et al.*, 2008). Moreover, it has been shown that $CD4^+$ T cells provide long-lasting tumor remission by sustaining $CD8^+$ T cell memory formation and preventing T cell exhaustion (Hunziker *et al.*, 2002; Moeller *et al.*, 2005; Shedlock and Shen, 2003). Additionally, tumor-specific $CD4^+$ T cells may directly provide cytotoxicity towards tumor cells (Xie *et al.*, 2010). However, using $CD4^+$ T cells in T cell therapy requires consideration of cytokine patterns of the transferred T cells to assure a sustained function of these T cells for anti-tumor immunity (Antony *et al.*, 2005) (see 3.2.2).

3.3.3 Application of Toll-like receptor (TLR) agonists as adjuvants

TLR ligands are potent activators of the innate immunity. TLR ligands are diverse conserved microbial molecules such as Lipopolysaccharide (LPS) or Flagellin present on the microbial cell surface. Synthetic nucleic acids mimicking microbial origin such as CpG oligodeoxynucleotides (CpG) or Poly (I:C) can similarly be recognized by distinct TLR. To date, 10 human TLR are well-described. These molecules are expressed on the cell surface or in the cell and show distinct expression patterns among immune cells (Dorner *et al.*, 2009; Schreibelt *et al.*, 2010; Zarember and Godowski, 2002). TLR ligands may not only induce a potent immune response against pathogens, but also break tolerance and therefore be an attractive immunotherapeutic agent or adjuvant for cancer vaccines (Kanzler *et al.*, 2007; Krieg, 2006). TLR ligands may achieve this by induction of proinflammatory cytokine secretion by DC supporting a Th1 response (Iwasaki and Medzhitov, 2004) and inhibiting Treg (Pasare and Medzhitov, 2003).

A well-established immunotherapeutic TLR ligand is Imiquimod. This synthetic TLR7 ligand is approved for topical application in treatment of basal carcinoma (Beutner *et al.*, 1999). Imiquimod provides anti-tumor immunity by sustaining a Th1 response against cancer cells. In 2007, Stary et al. showed that the effective treatment with Imiquimod does not only result from an improved Th1 induction by DC, but also by direct cytotoxicity of DC towards tumor cells (Stary *et al.*, 2007). Other TLR ligands are actually under investigation as immunotherapeutic agents. TLR9 ligand CpG is broadly investigated as immunotherapeutic agent and as adjuvant for vaccination (Krieg, 2006).

However, application of most TLR ligands has shown limited success in clinical trials (Krieg, 2007). Application of CpG as monotherapeutic treatment of several cancers demonstrated only partial success (Link *et al.*, 2006; Pashenkov *et al.*, 2006). In addition, CpG also did not meet expectations as adjuvant of vaccinations. Appay et al. observed an increase of Treg in

the tumor environment after vaccination with Melan-A peptide and CpG as adjuvant (Appay *et al.*, 2006). Actually, the role of TLR ligands with regard to Treg remains ambiguous since TLR ligands may either inhibit or activate this cell population (Conroy *et al.*, 2008).

3.4 Aim of the PhD thesis

The aim of this PhD project was the isolation and characterization of tumor-specific $CD4^+$ T cells from the self-restricted system for potential clinical application. In previous work, Formin-like protein 1 (FMNL1) has been shown to be an interesting tumor-associated antigen expressed in hematopoietic cells and overexpressed in CLL cells and lymphoblastoid cell lines (LCL) (Krackhardt *et al.*, 2002b). Thus, we used DC pulsed with FMNL1 peptides or protein for stimulation of autologous untouched peripheral blood mononuclear cells (PBMC) or autologous naïve T cells. We aimed to characterize in detail the reactivity of isolated T cells and TCR in response to tumor cells and healthy tissue in order to estimate their potential as candidates for novel anti-cancer treatments.

4 MATERIAL

4.1 Technical equipment

Centrifuge	Hettich, Kirchlengern, Germany
Counting chamber	Brand, Wertheim, Germany
Fluorescence Activated Cell Sorting (FACS) Calibur	Becton Dickinson and Company, New Jersey, USA
Fluorescence Microscope	Leica Microsystems, Wetzlar, Germany
Gammacell 40	Atomic energy of Canada limited, Ottawa, Kanada Acquisition date: 15.06.1979, 125,8 R/min Half-life: 30 years
γ-Counter (TopCount NXT)	Perkin Elmer, Waltham, USA
Gelelectrophoresis chamber	Harnischmacher, Kassel, Germany
Gelprint 2000i	Biophotonics, Ann Arbor, USA
Incubator	Heraeus Holding, Hanau, Germany
InGenius System IG-E	Synoptics, Cambridge, UK
LSR II	Becton Dickinson and Company, New Jersey, USA
Magnet agitator	Janke & Kunkel, Staufen, Germany
Microscope	Zeiss, Jena, Germany
MilliQ System	Millipore GmbH, Schwalbach, Germany
MoFlow™	Dako, Hamburg, Germany
Multichannel pipets	Eppendorf-Netheler-Hinz, Hamburg, Germany
Nanodrop ND-1000	PEQLAB Biotechnologie, Erlangen, Germany
OctoMACS separator	Miltenyi Biotech, Bergisch Gladbach, Germany
Pipetboy	Integra Biosciences, Fernwald, Germany
Pipets	Eppendorf-Netheler-Hinz, Hamburg, Germany
Sterile bench	BDK, Sonnenbühl-Genkingen, Germany
Sunrise Photometer	Tecan, Groot-Bijgaarden, Belgien
Thermocycler	Biometra, Göttingen
Thermomixer	Eppendorf, Hamburg
UV-Transilluminator	Ltf Labortechnik, Wasserburg, Germany
Vortexer	Bender & Hobe, Switzerland
Waterbath	Memmert, Schwabach, Germany

XCell II™ Blot Module	Invitrogen Corporation, Karlsruhe, Germany

4.2 Consumable supplies

Cell culture flasks	Becton Dickinson and Company, New Jersey, USA
Cryo tubes (1.8 ml)	Nunc, Langenselbold, Germany
EIA / RIA plates	Corning, New York, USA
Falcons (50 ml, 15 ml)	Becton Dickinson and Company, New Jersey, USA
Gloves latex	Rösner-Mautby Meditrade GmbH, Kiefersfelden, Germany
Gloves nitrile	Kimberly-Clark Corporation, Neenah, USA
Luminex Bio-Plex System	Bio-Rad, Munich, Germany
Luma plates	Perkin Ellmer, Waltham, USA
MS columns	Miltenyi Biotech, Bergisch Gladbach, Germany
Non treated PS 24 well plates	Becton Dickinson and Company, New Jersey, USA
Parafilm M	Brand, Wertheim, Germany
Petri dishes	Greiner Bio-One, Kremsmünster, Austria
Round bottom FACS tubes	Becton Dickinson and Company, New Jersey, USA
Sealing foil	Neolab, Heidelberg, Germany
Serological Pipets (5 ml, 10 ml, 25 ml)	Corning, New York, USA
Sterile filters	Techno Plastic Products, Trasadingen, Switzerland
Syringes 50 ml	Becton Dickinson and Company, New Jersey, USA
Tips (0.1–10 µl, 10–200 µl, 100–1000 µl)	Greiner Bio-One International AG, Kremsmünster, Austria
Tissue culture dishes (145 x 20mm)	Becton Dickinson and Company, New Jersey, USA
Tissue culture plates	Becton Dickinson and Company, New Jersey, USA
Tubes (1.5 ml)	Sarstedt, Nümbrecht, Germany
Tubes (1.5 ml, 0.5 ml)	Eppendorf-Netheler-Hinz, Hamburg, Germany

4.3 Reagents and chemicals

100 bp DNS ladder	New England Biolabs, Ipswich, UK
1-Bromo-3-Chloro-Propan	Sigma Aldrich Chemie, Taufkirchen, Germany
Agarose	Biozym, Oldendorf, Germany
AIM-V	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Ampicillin	Sigma Aldrich Chemie, Taufkirchen, Germany

$CaCl_2$	Merck, Darmstadt, Germany
Carboxyfluorescein succinimidyl ester (CFSE)	Invitrogen Corporation, Karlsruhe, Germany
CD19 MicroBeads	Miltenyi Biotech, Bergisch Gladbach, Germany
CD45RO MicroBeads	Miltenyi Biotech, Bergisch Gladbach, Germany
Chloroform-Isoamyl alcohol 24:1	Sigma Aldrich Chemie, Taufkirchen, Germany
Chloroquin	Sigma Aldrich Chemie, Taufkirchen, Germany
CpG	Invivogen, Toulouse, France
Cr^{51}	Hartmann Analytic, Brauschweig, Germany
Diethyl pyrocarbonate water (DEPC water)	Invitrogen Corporation, Karlsruhe, Germany
DMEM	Gibco, Invitrogen Corporation, Karlsruhe, Germany
DMSO	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
dNTP	Fermentas, St. Leon-Rot, Germany
D-PBS	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Dithiothreitol (DTT)	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Ethanol	Merck, Darmstadt, Germany
Ethidium Bromide	Merck, Darmstadt, Germany
F12-Medium	Sigma Aldrich Chemie, Taufkirchen, Germany
Far Red	Invitrogen Corporation, Karlsruhe, Germany
Fetal calf serum (FCS)	PAA Laboratories GmbH, Pasching, Austria
Ficoll	Biochrom, Berlin, Germany
Flagellin	Invivogen, Toulouse, France
Fungizone	Invitrogen Corporation, Karlsruhe, Germany
G 418	PAA Laboratories GmbH, Pasching, Austria
Gentamycine	PAA Laboratories GmbH, Pasching, Austria
Glucose	Merck, Darmstadt, Germany
H_3PO_4	Merck KGaA, Darmstadt, Germany
Heparine	B. Braun Melsungen, Germany
HEPES	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Human serum (HS)	Helmholtz Zentrum München, Munich, Germany
HyperLadder I	BIOLine GmbH, Luckenwalde, Germany
IMDM	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Insulin-Transferrin	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Isopropanol	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
Thermococcus kodakaraensis	

Polymerase (KOD)	Novagen, Merck, Darmstadt, Germany
Gel Loading Dye (6 x)	Fermentas, St. Leon-Rot, Germany
Lysogeny Broth (LB) Agar	Invitrogen Corporation, Karlsruhe, Germany
LB Broth Base	Invitrogen Corporation, Karlsruhe, Germany
L-Glutamine	Gibco, Invitrogen Corporation, Karlsruhe, Germany
LPS	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Milk powder	Frema, Lüneburg, Germany
Na_2CO_3	Merck, Darmstadt, Germany
Na_2HCO_3	Merck, Darmstadt, Germany
$Na_2HPO_4*2H_2O$	Merck, Darmstadt, Germany
NaCl	Merck, Darmstadt, Germany
NaN_3	Merck, Darmstadt, Germany
Ni-NTA Agarose	Qiagen, Hilden, Germany
Non Essential Amino Acids	Gibco, Invitrogen Corporation, Karlsruhe, Germany
NP 40	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
NuPAGE LDS sample buffer	Invitrogen Corporation, Karlsruhe, Germany
NuPAGE MOPS running buffer	Invitrogen Corporation, Karlsruhe, Germany
NuPAGE Novex BisTris Gel (4-12%)	Invitrogen Corporation, Karlsruhe, Germany
NuPAGE Transfer buffer	Invitrogen Corporation, Karlsruhe, Germany
Oligo(dT)-Primer	Promega, Madison, USA
OneShot TOP10	Invitrogen Corporation, Karlsruhe, Germany
Paraformaldehyde (PFA)	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
PBS Dulbecco's	Biochrom, Berlin, Germany
Penicilline Streptomycin,	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Pfu-Polymerase	Fermentas, St. Leon-Rot, Germany
Phenol, äquilibriert	Roth, Karlsruhe, Germany
Poly (I:C)	Invivogen, Toulouse, Germany
Precision Plus Standard All Blue	Bio-Rad, Munich, Germany
Propidiumiodide	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Protaminsulfate	MP Biomedicals, Illkirch, France
Restriction enzymes	New England Biolabs, Ipswich, UK
RetroNectin	TaKaRa, Japan
RNase Out	Invitrogen Corporation, Karlsruhe, Germany
RPMI 1640 –L-Glutamine	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Sheath fluid	Dako, Glostrup, Denmark
Sodium Pyruvate	Gibco, Invitrogen Corporation, Karlsruhe, Germany
S.O.C. medium	Invitrogen Corporation, Karlsruhe, Germany

Sterilium	Cutasept F, Bode Chemie, Hamburg, Germany
SuperScript II Rev. Transkriptase	Invitrogen Corporation, Karlsruhe, Germany
T4-Ligase	Fermentas, St. Leon-Rot, Germany
TAE (Tris Acetate Ethylenediamine-tetraacetic acid (EDTA)) Buffer	Invitrogen Corporation, Karlsruhe, Germany
Taq-Polymerase	Invitrogen Corporation, Karlsruhe, Germany
TMB Substrate Reagent Set	Becton Dickinson and Company, New Jersey, USA
Tri Reagent	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany
Trypan blue	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Trypsine EDTA 0,5 %	Gibco, Invitrogen Corporation, Karlsruhe, Germany
Tween 20	Sigma Aldrich Chemie GmbH, Taufkirchen, Germany

4.4 Cytokines, antibodies and peptides

Cytokines:

Interferone-γ (IFN-γ)	Peprotech, London, UK
IL-2	Chiron Vaccines International, Marburg, Germany
IL-4	Peprotech, London, UK
IL-6	Peprotech, London, UK
IL-7	Peprotech, London, UK
IL-15	Peprotech, London, UK
Tumor necrosis factor-α (TNF-α)	Peprotech, London, UK
CD40L	Peprotech, London, UK

Antibodies:

Antibodies	Clone	Isotype	Conjugation	Company
IgG 2b κ	27-35	IgG 2b κ	Fluorescein isothiocya-nate (FITC)	Becton Dickinson
IgG1 κ	MOPC-21	IgG1 κ	FITC	Becton Dickinson
IgG1 κ	MOPC-21	IgG1 κ	Phycoery-thrin (PE)	Becton Dickinson
α-human (α-h)CD3	UCHT1	IgG1 κ	FITC	Becton Dickinson
α-hCD4	RPA-T4	IgG1 κ	FITC	Becton Dickinson
α-hCD4	RPA-T4	IgG1 κ	Pacific Blue (Pac Blu)	Becton Dickinson

Antibodies	Clone	Isotype	Conjugation	Company
α-hCD5	BL1a	IgG2a	FITC	Becton Dickinson
α-hCD8	RPA-T8	IgG1 κ	PE	Becton Dickinson
α-hCD8	HIT8a	IgG1 κ	FITC	Becton Dickinson
α-hCD8	RPA-T8	IgG1 κ	Pac Blu	Becton Dickinson
α-hCD11c	HL-3	IgG1 κ	PE	Becton Dickinson
α-hCD14	M5E2	IgG2a	PE	Becton Dickinson
α-hCD19	HIB19	IgG1 κ	FITC	Becton Dickinson
α-hCD25	2A3	IgG1 κ	PE	Becton Dickinson
α-hCD28	CD28.2	IgG1 κ	PE	Becton Dickinson
α-hCD45RO	UCHL1	IgG2a	PE	Becton Dickinson
α-hCD56	B159	IgG1 κ	PE	Becton Dickinson
α-hCD80	MAB104	IgG1	PE	Immunotech (Coulter)
α-hCD83	HB15e	IgG1 κ	FITC	Becton Dickinson
α-hCD86	2331	IgG1 κ	PE	Becton Dickinson
α-hCD107a	H4A3	IgG1 κ	FITC	Becton Dickinson
α-hCD107b	H4B4	IgG1 κ	FITC	Becton Dickinson
α-hCD152	BNI3	IgG2a	PE	Acris
α-hCD178	NOK-1	IgG1 κ	PE	Biolegend
α-hFoxP3	3G3	IgG1	Allophyco-cyanin (APC)	Miltenyi Biotech
α-HLA-A,B,C	W6/32	IgG2a κ	-	Becton Dickinson
α-HLA-DR	L243	IgG2a κ	-	BioLegend
α-CD3	Okt-3	IgG1	-	
α-CD28	CD28.2	IgG1 κ	-	Becton Dickinson
goat -α-mouse IgG1 κ	-	-	PE	Invitrogen
α-hVβ9	FIN9	IgG1	PE	Immunotech (Coulter)
α-h αß TCR	T10B9.1 A-31	IgM κ	FITC	Becton Dickinson
α-mouse αß TCR (TCRmu)	H57-597	-	FITC	Beckman Coulter
TCRmu	H57-597	-	PE	Beckman Coulter

Table 4-1: Antibodies

Peptide library of FMNL1:

The peptide library included 142 peptides, covering the whole protein FMNL1 and was purchased from Biotrend, Cologne, Germany. The peptides were 16 amino acids long and had 8 aa overlap with the following peptide (Figure 4-1).

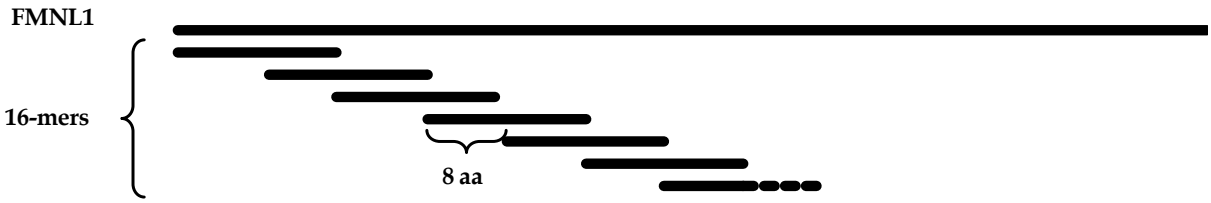

Figure 4-1: Peptide library of FMNL1

The peptide library used for T cell priming covers the whole FMNL1 protein and comprises 142 peptides with 16 aa length. The peptides have an overlap of 8 aa.

4.5 Kits

Dynabeads untouched human CD4 T cells	Invitrogen Corporation
Dynabeads untouched human CD8 T cells	Invitrogen Corporation
Human IFN-γ Enzyme-linked immunosorbent assay (ELISA) Set	Becton Dickinson and Company
Bio-Plex „Cytokine Reagent Kit"	Bio-Rad, Munich
Human Th1/Th2 11-Plex Flow Cytomix	Bender Medsystems
NucleoSpin Gel purification kit	Macherey Nagel
JETstar Plasmid Purification MAXI KIT	Genomed
Silver Quest Silver Staining Kit	Invitrogen Corporation
BCA (Bicinchoninic Acid) Protein Assay Kit	Pierce
FoxP3 Detection Kit	Miltenyi Biotec

4.6 Buffers and solutions

Buffer / Solution	Additives	Stock concentration	End concentration	Volume
FACS buffer	D-PBS			500 ml
	Δ FCS	100 %	1 %	5 ml
Δ FCS or Δ HS	Heat inactivation:			

Buffer / Solution	Additives	Stock concentration	End concentration	Volume
	20 min at 58 °C			
0,1 M Sodium-carbonate buffer [ELISA]	$NaHCO_3$	84.01 g/mol	0.1 M	8.40 g
	Na_2CO_3	105.99 g/mol	0.03 M	3.56 g
	H_2O pH 9,5			add 1 l
Washing buffer [ELISA]	PBS	10 x	1 x	2 L
	Tween-20	100%	0.05 %	1 ml
2x HBSS-buffer [Ca-phosphate transfection]	NaCl	58.44 g/mol	280 mM	1.64 g
	KCl	74.56 g/mol	10 mM	0.075 g
	Glucose	180.16 g/mol	12 mM	0.216 g
	HEPES	238.31 g/mol	50 mM	1.1916 g
	$Na_2 HPO_4*2H_2O$	177.99 g/mol	1.5 mM	0.0267 g
	H_2O dest			add 100 ml
$CaCl_2$ [Ca-phosphate transfection]	$CaCl_2$	110.99 g/mol	2 M	2.22 g
	H_2O bidest			10 ml
Milk powder [ELISA]	Milk powder	100 %	1 %	1 g
	PBS			100 ml
Milk powder [Western Blot]	Milk powder	100 %	5 %	5 g
	PBS			100 ml
DNA agarose gel	TAE buffer	10x	1x	20 ml
	H_2Odest			add 120 ml
	Agarose	100 %	1,7 %	2 g
	Ethidiumbromide	10 µg/µl	0.7 µg/ml	8 µl
Lysis buffer [Protein purification]	Tris HCl pH 7.4	1 M	50 mM	2.5 ml
	NaCl	1 M	150 mM	6.67 ml
	Imidazol	68,08 g/mol	10 mM	0.034 g
	NP40	100 %	0.5 %	0.25 g
	PMSF	0.1 M	1 mM	0.5 ml
	Complete	25 x	1 x	2 ml
	H_2O dest			Add 50 ml
Elution buffer [Protein purification]	Imidazol	0.5 M	0.5 M	950 µl
	PMSF	100 mM	1 mM	10 µl
	Complete	25 x	1 x	40 µl
RIPA buffer [Western Blot]	Tris HCl pH 7.4	1 M	10 mM	1 ml
	NaCl	1 M	10 mM	1 ml
	Sodium dodecyl sulfate (SDS)	100 %	1 %	1 ml
	NP40	100 %	1 %	1 ml
	Deoxycholic	100 %	0,5 %	0,5 ml
	H_2O dest			Add 100 ml
TBST	TBS buffer	10 x	1 x	2 L
	Tween 20	100%	0.05 %	1 ml
Lysis buffer	RIPA buffer			4750 µl

Buffer / Solution	Additives	Stock concentration	End concentration	Volume
[Western Blot]	PMSF	0.1 M	1 mM	200 µl
	Complete	25 x	1 x	50 µl

Table 4-2: Buffers and solutions
Buffers and solutions used for ELISA, transfection, western blot and protein purification.

4.7 Cells and cell culture media

4.7.1 Primary cells and donors

Cells	Description	Culture medium	Source
PBMC	Peripheral blood leukocytes, recovered from whole blood by Ficoll density centrifugation	T cell medium	Healthy donors
B cells	Recovered by CD19 MicroBead labelling and sorting through a coloumn	B cell medium	Healthy donors
DC	Recovered by plate adherence of monocytes with subsequent treatment with cytokines to finally recover mature DC	DC medium	Healthy donors
CLL	PBMC of patients with chronic lymphatic Leukemia, containing 50 % to 95 % CD5+CD19+ cells	DC medium	Hämato-Onkologische Praxis Prof. Dr. C. Salat, Munich
PBMC pool	PBMC pooled from at least four healthy male caucasic donors; Usage for restimulation of T cells and cloning by limiting dilution	T cell medium	Institute of Molecular Immunology, Helmholtz Zentrum München

Table 4-3: Primary cells

Donor	HLA-DRB1 alleles
I	0101, 1101
II	01, 15

Table 4-4: HLA-DR types of blood donors

4.7.2 Cell lines

Cell line	Description	Culture medium	Source / Reference
BJAB	EBV-negative Burkitt lymphoma cell line	RPMI (10 % Δ FCS)	Joseph Mautner, Helmholtz Zentrum München
C1R	human plasma leukemia cell line	RPMI (10 % Δ FCS)	S. Stevanovic
TCD40L-fibroblasts	NIH3T3 fibroblasts, expressing CD40L	TCD40L-Medium with 400 µg/ml G418	Boston, USA (Krackhardt *et al.*, 2002a)
CD40L	Murine fibroblasts	B cell medium	Andreas Moosmann, Helmholtz Zentrum München
293T	Human embryonic kidney cells	DMEM (10 % Δ FCS)	Joseph Mautner, Helmholtz Zentrum München
293A	Human embryonic kidney cells**,** transformed with sheared human adenovirus type 5 DNA	DMEM (10 % Δ FCS)	Invitrogen
LCL	EBV-transformed B cell lines	RPMI (10 % Δ FCS)	Joseph Mautner, Helmholtz Zentrum München

Table 4-5: Cell lines

4.7.3 Cell culture media

Medium	Additives	Stock concentration	End concentration	Volume
Freezing medium	Δ FCS	100 %	90 %	1.62 ml
	DMSO	100 %	10 %	180 µl
Medium for TCD40L fibroblasts	F12 + DMEM			500 ml
	Δ FCS	100 %	10 %	50 ml
	L-Glutamine	200 mM	2 mM	5 ml
	Non essential amino acids	100 x	1 x	5 ml
	HEPES; pH 7,4	1 M	10 mM	5 ml
	Gentamycine	10 mg/ml	16.6 µg/ml	0.83 ml
	G418	50 mg/ml	400 µg/ml	4 ml
RPMI (10 % Δ FCS)	RPMI-1640 – L-Glutamine			500 ml
	Δ FCS	100 %	10 %	50 ml
	L-Glutamine	200 mM	2 mM	5 ml
	Non essential amino acids	100 x	1 x	5 ml
	Sodium-Pyruvate	100 mM	1 mM	5 ml
	Penicillin-Streptomycin	P.: 10000 U/ml S.: 10000 µg/ml	P.: 100 U/ml S.: 100 µg/ml	5 ml

Medium	Additives	Stock concentration	End concentration	Volume
B cell medium	RPMI-1640 – L-Glutamine			500 ml
(also used for	L-Glutamine	200 mM	2 mM	5 ml
CD40L culture)	Sodium Selenite	1000000 x	1 x	0.5 µl
	Penicillin-Streptomycin	P.: 10000 U/ml S.: 10000 µg/ml	P.: 100 U/ml S.: 100 µg/ml	5 ml
DC medium	IMDM			500 ml
	Insulin-Transferrin	2.5 mg/1,5 ml	50 µg/ml	1.5 ml
	Δ HS	100 %	10 %	50 ml
	L-Glutamine	200 mM	2 mM	5 ml
	Gentamycine	10 mg/ml	16.6 µg/ml	0.83 ml
T cell medium	RPMI-1640 – L-Glutamine			500 ml
	Δ HS	100 %	10 %	50 ml
	L-Glutamine	200 mM	2 mM	5 ml
	Non essential amino acids	100 x	1 x	5 ml
	Sodium-Pyruvate	100 mM	1 mM	5 ml
	Penicillin-Streptomycin	P.: 10000 U/ml S.: 10000 µg/ml	P.: 100 U/ml S.: 100 µg/ml	5 ml
	HEPES; pH 7,4	1 M	10 mM	5 ml
	Gentamycine	10 mg/ml	16.6 µg/ml	0,83 ml
DMEM	DMEM (4,5 g/l Glukose)			500 ml
(10 % or	Δ FCS	100 %	10 % oder 3 %	50 ml/15 ml
3 % Δ FCS)	L-Glutamin	200 mM	2 mM	5 ml
	Non essential amino acids	100 x	1 x	5 ml
	Sodium-Pyruvat	100 mM	1 mM	5 ml
	Penicillin-Streptomycin	P.: 10000 U/ml S.: 10000 µg/ml	P.: 100 U/ml S.: 100 µg/ml	5 ml

Table 4-6: Cell culture media
Cell culture media used for culturing different cell lines and primary cells.

4.8 DNA vectors, enzymes and primer

4.8.1 Vectors

Vector	Description	Resistance	Source
peGFP	Control vector with green fluorescent protein (GFP)	Ampicilline	Clontech, Saint-Germain-en-Laye, France
pCMV-CHis	Vector bearing a potent cytomegalovirus vector (CMV) and a poly histidine sequence	Ampicilline	
pALF10A1 (“env”)	10A1 virus envelope gene of the murine Leukemia virus	Ampicilline	Wolfgang Uckert (Stitz *et al.*, 2000)
pcDNA3.1-MLV g/p	Group specific antigen and	Ampicilline	Wolfgang Uckert,

Vector	Description	Resistance	Source
("gag-pol")	polymerase genes		Berlin, Chrisopher Baum, Hannover
pMP71G$_{PRE}$	Retroviral vector with eGFP reporter gene	Ampicilline	Wolfgang Uckert, (Engels *et al.*, 2003)
with insert:			
pCMV-CHis FMNL1β	pCMV-CHis with the β isoform of FMNL1	Ampicilline	Kindly provided by E. Eppinger
pMP71G$_{PRE}$ Aa2.2α	pMP71G$_{PRE}$ TCR Aa2.2 α-chain, without eGFP	Ampicilline	Constructed in this work
pMP71G$_{PRE}$ Aa2.2β	pMP71G$_{PRE}$ TCR Aa2.2 β-chain, without eGFP	Ampicilline	Constructed in this work
pMK-RQ	pMK-RQ with TCR Aa2.2 β-chain-P2A-α-chain	Kanamycine	GeneArt
pMP71G$_{PRE}$ and Aa2.2 βP2Aα	pMP71G$_{PRE}$ TCR Aa2.2 β-chain-P2A-α-chain, without eGFP	Ampicilline	Constructed in this work
pINCO GFP	pINCO with GFP	Ampicilline	Kindly provided by J. Mautner
pCMV-CHis and HLA-DRB1*1	pCMV-CHis with human HLA-DRB1*1, without GFP. Isolation from an HLA-DRB1*1 monoallelic LCL kindly provided by J. Mautner	Ampicilline	Constructed in this work
pINCO and HLA-DRB1*1	pINCO with human HLA-DRB1*1, without GFP.	Ampicilline	Constructed in this work
pINCO and HLA-DRB1*11	pINCO with human HLA-DRB1*11, without GFP	Ampicilline	Kindly provided by J. Mautner

Table 4-7: Vectors

4.8.2 Enzymes

Enzymes were provided from New England Biolabs and Fermentas

- Restriction enzymes: BamHI, EcoRI, MfeI, NotI, NotI-HF, XhoI
- T4 Ligase
- KOD Polymerase
- Taq Polymerase

4.8.3 Primer

Primers were supplied at Sigma-Aldrich, Taufkirchen, Germany.
All primers are indicated in 5’ → 3’ direction.

4.8.3.1 Primer for EBV analysis

Primer for the chloramphenicol-acetyltransferase on the mini-EBV vector:
cam up: 5’ – TTC TGC CGA CAT GGA AGC CAT C – 3’
cam down: 5’ – GGA GTG AAT ACC ACG ACG ATT TCC – 3’
Primer for glycoprotein 85 expressed by wildtype EBV:
gp85c: 5' – TGG TCA GCA GCA GAT AGT GAA CG – 3'
gp85d: 5' – TGT GGA TGG GTT TCT TGG GC – 3'
Primer for beta globin (GH20) expressed by human cells:
GH20: 5’ – GAA GAG CCA AGG ACA GGT AC – 3'
PC04: 5' – CAA CTT CAT CCA CGT TCA CC – 3'

4.8.3.2 Primer of the Vαβ Repertoire

Nomenclature of primers follows the nomenclature of B. Arden (Arden *et al.*, 1995).
TCR-α-chain – repertoire (Steinle *et al.*, 1995)
Concentration: 2,5 pmol/µl, unless otherwise noted:

P-5’αST	CTG TGC TAG ACA TGA GGT CT	
P-3’αST	CTT GCC TCT GCC GTG AAT GT	
3’T-Cα	GGT GAA TAG GCA GAC AGA CTT GTC ACT GGA	c = 5 pmol/µl
PANVα1	AGA GCC CAG TCT GTG ASC CAG	S = C/G
PANVα1.1	AGA GCC CAG TCR GTG ACC CAG	R = A/G
Vα2	GTT TGG AGC CAA CRG AAG GAG	
Vα3	GGT GAA CAG TCA ACA GGG AGA	
Vα4	TGA TGC TAA GAC CAC MCA GC	
Vα5	GGC CCT GAA CAT TCA GGA	
Vα6	GGT CAC AGC TTC ACT GTG GCT A	
Vα7	ATG TTT CCA TGA AGA TGG GAG	
Vα8	TGT GGC TGC AGG TGG ACT	
Vα9	ATC TCA GTG CTT GTG ATA ATA	
Vα10	ACC CAG CTG CTG GAG CAG AGC CCT	
Vα11	AGA AAG CAA GGA CCA AGT GTT	
Vα12	CAG AAG GTA ACT CAA GCG CAG ACT	
Vα13	GAG CCA ATT CCA CGC TGC G	

Vα14.1	CAG TCC CAG CCA GAG ATG TC
Vα14	CAG TCT CAA CCA GAG ATG TC
Vα15	GAT GTG GAG CAG AGT CTT TTC
Vα16	TCA GCG GAA GAT CAG GTC AAC
Vα17	GCT TAT GAG AAC ACT GCG T
Vα18	GCA GCT TCC CTT CCA GCA AT
Vα19	AGA ACC TGA CTG CCC AGG AA
Vα20	CAT CTC CAT GGA CTC ATA TGA
Vα21	GTG ACT ATA CTA ACA GCA TGT
Vα22	TAC ACA GCC ACA GGA TAC CCT TCC
Vα23	TGA CAC AGA TTC CTG CAG CTC
Vα24	GAA CTG CAC TCT TCA ATG C
Vα25	ATC AGA GTC CTC AAT CTA TGT TTA
Vα26	AGA GGG AAA GAA TCT CAC CAT AA
Vα27	ACC CTC TGT TCC TGA GCA TG
Vα28	CAA AGC CCT CTA TCT CTG GTT
Vα29	AGG GGA AGA TGC TGT CAC CA
Vα30	GAG GGA GAG AGT AGC AGT
Vα31NEU	TCG GAG GGA GCA TCT GTG ACT A
Vα32	CAA ATT CCT CAG TAC CAG CA

TCR-β-chain – degenerated Primer (Zhou *et al.*, 2006a)
Concentration: 5 pmol/µl

VP1	GCI ITK TIY TGG TAY MGA CA
VP2	CTI TKT WTT GGT AYC IKC AG
CP1	GCA CCT CCT TCC CAT TCA C

4.8.3.3 Primer for TCR cloning

The restriction enzyme sites are highlighted in red. The Kozak sequence in front of the targeted gene sequence is marked in blue.

<u>Aa2.2 Alpha chain:</u>

5’ TRAV6-1 NotI: 5’ – TAGCGGCCGCACCATGGAGTCATCCCTGGGAGGTG – 3’

3’ TRAC MfeI: 5’ – TGCAATTGTCAGCTGGACCACAGCCGCAGC – 3’

<u>Aa2.2 Beta chain:</u>

5’ TRBV3-1 NotI: 5’ – TAGCGGCCGCACCATGGGCTGCAGGCTCCTCTGCTG – 3’

3’ TRBC2 EcoRI: 5’ – TGGAATTCCTAGCCTCTGGAATCCTTTCTCTTG – 3’

Bb5.14 Alpha chain:

5’ TRAV29/DV5 NotI: 5’ – TAGCGGCCGCACCATGCTCCTGGGGGCATCAG – 3’

3’ TRAC EcoRI: 5’ – TGGAATTCTCAGCTGGACCACAGCCGCAGC – 3’

Bb5.14 Beta chain:

5’ TRBV6-4 NotI: 5’ – TAGCGGCCGCACCATGAGAATCAGGCTCCTGTG – 3’

3’ TRBC2 EcoRI: 5’ – TGGAATTCTCAGAAATCCTTTCTCTTGACC – 3’

4.8.3.4 Primer for cloning of HLA-DRB1*1

HLA-DRB1*1:

5’ HLA-DRB1*1 BamHI: 5’ – TAGGATCCACCATGGTGTGTCTGAAGCTCCCTG – 3’

3’ HLA-DRB1*1 XhoI: 5’ – TGCTCGAGTCAGCTCAGGAATCCTGTTGGCTG – 3’

4.9 Computer and Online programs

Analysis of flow cytometric data:

FlowJo, Tree Star, Ashland, USA

Analysis of DNA data:

Sequencher 4.5, Gene codes, Ann Arbor, USA

Clone Manager 7, Scientific & Educational Software, Cary, USA

Analysis of TCR sequences:

IMGT - http://imgt.cines.fr

Analysis of Flow Cytomix Data:

Flow Cytomix Pro 2.4

Prediction algorithms:

HLA-DR binding motifs:

www.syfpeithi.de

http://www.imtech.res.in/raghava/propred/

Proteasomal sites:

www.paproc2.de

5 METHODS

5.1 Cell culture methods

5.1.1 General cell culture methods

Cells were cultured at 37°C, 5 to 6 % CO_2 and 95 % humidity in incubators. Cell culture was performed under sterile conditions and work with retroviruses, EBV^+ cell lines or human blood was accomplished under biosafety level 2.

5.1.1.1 Thawing and freezing of cells

For freezing, cells were centrifuged at 1500 rpm for 5 min, taken up in 1800 µl Δ FCS 10 % DMSO and kept in -80 °C freezer for one day before long term storage in N_2.
For thawing, cells were shortly incubated at 37°C in the water bath and then quickly taken up in Medium, centrifuged (1500 rpm, 5 min) and resuspended in the corresponding culture medium.

5.1.1.2 Culture of cell lines

Suspension and adherent cell lines were split every 3 to 4 days. For adherent cell splitting, old medium was removed and cells were washed with 5-10 ml PBS. Then, cells were incubated for 3 to 10 minutes at room temperature (RT) or at 37°C in trypsine to remove the cells from the cell culture flask surface. After transfer in a 50 ml tube, cells were washed with medium and centrifuged for 5 min at 1500 rpm. After resuspension in fresh medium, cells were recultured in a new cell culture flask.

5.1.1.3 Cell counting

To determine the cell number, cells were diluted in trypan blue. Trypan blue can only stain dead cells, since the compound cannot penetrate intact cell membranes. Therefore, it is

possible to distinguish unstained bright alive cells from blue dead cells. Living cells were counted under the microscope in four squares of a Neubauer counting chamber. Cell concentrations were then calculated as follows:

$$c\ [\text{cells/ml}] = \text{number of cells} / \text{Number of squares} \cdot \text{dilution factor} \cdot 10^4$$

5.1.2 PBMC isolation from whole blood

PBMC were isolated from whole blood from healthy donors by Ficoll density gradient centrifugation. Therefore, blood was diluted 1:1 with RPMI and 35 ml were carefully pipetted on 15 ml Ficoll per 50 ml tube and centrifuged for 30 min at 2000 rpm with low acceleration and without brake. Then PBMC were extracted from the buffy coats and transferred in new 50 ml tubes and washed twice with RPMI. Thereafter, cells were resuspended in T cell medium, in DC medium for plate adherence (see 5.1.3), in PBS with 2 % ΔFCS for sorting with a Dynabeads Kit (see 4.5) to isolate $CD4^+$ or $CD8^+$ T cells following manufacturers' instructions or for sorting with MACS Beads (see 4.3) to isolate $CD19^+$ or $CD45RO^-$ cells following the manufacturers' instructions.

5.1.3 Dendritic cell and macrophage enrichment

In a first step, monocytes ($CD14^+$ cells) were enriched by plate adherence (Day 0). Therefore, PBMC were resuspended in DC medium and plated in 6 well plates with 3 ml total volume at a concentration of 5 x 10^6 cells/ml. The plates were incubated for 2h in the incubator, for adherence of monocytes. During this time, two aliquots of DC medium were prewarmed at 37°C for washing and culturing of the monocytes. After two hours of incubation, wells were carefully rinsed with the medium. This medium was removed and replaced by prewarmed new medium for another rinsing step in order to remove non-adherent lymphocytes. Finally, this medium was also removed and replaced by fresh prewarmed medium. To support differentiation of monocytes towards DC, IL-4 (c = 20 ng/ml) and GM-CSF (c = 100 ng/ml) were added to the medium. To induce differentiation towards macrophages, medium was supplemented either with 20 ng/ml human M-CSF for M1 or 100 ng/ml human GM-CSF for M2 macrophages. At day 3, the same cytokines were added to the cultures. On day 6 DC were matured by addition of 15 ng/ml IL-6, 10 ng/ml TNF-α, 10 ng/ml IL-1β and macrophages were polarized to M1 or M2 macrophages by addition of 100 U/ml IFN-γ and 1 ng/ml LPS for M1 or 20 ng/ml IL-4 for M2 (Leidi *et al.*, 2009). Cells were ready to use on day 7.

5.1.4 Enrichment and culture of EBV$^-$ B cells and mini-LCL

For unlimited availability of autologous professional antigen presenting cells, we established B cell lines from the same donor as for the generation of reactive T cell lines.

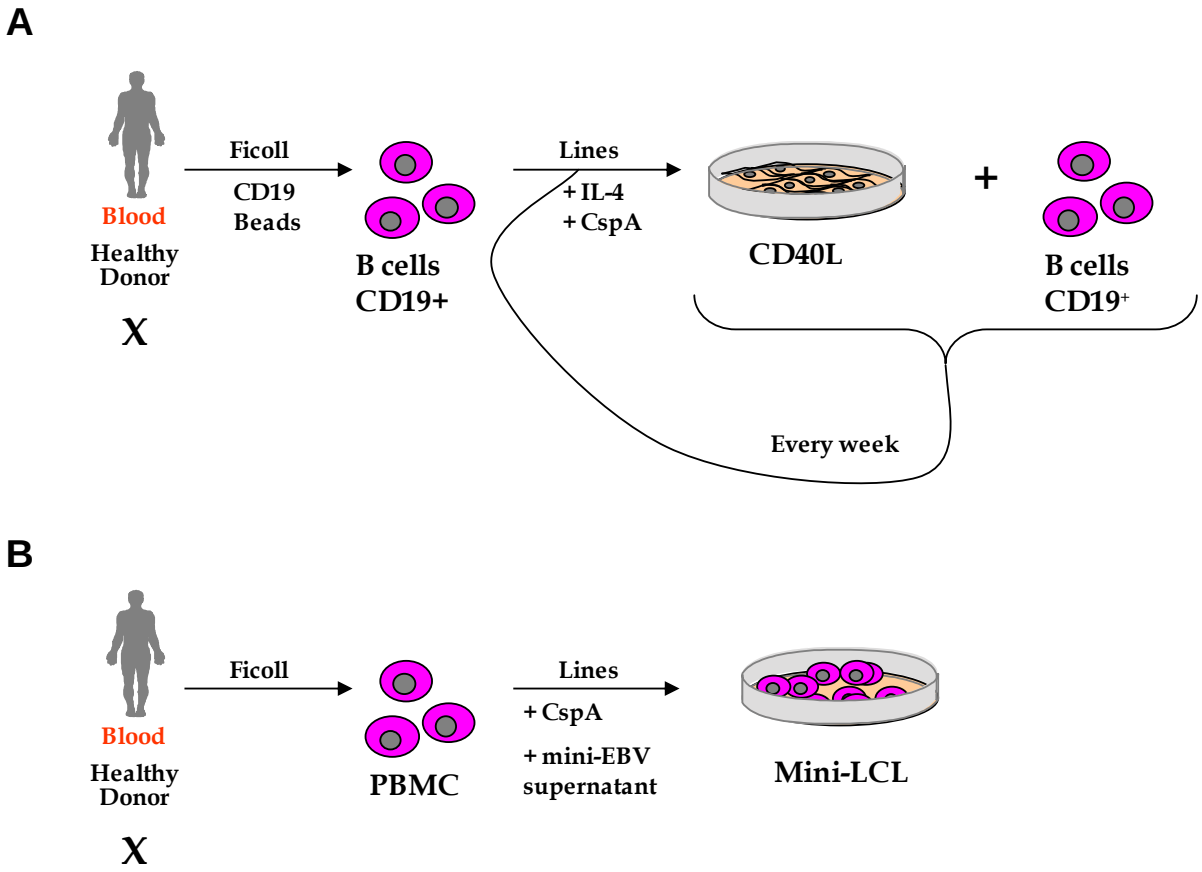

Figure 5-1: Generation of autologous B cell lines

(A) EBV$^-$ B cell lines were isolated from sorted B cells and were cultured with IL-4 on CD40L expressing fibroblasts. (B) PBMC were transduced with Mini-EBV particles to generate Mini-LCL.

5.1.4.1 Generation and culture of EBV$^-$ B cell lines

EBV$^-$ B cell lines were generated as first described by Wiesner et al. (Wiesner *et al.*, 2008). Therefore, CD40L-transfected fibroblasts were irradiated with 10000 rad and per 96 well flat bottom plate 1 x 10^6 fibroblasts were plated out in 200 µl / well. Two days later, PBMC were isolated as previously described (see 5.1.2) and B cells were further isolated by CD19 Micro Bead labelling and magnetic bead enrichment following the manufacturers' instructions (Miltenyi Biotec). B cells were then counted and plated out in different concentrations on CD40L feeder cells. 2500, 5000, 10000 or 250000 cells were plated on CD40L feeder cells with 2 ng/ml IL-4 and 1 µg/ml Cyclosporine A (CspA) (Figure 5-1). After 1 week, cells were

restimulated with CD40L and IL-4. CD40L feeder cells were prepared and plated out in fresh 96 well flat bottom plates. CspA was added at the time point of each restimulation for 6 to 8 weeks in order to remove remaining T cells from the B cell culture. As soon as B cell lines were expanded to 4 to 6 wells of a 96 well plate, wells were rejoined in one well of a 12 well plate containing CD40L feeder.
After approximately one month of culture, the B cell lines were analysed for EBV expression by polymerase chain reaction (PCR) (see 5.3.2.1). In case of EBV negativity, B cells were used for T cell stimulation assays.

5.1.4.2 Generation and culturing of Mini-LCL lines

PBMC were isolated as previously described (see 5.1.2). 0.5 x 10^6 cells were then plated out in a 96 well flat bottom plate in 100 µl medium/well containing CspA (1 µg/ml). Thereafter, 100 µl of Mini-EBV supernatant was added (Figure 5-1). The next day, 100µl of supernatant was removed and replaced by 100 µl CspA (1 µg/ml) containing medium. Twice a week, half of the medium was exchanged. As soon as Mini-LCL aggregates were visible, cells were expanded. After expansion of Mini-LCL, expression of EBV and marker for the Mini-EBV plasmid (chloramphenicol-acetyltransferase) were analysed by PCR (see 5.3.2.2).
Wildtype EBV^- and chloramphenicol-acetyltransferase$^+$ Mini-LCL lines were expanded and frozen until use. Mini-LCL lines were kindly provided by A. Moosmann.

5.1.5 Protein, peptide or TLR pulsing of PBMC, EBV^- B cells and Mini-LCL

PBMC, EBV^- B cells and Mini-LCL were pulsed with peptide, protein or TLR ligand. Cells were counted and taken up in AIM-V medium with a final concentration of 2 x 10^6 cells / ml. Then, either 1 µg/ml FMNL1 protein or 10 µmol/ml peptide pool were added to the cells. In case of TLR ligands, cells were treated with 1 µg LPS, Flagellin, Poly (I:C) or 0.5 µM CpG oligodeoxynucleotides (CpG) were added to the cells. Cells were incubated for 2h at 37 °C in the incubator. Thereafter, cells were washed and taken up in the appropriate volume of the adequate medium.

5.1.6 CLL Culture

CLL cells were cultured in 6 well plates on irradiated (90 Gy) TCD40L cells and addition of IL-4 (5 ng/ml). CLL cells were activated by CD40 stimulation for 3 days before usage in stimulation assays.

5.1.7 Transfection of T293 with FMNL1

293T were plated out 16h before transfection in a concentration of 1.5 x 10^6 in a cell culture dish (145 x 20 mm). On the next day, 2.22 g $CaCl_2$ were solved in 10 ml aqua bidest and 10 ml of 2 x HBSS buffer were adjusted with NaOH (5 M) to pH 7.06. Both solutions were filtered through a 0.22 µm filter. 293T medium was changed to medium, containing 3 % ΔFCS. Thereafter, target DNA was solved in 1 ml H_2O containing 200 mM $CaCl_2$, 0.25 µM chloroquine and 20 µg plasmid.
To produce complexes for transfection, the prepared plasmid mix was added dropwise into 1 ml 2 x HBSS buffer, which was agitated at the same time by producing bubbles with a pipet. This mixture was then incubated for 30 min at RT, before it was added dropwise to 293T cells. After slightly swaying the dishes, cells were incubated for 6h in the incubator. Finally, after again slightly swaying the plates, the medium with the complexes was carefully removed and replaced by normal medium (DMEM with 10% ΔFCS). After 48 h, cells were collected by knocking on the dishes to remove the cells from the plate. Cells were washed twice in PBS and counted. 1 x 10^6 cells were used for FACS analysis and 5 x 10^6 cells were stored as pellets at -80 °C for Western blot (see 5.2.3). The remaining transfected cells were used for protein purification (see 5.2.1).

5.1.8 $CD4^+$ T cell culture

5.1.8.1 T cell stimulation

DC were generated as previously described (see 5.1.3). On day 5 of DC culture, cells were scraped from 6 well plates and were replated on 48 well plates with 0.05 x 10^6 cells / 0.5 ml / well. 1 µg/ml FMNL1 (Table 5-1B) or alternatively 10 µmol/ml of the peptide pools (Table 5-1A / B) were added per well. Remaining DC were frozen at -80 °C for subsequent restimulations.

On day 6, cultures were supplemented either with cocktail A (50 U/ml IFN-γ, 10 ng/ml TNF-α, 1 µg/ml CD40L) or cocktail B (15 ng/ml IL-6, 10 ng/ml TNF-α, 10 ng/ml IL-1β).

A.

Pool	Peptides
A	1-20
B	21-40
C	41-60
D	61-80
E	81-100
F	101-121
G	122-142

B.

Stimulation with	Stimulation of					
	a. PBMC			b. $CD45RO^-$		
DC cocktail A + FMNL1	1	2	3	4	5	6
DC cocktail B + FMNL1	1	2	3	4	5	6
DC cocktail B + Pool A	1	2	3	4	5	6
DC cocktail B + Pool B	1	2	3	4	5	6
DC cocktail B + Pool C	1	2	3	4	5	6
DC cocktail B + Pool D	1	2	3	4	5	6
DC cocktail B + Pool E	1	2	3	4	5	6
DC cocktail B + Pool F	1	2	3	4	5	6
DC cocktail B + Pool G	1	2	3	4	5	6

Table 5-1: Peptide pools and stimulation conditions for generation of FMNL1-reactive T cell lines

(A) Constitution of FMNL1 peptide pools: each peptide pool comprised 21 to 22 peptides. (B) PBMC (a.) or $CD45RO^-$ (b.) cells were primed with DC matured with cocktail A (IFN-γ, TNF- α, CD40L) or B (IL-6, TNF-α, IL-1β) and pulsed previously with FMNL1 protein or one of the FMNL1 peptide pools. Three lines were prepared for each condition.

On day 7, PBMC of the same donor were isolated and $CD45RO^-$ cells were isolated using the flow through after magnetic bead depletion of $CD45RO^+$ cells following the manufacturers' instructions (Miltenyi Biotec). 0.5 x 10^6 PBMC or $CD45RO^-$ cells were plated on the DC after removal of DC medium using 800 µl T cell medium per well. The ratio of DC : PBMC was chosen 1 : 10. This procedure was repeated for conditions stimulated with FMNL1 protein every 7 days using previously frozen DC samples at a DC : PBMC ratio of 1:100. In case of conditions stimulated with the peptide pool, autologous PBMC were thawn, pulsed with the respective peptide pools and irradiated with 6000 rad. Finally 1 x 10^6 cells were added per well.

5.1.8.2 T cell cloning by limiting dilution

0.1 x 10^6 PBMC / well originating from the PBMC Pool (see 4.7.1) were irradiated with 60 Gy and used as feeder for T cell cloning. In addition, Mini-LCL (0.01 x 10^6 cells/ well) derived from the same donor as the T cell lines as well as Mini-LCL (0.01 x 10^6 cell / well) derived from another donor were pulsed with protein or the corresponding peptide pool (Table 5-1) and irradiated with 10000 rad. For cloning, T cell lines were plated out in a concentration of 1 cell / well together with the PBMC Pool and Mini-LCL for stimulation. IL-2 (50 U / ml), IL-7 (5 ng / ml) and IL-15 (5 ng / ml) were added to the culture. T cell clones were restimulated in a similar way every 14 days. Il-2 (50 U / ml) was added every 3 to 4 days.

5.1.9 Unspecific stimulation of PBMC, $CD4^+$ and $CD8^+$ T cells

5.1.9.1 Activation of PBMC using IL-2 and α-CD3

PBMC were activated after isolation (5.1.2) by culturing in T cell medium complemented with IL-2 (50 U/ml) and with α-CD3 (50 ng/ml). 1 x 10^6 were plated out in 24 well plates and incubated for 2 days before transduction (5.5.2) and for 3 days when used as targets in stimulation assays (5.7.1).

5.1.9.2 Activation of $CD4^+$ and $CD8^+$ T cells with IL-2, α-CD3 and α-CD28

$CD4^+$ and $CD8^+$ T cells were isolated by negative isolation using magnetic beads (see 4.5) following the manufacturers' instructions (Invitrogen). Before transduction (5.5.2), cell populations were activated in 24 well plates coated with α-CD3 and α-CD28 antibodies. For coating, untreated 24 well plates were incubated for 2h at 37 °C with 0.5 ml PBS supplemented with α-CD3 (5 µg/ml) and α-CD28 (1 µg/ml). Thereafter, blocking was performed using 0.5 ml 2 % Bovine Serum Albumine (BSA) per well for 30 min at 37 °C. Finally, wells were washed with PBS and cells were plated on the wells in T cell medium supplemented with 50 U/ml IL-2. Cells were activated for 2 days.

5.2 Biochemical Methods

5.2.1 FMNL1 purification

293T cells transfected with FMNL1 (see 5.1.7) were centrifuged with 1500 rpm for 10 min. The pellet was vortexed and resuspended in 20 ml lysis buffer (see 4.6). Cells were destroyed in an ultrasonic bath for 2 min. To remove cell membranes and nucleic acids, lysates were centrifuged for 20 min at 5000 rpm and 4°C. During this time 0.5 ml Ni-NTA agarose was washed in lysis buffer in a 50 ml tube. The supernatant of the lysate was transferred to the tube with the Ni-NTA agarose and shaked overhead at 4°C over night (Figure 5-2).

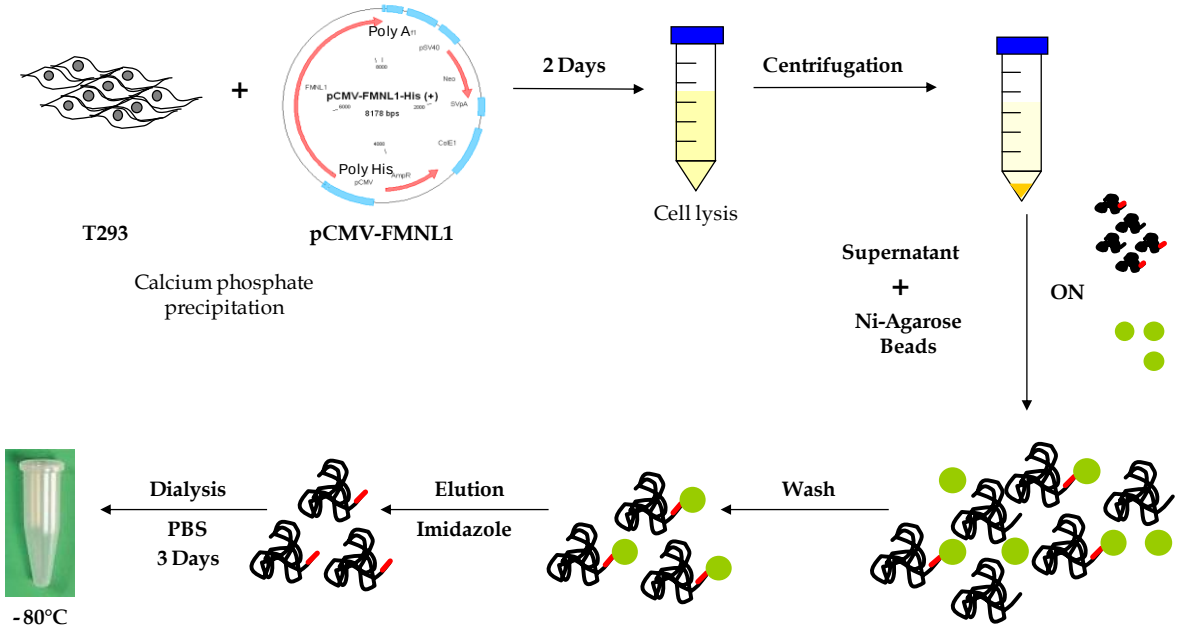

Figure 5-2: FMNL1 His Tag purification

FMNL1 was expressed in 293T cells. 2 days later, these cells were lysed and recombinant FMNL1 bearing a C-terminal His Tag sequence was bound by Ni-NTA agarose beads specific for the His-tag sequence. Beads were then washed to remove irrelevant proteins. FMNL1 was released from the beads through Imidazole treatment. Since Imidazole is toxic, the protein was dialysed for 3 days in PBS. Finally FMNL1 was aliquotted and stored at -80°C.

The next day, the probe was centrifuged for 5 min at 2500 rpm and 4 °C. The supernatant was carefully removed and FMNL1 bound to the Ni-NTA agarose was resuspended in 5 ml lysis buffer (without pipeting). This solution was transferred to a 15 ml tube. The former tube was washed twice with another 5 ml lysis buffer, which was then added to the transferred solution, to take nearly all Ni-NTA agarose. The 15 ml tube was then centrifuged again at 2500 rpm and 4 °C for 5 min. The supernatant was discarded and 250 µl elution buffer (see

4.6) was added and mixed with the Ni-NTA agarose by slightly swaying. After 1 min, the Ni-NTA agarose was spinned down at 2500 rpm and stored at 4 °C for 5 min. The supernatant was transferred into a 1 ml glass tube. The elution procedure was repeated twice with 250 µl again, so that the final volume was 750 µl. A dialysis membrane was fixed on the top of the tube, the impermeability was tested and the tube was incubated over night in PBS (500 ml) at 4 °C while stirring (Figure 5-2). On each of the next two days, the volume of the protein suspension was tested, fresh protease inhibitors (PMSF and Complete) were added, the dialysis membrane was renewed and the tube was put in fresh PBS. After 3 days of dialysis, the protein concentration was analyzed (see 5.2.2) and the protein was stored at -80 °C.

5.2.2 Determination of the protein concentration

For Western Blot, protein concentrations of the purified FMNL1 or of total protein lysates were determined. Therefore, the cell pellet was thawn on ice, lysed in 1 ml Ripa buffer by resuspension and following incubation for 30 min on ice (see 4.6). The sample was then centrifuged for 10 min at 12000 rpm and 4 °C. The supernatant containing the proteins was transferred to a new tube.
The protein concentration was determined by a Bicinchoninic Acid protein assay following the manufacturers' instructions (Pierce).

5.2.3 Western Blot of FMNL1

25 µg of the total protein lysate and 0.5 µg of the purified protein were prepared for Western Blot with loading buffer (LDS) and DTT:

Total sample volume = (Volume lysate / 70) x 100

Volume LDS = Total sample volume / 4

Volume DTT = Total sample volume / 100 x 5

Samples were vortexed and subsequently incubated for 5 min at 95 °C for denaturation and reduction of proteins. The NuPAGE Novex Bis-Tris Gel was clamped into the running chamber filled with *NuPAGE MOPS* SDS Running Buffer (diluted 1:25 in aqua bidest). Samples as well as 7 µl of the protein ladder (Precision Plus Standard All Blue) were applied on the gel and run for 1h at 200 Volt and 4 °C. During this time, 25 ml *NuPAGE transfer* buffer was mixed with 50 ml Methanol and diluted with 425 ml aqua bidest. The nitrocellulose membrane and the gel were then equilibrated for 5 to 10 min in the prepared transfer buffer.

Filters and sponges were moisturized in the buffer. Thereafter, the different components were stacked in the transfer chamber without air bubbles as follows: first, two sponges, second, one filter, third, the gel, fourth, the nitrocellulose membrane, fifth, one filter and lastly two sponges. The transfer buffer was then filled in the chamber and the outer chamber was filled with water to cool the chamber during the transfer procedure. The transfer took place at 30 V and 4 °C for 2h. Successful transfer of the protein on the membrane was tested by Ponceau staining. For this, the membrane was stained with Ponceau solution for 1 min, the color excess was removed with aqua bidest. Proteins on the membrane appeared in red. The gel was shortly discolored with aqua bidest, before the membrane was equilibrated in TBST buffer (TBS buffer with 0.05 % Tween) by shaking the membrane twice for 15 s and twice for 5 min in TBST. Thereafter, the membrane was blocked, shaked for 1 h at RT in 5 % milk powder in TBST buffer. After washing by shaking twice for 15 s and thereafter twice for 5 min in fresh TBST, the membrane was agitated for 1 h at RT in 1 % milk powder in TBST buffer with FMNL1-specific antibody 6F2 (rat-α-human 1:10). Then, the membrane was washed intensely in TBST buffer (twice for 15 s and four times for 10 min) and shaked for 1 h with the second antibody in 1 % milk powder in TBST buffer (peroxidase-conjugated goat-α-rat 1:4000). Afterwards the membrane was intensively washed twice for 15 s and four times for 10 min in TBST). To analyze the membrane, 1 ml of substrate (Western Lightning Chemiluminescence Reagent) was freshly prepared (solution A : solution B = 1:1) and incubated for 1 min on the membrane. The membrane was then placed in the cassette. In the dark room, a film was placed into the cassette and incubated between 10 s and 5 min before development, depending on the signal strength. Finally the membrane was shortly washed in TBST and stained again in Ponceau staining for 1 min. The Ponceau solution excess was removed by shortly adding some water. The so stained membrane was dryed and scanned for documentation.

5.2.4 Silver stain

The Silver stain was performed with the Silver Quest Silver Staining Kit (see 4.5) following the manufacturers' instructions (Invitrogen Corporation). Bands were cut, prepared for digestion and analysed by mass spectrometry (by Elfriede Eppinger and Hakan Sarioglu).

5.3 Molecular Biology

5.3.1 Standard methods

5.3.1.1 RNA isolation

A pellet of approximately 0.1 x 10^6 cells was resuspended in 200 µl Tri-Reagent, vortexed and incubated for 5 min at RT. Thereafter, 10 µg of Yeast-tRNA and 40 µl 1-Bromo-3-Chloro-Propan were added, followed by incubation for 10 min at RT and centrifugation for 15 min at 4°C and 12000 rpm. The upper, aqueous, RNA-containing phase was transferred into a new tube and precipitated with 100 µl Isopropanol. For this step, the tube was incubated at least over night at -20°C. Thereafter, the tube was centrifuged for 20 min at 4°C and 12000 rpm, the supernatant was removed and the pellet was washed in 75% ethanol (by centrifugation for 5 min at 4°C and 12000 rpm). The supernatant was again discarded and the remaining pellet was resolved in 20 µl of DEPC water. RNA concentration was measured on a spectrometer (Eppendorf) and the RNA was stored at -80°C until use.

5.3.1.2 cDNA synthesis

For each reaction tube, the following reagents were added to 1 µg of RNA:
1 µl dNTP (10 mM), 1 µl Oligo d(T) Primer, addition of DEPC water to a final volume of 12 µl. This mix was incubated at 65°C for 5 min and cooled down on ice again. Then, 4 µl 5x First Strand Buffer, 2 µl DTT (0.1 M) and 1 µl RNaseOUT (40 U/ml) were added and the tube was then incubated at 42 °C for 2 min. Finally, 1 µl of the Superscript II Reverse Transcriptase was added and the synthesis was performed at 42°C for 50 min. Finally, the enzyme was inactivated at 70°C for 15 min. The reaction was accomplished in a thermocycler (Biometra). The cDNA was stored at -20°C until use.

5.3.1.3 Gel electrophoresis and Gel extraction

Depending on the expected DNA size, a 1% to 1.5% agarose gel (Agarose in TAE buffer) with 0.7 µg/ml Ethidium Bromide was prepared. The compact gel was then loaded in the electrophoresis chamber filled with TAE buffer. DNA was diluted 5:1 with loading dye and

applied on the gel, as well as a 10bp or 1 kbp DNA ladder. The gel was run at 50 to 120 Volt. Finally, DNA size was analyzed and documented by an UV-transilluminator. When indicated, bands of interest were cut and the DNA content was isolated with the NucleoSpin Gel-Extraction Kit (see 4.5) following the manufacturer's instructions (Macherey-Nagl).

5.3.1.4 Purification of digested inserts and vectors

PCR products are separated from very small DNA fragments after digestion by phenol chloroform extraction. Therefore, 500 µl of equilibrated phenol was added to the digested product and vortexed. After centrifugation at 14000 rpm for 2 min, the upper aqueous phase was transferred into a new tube and 500 µl chloroform isoamyl alcohol was added and the mixture was vortexed. After centrifugation at 14000 rpm for 2 min, the upper phase was again collected and transferred in a fresh tube. Thereafter, the solution was mixed with 10 µl sodium acetate (3 M) and 1 ml ethanol (99%). After a last centrifugation step at 14000 rpm and 4 °C for 20 min, the supernatant was discarded and the DNA pellet was dryed at the air and rehydrated in DEPC water. DNA concentration was measured on a spectrometer.
The vector was purified after digestion by gel purification (see 5.3.1.3).

5.3.1.5 Ligation

Digested and purified inserts as well as targeted digested and purified vectors were pooled for ligation in an appropriate ratio (Insert : vector). The amount of DNA applied to the reaction was calculated as follows: insert [ng] = 3 · insert [bp] · vector [ng] / vector [bp]:

Insert	300 ng
Vector	x ng
Ligationbuffer (10 x)	1 µl
T4 Ligase	1 µl (5 Weiss Units)
DEPC water	add 10 µl

Reaction	16 °C over night
Inactivation	65 °C 10 min
Cooling	4 °C

5.3.1.6 Transformation of *E.coli*

Ligation products or purified vectors were transferred into chemically competent *E.coli* (OneShotTop10) by application of a heat shock. The bacteria were thawn on ice, then 2 µl of ligation product or 1 µl of vector were added. After 30 min on ice, bacteria were incubated at 42 °C for 45 s. The tube was then immediately cooled down on ice. Thereafter, 250 µl of S.O.C medium were added and bacteria were incubated at 37°C on a heat block shaking at 225 rpm. 50 µl and 100 µl of bacterial suspension were plated out on LB agar plates with antibiotics corresponding to the resistance of the construct. Finally the plates were incubated at 37°C over night.

5.3.1.7 Plasmid extraction from bacteria

For plasmid expansion and purification, 5 to 15 tubes with 5 ml LB medium (containing the respective antibiotics) were inoculated with individually picked colonies from the bacteria plates and shaked over night at 250 rpm. On the next day, 1.5 ml of each of these starter cultures were used for plasmid purification with the JETstar Plasmid Purification MAXI KIT according to the manufacturer's instructions.
For large scale plasmid purification, 300 ml of LB medium (containing the respective antibiotics) were inoculated with 100 µl to 1 ml of starter culture and shaked over night at 250 rpm in an Erlenmeyer flask. On the next day, JETstar Plasmid Purification MAXI KIT was applied according to the manufacturer's instructions for maxi-preparation, to purify DNA.

5.3.1.8 Test digestion and sequencing

After purification, DNA constructs were analyzed by test digestion with enzyme(s) preferably cutting within the insert and the vector, producing DNA sizes being analyzable in one gel. When expected bands appeared in the gel, the constructs were sent to MWG (Ebersberg) for sequencing. The PCR products of the TCR repertoire analysis were sent to Sequiserve (Vaterstetten).

5.3.2 Analysis of EBV$^-$ B cells and Mini-LCL

5.3.2.1 Analysis of CD40L activated B cells

After approximately 1 month of expansion, B cells original from one well (12 well plate, approx. 1 mm^3 of cells) were harvested and DNA was isolated by alkaline lysis. Therefore, the B cell pellet was resuspended in 25 µl NaOH (50mM) and incubated for 10 min at 95 °C. After cooling, the sample was neutralized with 4 µl Tris HCl pH7 (1M). The sample was centrifuged for 1 min at maximal speed immediately before adding the DNA to the PCR. Two PCR were performed with one sample: one PCR to prove presence of DNA in the sample sample by using primers encoding a house keeping gene (beta-globin primer) and one PCR to analyze the presence of EBV (glycoprotein 85).

PCR mix beta globin:	Buffer (10x)	5.0 µl
	dNTP (10 mM)	1 µl
	MgCl2 (50 mM)	1.5 µl
	GH20 (2,5 pmol/µl)	2 µl
	PC04 (2,5 pmol/µl)	2 µl
	Taq-polymerase (5 U/µl)	0.25 µl
	DNA (from B cells)	1 µl
	DEPC water	add 50 µl

PCR program:	1.	Initial denaturation	96 °C	2 min
	2.	Denaturation	96 °C	45 s
	3.	Annealing	55 °C	45 s
	4.	Elongation	72 °C	45 s
	5.	Terminal elongation	72 °C	10 min
	6.	Cooling	14 °C	
	30 cycles from step 2 to step 4			

PCR mix gp85:	Buffer (10x)	5.0 µl
	dNTP (10 mM)	1 µl
	MgCl2 (50 mM)	1.5 µl
	gp85c (2,5 pmol/µl)	2 µl
	gp85d (2,5 pmol/µl)	2 µl
	Taq-polymerase (5 U/µl)	0.25 µl
	DNA (from B cells)	1 µl
	DEPC water	add 50 µl

PCR program:	1.	Initial denaturation	96 °C	2 min
	2.	Denaturation	96 °C	45 s
	3.	Annealing	59 °C	45 s
	4.	Elongation	72 °C	45 s
	5.	Terminal elongation	72 °C	10 min
	6.	Cooling	14 °C	
	30 cycles from step 2 to step 4			

The PCR products were analyzed on a 1.5% gel electrophoresis. The beta-globin product has a size of 260 bp and the gp85 product of 461 bp. EBV^- B cell lines were expanded and frozen until use.

5.3.2.2 Analysis of Mini-LCL

Mini-LCL original from one well (12 well plate, approx. 1 mm^3 of cells) were harvested and DNA was isolated by alkaline lysis as previously described (see 5.3.2.1). Three PCR reactions were performed with one sample: one PCR to prove the presence of DNA (beta-globin primer) in the sample (see 5.3.2.1), one to analyze the presence of wildtype EBV (glycoprotein 85; see 5.3.2.1) and one PCR to analyze the presence of the Mini-EBV plasmid marker (chloramphenicol-acetyltransferase):

PCR mix chloramphenicol-acetyltransferase:

Buffer (10x)	5.0 µl
dNTP (10 mM)	1 µl
MgCl2 (50 mM)	1.5 µl
cam-up (2,5 pmol/µl)	2 µl
cam-down (2,5 pmol/µl)	2 µl
Taq-polymerase (5 U/µl)	0.25 µl
DNA (from B cells)	1 µl
DEPC water	add 50 µl

PCR program:	1.	Initial denaturation	96 °C	2 min
	2.	Denaturation	96 °C	45 s
	3.	Annealing	59 °C	45 s
	4.	Elongation	72 °C	45 s
	5.	Terminal elongation	72 °C	10 min
	6.	Cooling	14 °C	
	30 cycles from step 2 to step 4			

The PCR products were analyzed on a 1.5% gel electrophoresis. The beta-globin product has a size of 260 bp, gp85 product of 461 bp and the chloramphenicol-acetyltransferase of 294

bp. Wildtype EBV$^-$, chloramphenicol-acetyltransferase$^+$ mini-LCL lines were expanded and frozen until use.

5.3.3 Vαβ-Repertoire analysis of T cell clones

To analyze the TCR Vα-repertoire, RNA was isolated from T cell clones (see 5.3.1.1), cDNA was generated (see 5.3.1.2) and this cDNA was then used for PCR reactions with 34 different 5'-Vα-primers coding for variable TCR regions. Primers P5'-(α) ST and P3'-(α) ST were localized in the constant region and served thereby as internal controls for the PCR and resulting in a PCR product of 550 bp length. 3'T-Cα primer was localized in the constant region, near to the variable region. Sequences of primers are listed in 4.8.3.2.

PCR mix:	5'Vαx (2,5 pmol/µl)	6 µl
	Buffer (10x)	4.0 µl
	dNTP (10 mM)	0.8 µl
	$MgCl_2$ (50 mM)	1.2 µl
	P-5'αST (2,5 pmol/µl)	2.8 µl
	P-3'αST (2,5 pmol/µl)	2.8 µl
	3'T-Cα (5 pmol/µl)	4.0 µl
	Taq-polymerase (5 U/µl)	0.4 µl
	cDNA (from T cells)	1 µl
	DEPC water	add 40 µl

PCR program:	1.	Initial denaturation	94 °C	2 min
	2.	Denaturation	94 °C	30 s
	3.	Annealing	56 °C	30 s
	4.	Elongation	72 °C	1 min
	5.	Terminal elongation	72 °C	10 min
	6.	Cooling	14 °C	
	38 cycles from step 2 to step 4			

To analyze the TCR Vβ-Repertoire, degenerated primer pairs were used instead of 38 Vβ-specific primers (Zhou *et al.*, 2006a), since cDNA material of T cell clones was limited. Using these primers, only two PCR reactions were necessary, covering 96 % of known Vβ-regions. The gene segments Vβ10.1 and Vβ16.1 (Arden *et al.*, 1995) could not be amplified with these primers.

PCR Mix:	Buffer (10x)	4.0 µl
	$MgCl_2$ (50 mM)	1.2 µl
	dNTP (10 mM)	0.8 µl
	VP1 or VP2 (5 pmol/µl)	16 µl
	CP1 (5 pmol/µl)	1.6 µl
	Taq-polymerase (5 U/µl)	0.5 µl
	cDNA (from T cells)	1.5 µl
	DEPC water	add 40 µl

PCR program:				
	1.	Initial denaturation	94 °C	2 min
	2.	Denaturation	94 °C	30 s
	3.	Annealing	50 °C	30 s
	4.	Elongation	72 °C	1 min
	5.	Terminal elongation	72 °C	10 min
	6.	Cooling	14 °C	
	40 cycles from step 2 to step 4			

5.3.4 TCR cloning into the retroviral vector pMP71G$_{PRE}$

TCR Aa2.2 and Bb5.14 were cloned into the retroviral vector pMP71G$_{PRE}$ for TCR transfer into new effector cells (PBMC, $CD4^+$, $CD8^+$ T cells).

5.3.4.1 TCR sequence amplification with simultaneous restriction enzyme site synthesis

Primers were designed with the requested restriction enzyme-sites (forward primer Not I, reverse primer EcoRI or MfeI (for Aa2.2 α); see 4.8.3.3) for the insertion into pMP71G$_{PRE}$. The forward primer additionally contained a Kozak sequence (see 4.8.3.3) prior to the start codon (ATG) of the specific TCR sequence.

PCR Mix:	Buffer for KOD (10x)	5 µl
	dNTP for KOD (2 mM)	5 µl
	$MgSO_4$ for KOD (25 mM)	3 µl
	α or β NotI (5 pmol/µl)	1 µl
	α or β MfeI or EcoRI (5 pmol/µl)	1 µl
	KOD (5 U/µl)	1 µl
	DMSO	2.5 µl
	cDNA (from T cell clone)	1 µl
	DEPC water	add 50 µl

PCR program:	1.	Initial denaturation	94 °C	2 min
	2.	Denaturation	94 °C	30 s
	3.	Annealing	55 °C	30 s
	4.	Elongation	72 °C	1 min 30 s
	5.	Terminal elongation	72 °C	10 min
	6.	Cooling	14 °C	
	35 cycles from step 2 to step 4			

This PCR reaction was performed 12 times to recover enough template DNA after gel purification (see 5.3.1.3). DNA concentration was measured with the nanodrop spectrometer (Eppendorf).

5.3.4.2 Digestion of TCR fragments and the retroviral vector

Inserts were then digested with NotI and EcoRI or Mfe I (for Aa2.2 α-chain):

PCR product	whole purified product	
Buffer NEB3 (10x)	10 µl	
BSA (100x)	1 µl	
NotI / NotI-HF (10 U/µl)	4 µl	(100 % activity in buffer NEB3/4)
EcoRI / MfeI (10 U/µl)	4 µl	(100 % activity in buffer NEB3/4)
DEPC water	add 100 µl	
Incubation	37 °C, 2h	
Cooling	4 °C	

Thereafter, inserts were purified by phenol-chloroform extraction (see 5.3.1.4) and DNA-concentration was measured with the nanodrop spectrometer (Eppendorf).

The vector pMP71$_{PRE}$ was digested as follows:

pMP71$_{PRE}$	20 µg	
Buffer NEB3 (10x)	10 µl	
BSA (100x)	1 µl	
NotI (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
EcoRI (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
DEPC water	add 100 µl	
Incubation	37 °C, 4h	
Cooling	4 °C	

After digestion, the vector was purified by gel extraction (see 5.3.1.3) and concentration was measured with the nanodrop spectrometer (Eppendorf).

After purification, TCR α or β chains, and pMP71$_{PRE}$ were ligated (see 5.3.1.5), transferred into bacteria (see 5.3.1.6) followed by plasmid purification (see 5.3.1.7). The accuracy of the recovered plasmid DNA was investigated by digestion and sequencing (see 5.3.1.8). Finally, larger amounts of the construct were isolated by maxi-preparation.

5.3.5 Optimized TCR cloning into the retroviral vector pMP71G$_{PRE}$

Since the single chain vectors did not result in successful TCR expression transduced cells, we performed several modifications:

- insertion of the Picorna virus-derived peptide element P2A between α and β chain for equimolar expression of the TCR chains (Leisegang *et al.*, 2008)
- replacement of constant chains by mouse constant chains (mu) for improvement of chain pairing (Cohen *et al.*, 2006)
- insertion of an additional disulfide bond (2S) for improvement of TCR pairing and expression (Cohen *et al.*, 2007; Voss *et al.*, 2010).

These modified sequences: TCR Aa2.2 NotI-β-mu2S-P2A-α-mu2S-EcoRI and TCR Bb5.14 NotI-β-mu2S-P2A-α-mu2S-EcoRI were sent to GeneArt for codon optimization (Scholten *et al.*, 2006). After codon optimization, the EcoRI cutting site within the Aa2.2 TCR alpha chain disappeared. Therefore, this construct could then also be cloned with EcoRI instead of Mfe I. The company sent the optimized TCR sequences cloned into a vector with canamycine resistance (pMK-RQ). Competent bacteria were transformed (see 5.3.1.6) with this construct for amplification. Subsequently, the plasmid was isolated by plasmid extension (see 5.3.1.7), gel extraction (see 5.3.1.3) and concentration was measured. The constructs were then digested with EcoRI and NotI:

pMK-RQ Aa2.2/Bb5.14	20 µg	
Buffer NEB3 (10x)	5 µl	
BSA (100x)	0,5 µl	
NotI (10 U/µl)	3 µl	(100 % activity in buffer NEB3)
EcoRI (10 U/µl)	3 µl	(100 % activity in buffer NEB3)
DEPC water	add 50 µl	
Incubation	37 °C, 2h	
Cooling	4 °C	

After digestion, Aa2.2 and Bb5.14 β-mu2S-P2A-α-mu2S were purified by gel extraction and the DNA concentration was determined. Aa2.2 and Bb5.14 β-mu2S-P2A-α-mu2S were

ligated into MP71G$_{PRE}$ (see 5.3.1.5), then amplified into TOP10 (see 5.3.1.6), isolated by plasmid preparation (see 5.3.1.7) and correct insertion was analyzed by digestion with restriction enzymes cutting within insert and vector. The size of resulting fragments was determined by gel electrophoresis (see 5.3.1.8). Finally, the plasmid constructs were sent to MWG for sequencing and large scale amplification was perfomed with a 300 ml bacterial culture followed by plasmid isolation with JETstar Plasmid Purification MAXI KIT following the manufacturer's instructions.

5.3.6 Cloning of HLA-DRB1*1 into pINCO

To HLA-DRB1*1 was cloned into pINCO using pCMV-CHis as transfer vector, in order to include a potent promoter (CMV) for HLA-DRB1*1 expression.

5.3.6.1 HLA-DRB1*1 sequence amplification with simultaneous restriction enzyme site synthesis

Primers were designed with the requested restriction enzyme-sites (forward primer Bam HI, reverse primer Xho I; see 4.8.3.4) for insertion into the primary vector pCMV-CHis. The forward primer comprised an additional Kozak sequence (see 4.8.3.4) prior to the specific HLA-DRB1*1 sequence. cDNA from a monoallelic LCL was used as template, to anticipate amplification of mixed HLA-DRB sequences.

PCR Mix:	Buffer for KOD (10x)	5 µl
	dNTP for KOD (2 mM)	5 µl
	$MgSO_4$ for KOD(25 mM)	3 µl
	HLA-DRB1*1 Bam HI (5 pmol/µl)	1 µl
	HLA-DRB1*1 Xho I (5 pmol/µl)	1 µl
	KOD (5 U/µl)	1 µl
	DMSO	2.5 µl
	cDNA (from monoallelic LCL)	1 µl
	DEPC water	add 50 µl

PCR program:				
	1.	Initial denaturation	94 °C	2 min
	2.	Denaturation	94 °C	30 s
	3.	Annealing	55 °C	30 s
	4.	Elongation	72 °C	30 s
	5.	Terminal elongation	72 °C	10 min
	6.	Cooling	14 °C	
	40 cycles from step 2 to step 4			

This PCR reaction was performed 12 times to recover enough template DNA by gel purification (see 5.3.1.3) and DNA concentration was measured with the nanodrop spectrometer (Eppendorf).

5.3.6.2 Digestion of insert, transfer vector pCMV and destination vector pINCO

The insert was digested by Bam HI and Xho I on the beforehand inserted cutting sites:

PCR product	whole purified product	
Buffer NEB3 (10x)	10 µl	
BSA (100x)	1 µl	
Bam HI (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
Xho I (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
DEPC water	add 100 µl	
Incubation	37 °C, 2h	
Cooling	4 °C	

The insert was then purified by phenol-chloroform extraction (see 5.3.1.4) and DNA-concentration was measured with the nanodrop spectrometer (Eppendorf).

pCMV-CHis was cut by Bam HI and Xho I:

PCR product	20 µg	
Puffer NEB3 (10x)	10 µl	
BSA (100x)	1 µl	
Bam HI (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
Xho I (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
DEPC water	add 100 µl	
Incubation	37 °C, 4h	
Cooling	4 °C	

The digested vector was purified by gel extraction (see 5.3.1.3) and DNA-concentration was measured with the nanodrop spectrometer (Eppendorf).

After purification, HLA-DRB1*1 and pCMV-CHis were ligated (see 5.3.1.5), transferred into bacteria (see 5.3.1.6) and plasmid purification was performed (see 5.3.1.7). Accuracy of the recovered plasmid DNA was investigated via test digestion and sequencing (see 5.3.1.8). This plasmid and pINCO were then digested with EcoRI and NotI:

Vector / Plasmid	10 - 20 µg	
Buffer NEB3 (10x)	10 µl	
BSA (100x)	1 µl	
NotI (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
EcoRI (10 U/µl)	4 µl	(100 % activity in buffer NEB3)
DEPC water	add 100 µl	

Incubation	37 °C, 4 h
Cooling	4 °C

Finally, the cut pieces of the insert and pINCO were recovered by gel purification (see 5.3.1.3), followed by DNA concentration measurement. Both components were ligated as described before (see 5.3.1.5), transferred into bacteria (see 5.3.1.6) and plasmid purification was performed (see 5.3.1.7). The accuracy of the recovered plasmid DNA was investigated by test digestion and sequencing (see 5.3.1.8). Finally, larger amounts of the construct were isolated by JETstar Plasmid Purification MAXI KIT following the manufacturer's instructions.

5.4 Adenovirus production and transduction of target cells

5.4.1 Adenovirus production

The primary adenoviral supernatant was produced by Yanyan Han. She provided me FMNL1/GFP and GFP containing adenoviral supernatants required for adenoviral supernatant production. 6 x 10^6 293A cells were seeded in 20 ml medium in tissue culture plates (145 x 20 mm). The next day, 200 µl of adenoviral supernatant was added to each plate. After 2 days, GFP-positive cells occurred and cells were harvested followed by cell destruction by freezing for at least 45 min in -80°C in 50 ml Falcon tubes. Thereafter, tubes were thawn in a 37°C water bath for 15 min. This procedure was repeated another two times. After the last thawing step, supernatants were centrifuged at 3000 rpm for 10 min. Supernatants were then transferred to a fresh tube to avoid contamination with cell debris. Supernatants were kept until use at -80°C.

5.4.2 Adenoviral transduction of EBV$^-$ B cells

1 x 10^6 B cells were taken up in 1 ml adenoviral supernatant, supplemented with 2 ng/ml IL-4 and rotated for 6h at 37 °C. Thereafter, one ml of B cell suspension was plated per well on CD40L feeder cells. On the next day, one ml IL-4-supplemented medium was added to each well. On day 3, B cells were used for stimulation assays and pellets were frozen for Western Blot analysis to examine successful adenoviral transduction.

5.5 Retroviral Transfer

5.5.1 Virus particle production

To produce virus particles for the transfer of targeted TCR or GFP, three vectors are necessary: pAlF10A1-GALV („env") coding for viral envelope proteins, pcDNA3.1-MLV („gag-pol") coding for viral group specific antigens and polymerase, and pMP71G$_{PRE}$ with the genes coding the TCR or the gene coding for GFP. These vectors were transferred by transfection by TransIT into 293T, which produced viral particles. In detail, 0.3-0.4 x 10^6 293T were plated in each well of a 6 well plate in a volume of 3 ml/well. The next day, 200 µl DMEM was mixed with 9 µl TransIT and incubated for 20 min at RT. Then, one µg of each of the three vectors was mixed with this solution and incubated again at RT for 30 min. Finally, this mix was added dropwise to one well in a 6-well plate. After slightly swaying the plates, the cells were kept for 48 h in the incubator.

5.5.2 Transduction of human PBMC, CD4$^+$ and CD8$^+$ T cells

Non-treated 24 well plates were coated with retronectin, to enhance uptake of retroviral particles by the targeted cells. Therefore, non-treated 24 well plates were filled with 400 µl retronectin (c_{end} = 12.5 mg/ml) and incubated for 2h at RT. Thereafter, retronectin was removed and wells were filled with 500 µl 2 % BSA solution. After another incubation of 30 min at 37 °C, wells were washed with 2 ml PBS with 2 % HEPES, refilled with 2 ml of PBS 2.5 % HEPES and kept at 4 °C until use (for a maximum of 1 week).
For transduction, PBMC, CD4$^+$ or CD8$^+$ T cells activated with Okt-3 and IL-2 were plated at a concentration of 1 x 10^6/well in a total volume of 1 ml T cell medium on retronectin coated plates. Viral supernatant was removed from 293T cells 48h after transfection (5.5.1) and

filtered through a 0.45 µm filter. One ml of the supernatant was added to prepared cells per well. Wells were supplemented with 100 U/ml IL-2, 4 µg/ml protamine sulfate and 1 % HEPES. Thereafter, plates were centrifuged for 90 min at 2000 rpm at 32 °C and subsequently kept in the incubator. The next day, cells were washed and transduced a second time in the same way with virus supernatant. Finally, the viral supernatant was replaced by T cell medium supplemented with 100 U/ml IL-2. The efficiency of the transduction was investigated by FACS analysis after three to four days.

5.6 Flow cytometry

5.6.1 Extracellular FACS staining

For FACS analysis cells were stained with fluorescence-labeled antibodies. Between 0.05 x 10^6 and 0.3 x 10^6 cells were added to one FACS tube and washed once with FACS buffer (PBS / 1 % ΔFCS). Cells expressing F_c receptors (e. g. monocytes) were incubated for 10 min with 100 µl human serum on ice, to block these receptors. Cells were then washed again, and 2 to 5 µl of antibody were added and incubated for 30 min in the dark and on ice. After one last washing step, 100 µl PBS with 1 % PFA in was added to all samples and thereafter samples were measured on the flow cytometer (LSR II).

5.6.2 CFSE and Far Red staining

Far Red and CFSE staining were performed following the same protocol. 10 µM Far Red or alternatively 5 µM CFSE were added to cells washed and resuspended in room temperature PBS + 2 % ΔFCS. This mixture was incubated for 10 min at 37 °C and staining was quenched by addition of at least the 5-fold ice cold buffer of the initial volume followed by incubation for 5 min on ice. Finally, cells were washed three times with buffer.

5.6.3 Degranulation Assay

0.1 x 10^6 TCR-transduced PBMC were incubated for 4h with 0.2 x 10^6 Far Red stained target cells or without target cells, 10 µg/ml Brefeldine A, 5 µl CD107a and 5 µl CD107b antibodies

were added in a total volume of 200 µl T cell medium at 37 °C. To stop the stimulation assay, 0.9 ml FACS buffer containing 0.1 % NaN_3 was added to the cells. After centrifugation, surface staining procedure was started (see 5.6.1). Cells were resuspended in 300 µl NaN_3 FACS buffer for measurement.

5.6.4 FoxP3 staining

FoxP3 staining was performed with the Treg detection Kit from Miltenyi Biotec following the manufacturers' instructions. Shortly, cell surface staining was performed (see 5.6.1), subsequently cells were fixed, permeabilized and stained with the Treg detection Kit from Miltenyi Biotec following the manufacturers' instructions. Finally cells were resuspended in 300 µl FACS buffer for measurement.

5.7 Functional Analysis

5.7.1 T cell stimulation assays

For T cell stimulation assays of original clones, we used an effector : target (E:T) ratio of 1 : 1, for assays with transduced T cells, we chose an E:T ratio of 5:1. First, target cells were prepared: cells were harvested, washed and counted. Occasionally, Mini-LCL or B cells were previously pulsed with peptide or with protein (see 5.1.5, without irradiation step). Thereafter target cells were diluted to a final concentration of 10000 cells / 100 µl in T cell medium and plated in a 96 well plate (100 µl per well, triplicates). T cells were subsequently harvested, washed, counted and diluted in T cell medium to a final concentration of 10000 cells / 100 µl (for transduced T cells the final concentration was 50000 cells / 100 µl). The cells were then added to target cells (100 µl / well). After 24 hours of incubation, supernatants (150 µl / well) were harvested and frozen at – 20 °C until analysis (IFN-γ ELISA, FlowCytomix).

5.7.2 IFN-γ ELISA

IFN-γ ELISA was performed using Human IFN-γ ELISA Set (Becton Dickinson). For coating, ELISA plates were covered with capture antibody (50 µl / well) diluted in coating buffer (per 1

ml coating buffer 4 µl of capture antibody was added) and incubated at 4 °C over night, sealed with an adherent foil. The next day, plates were washed three times with washing buffer (see 4.6). Thereafter, blocking was performed for 1 h with 300 µl of 1 % milk powder solution per well at RT. Plates were then washed again three times with washing buffer. Afterwards, 50 µl / well of standard dilution row was pipetted in duplicates on each plate with following concentrations: 1000 pg / ml, 500 pg / ml, 250 pg / ml, 125 pg / ml, 62.5 pg / ml, 31.25 pg/ml and 0 pg / ml (diluted in T cell medium). At the same time, 50 µl / well of samples were applicated on the plates in triplicates. Plates were then incubated for another hour at RT, before they were washed five times with washing buffer. For detection, 50 µl / well of Working Detector (per 1 ml 1 % milk powder 4 µl biotylinated detection antibody and 4µl Streptavidin-horseradish peroxidase conjugate) were added to all wells. After 1 h of incubation time at RT, the plates were washed seven times with washing buffer. Then, 100 µl / well of substrate solution (solution A 1 : 1 solution B of the TMB Substrate Reagent Set) was added to all wells and plates were incubated for 15 to 25 min in the dark at RT. Finally, the reaction was stopped by addition of 50 µl stop solution (H_3PO_4, 1 M) to each well and plates were measured at 450 nm by a microplate reader (Tecan).

5.7.3 Chromium release assay

The chromium release assay was used to detect antigen-specific cytotoxicity of TCR-transduced effector cells at an E:T ratio of 15 : 1. Target cells were harvested, washed and counted. B cells were eventually previously pulsed with LPS (see 5.1.5, without irradiation step). Thereafter, target cells were diluted to a final concentration of 1 x 10^6 / 100 µl in Δ FCS and labelled with 100 µCi Cr^{51} for 1 h at 37 °C. During this incubation step, T cells were harvested, washed, counted, diluted in T cell medium to a final concentration of 30000 cells / 50 µl T cell medium and plated in a 96 well plate. Target cells were then washed three times, counted and diluted to 2000 cells / 50 µl T cell medium and subsequently added to the prepared T cells. As controls, targets were plated in medium without T cells (for calculation of spontaneous lysis; Spont) and directly on a Luma plate (for calculation of maximal lysis; Max). Finally, after 4 hours of incubation, supernatants (50 µl / well) were harvested and transferred to Luma plates. After drying of the membranes, radioactivity of the wells was measured by a γ-counter (Perkin Elmer). For analysis, the obtained values were normalized by the following calculation, to obtain the final measured cytotoxicity:

$$\frac{(\text{value} - \text{spont})}{(\text{max} \div 2 - \text{spont})}$$

5.7.4 Flow Cytomix

Multiplex analysis was performed with human Th1/Th2 11-Plex Flow Cytomix from Bender Medsystems following the manufaturers' instructions. Shortly, 24h stimulation assay supernatants (see 5.7.1) were incubated with IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-22, IFN-γ, TNF-α antibodies and 13 different fluorescent beads. After the staining procedure, samples were analyzed on a FACS Calibur.

6 RESULTS

6.1 Prerequisites and priming procedure for generation of tumor reactive self-restricted CD4$^+$ T cells

6.1.1 FMNL1 protein production and purification

We aimed to isolate tumor-reactive CD4$^+$ T cells from the self-restricted T cell repertoire. Since it has been shown that FMNL1 represents an interesting tumor-associated antigen, restrictedly expressed in hematopoietic cells and over expressed in leukemic cells (Krackhardt *et al.*, 2002b), we targeted this antigen.

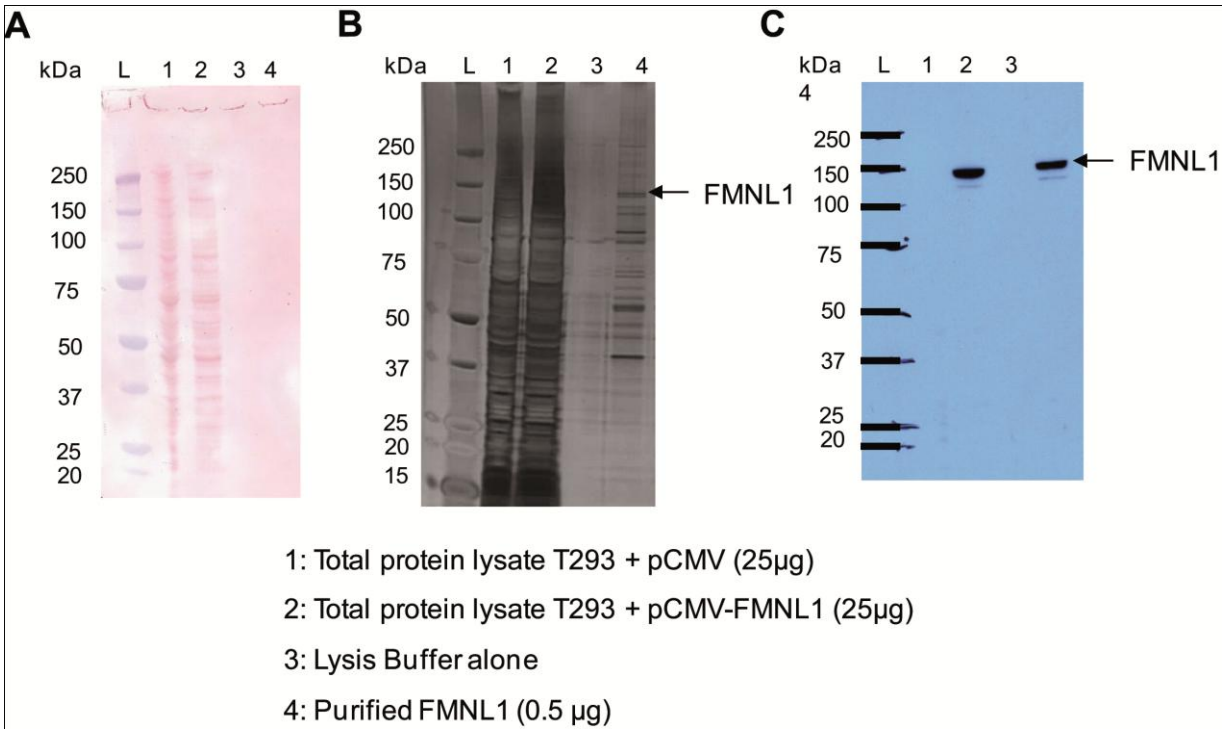

Figure 6-1: Protein analysis of recombinant FMNL1

Protein expression and purification was investigated by Ponceau staining (A), Silver staining (B) and Western Blot (C). (A) Ponceau staining was performed to verify the successful transfer of protein to the membrane and to investigate the remaining background with irrelevant proteins after purification (B) Silver staining was performed to investigate the remaining background with irrelevant proteins after purification. Significant bands from lane 4 were cut and used for mass spectrometry. Lane 3 was loaded with lysis buffer alone to analyze purity of the buffer and to visualize the background of the gel. (C) Western Blot analysis was performed with an FMNL1-specific antibody, to prove presence of FMNL1 in the lysate of FMNL1-transfected 293T cells (lane 2) and the purified protein suspension (lane 4).

We were able to express recombinant FMNL1 in T293 cells and isolated it by His-tag purification (see 5.2.1). Ponceau staining showed that after purification of the lysate, protein background disappeared (line 4, Figure 6-1A). However, Silver staining is more sensitive and demonstrated that additional bands are present after purification of recombinant FMNL1 (Figure 6-1B). However, these bands did result in significant hits by mass spectrometry analysis (performed by Elfriede Eppinger and Hakan Sarioglu, data not shown). Western Blot analysis demonstrated that FMNL1 was successfully expressed and purified (Figure 6-1C). Finally, we used this protein to pulse DC for the priming procedure.

6.1.2 Generation of autologous B cell lines

MHC class II is constitutively expressed only on professional antigen presenting cells, namely on DC, macrophages and B cells. We established autologous B cell lines in order to have an unlimited source of autologous antigen presenting cells available to investigate T cell lines and clones for their reactivity. We decided to use two kind of autologous B cell lines: EBV^- B cell lines and Mini-LCL lines. EBV^- B cell lines were grown in IL-4-supplemented medium on mouse fibroblasts which are stably transfected with CD40L. These B cell lines were were generated as described (5.1.4) and tested for expression of the EBV protein gp85 by PCR (Figure 6-2).

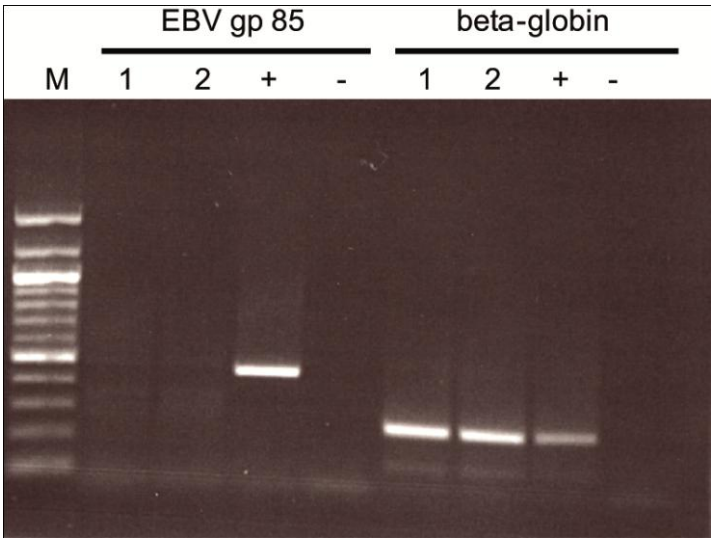

Figure 6-2: EBV gp85 cannot be detected in autologous B cell lines

DNA isolated from autologous B cell lines derived from donor I was investigated for EBV expression by PCR using a primer pair specific for the EBV protein gp85 (4 lanes besides the marker). As control for successful PCR extraction, a second primer pair specific for the cellular housekeeping gene β-globin was used. 1 and 2 are two B cell lines from donor I, + is DNA from an LCL and – represents the water control.

Lines 1 and 2 were EBV$^-$ and could therefore be used as target cells (Figure 6-2). For establishment of Mini-LCL lines, B cells were transduced with Mini-EBV supernatant (5.1.4). Mini-EBV encloses all latent EBV genes, leading to immortalization of B cells (Kempkes *et al.*, 1995a; Kempkes *et al.*, 1995b). Therefore, Mini-LCL present only a restricted panel of EBV peptides on their surface. Mini-LCL lines were kindly provided by A. Moosmann.

6.1.3 Generation of tumor reactive self-restricted CD4$^+$ T cells

DC were used for T cell priming. Therefore, we first generated DC from blood monocytes derived from donor I (HLA-DRB1*0101, HLA-DRB1*1101; Figure 6-3).

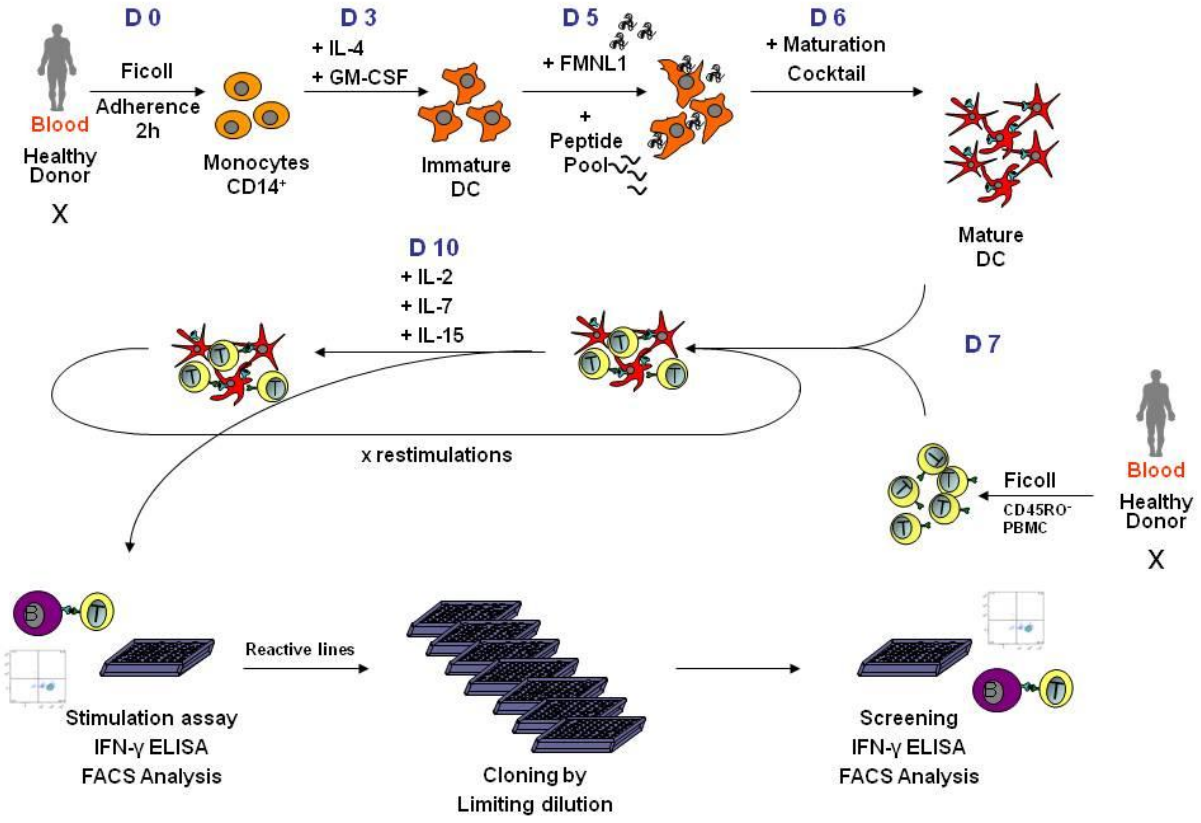

Figure 6-3: Stimulation protocol for the generation of self-reactive T cell clones
PBMC or CD45RO$^-$ cells derived from donor 1 were primed and restimulated with autologous DC matured with FMNL1 protein and IFN-γ, TNF-α, CD40L (cocktail A) or IL-6, TNF-α and IL-1β (cocktail B). After 4 weekly stimulations, T cell lines were restimulated 2 to 4 times every two weeks with autologous PBMC pulsed with FMNL1 protein. T cell lines were analyzed for specific reactivity in response to autologous antigen presenting cells by 24h co-culture followed by IFN-γ ELISA. Reacting T cell lines were cloned by limiting dilution resulting in the isolation of the peptide-specific T cell clones from the Ga3 T cell line and T cell clones Aa2.2 and Bb5.14.

On day 5, we added FMNL1 protein or one of the FMNL1 peptide pools described in Table 5-1 to the culture. We used two different cytokine cocktails for DC maturation on day 6: cocktail A comprising IFN-γ, TNF-α and CD40L or cocktail B including IL-6, TNF-α and IL-1β (Table 5-1B and Figure 6-3). Untouched PBMC or alternatively naïve T cells ($CD45RO^-$ sorted PBMC) were isolated from the same donor and costimulated with peptide- or protein-pulsed matured DC (Table 5-1B and Figure 6-3). IL-2, IL-7 and IL-15 were added primarily after three days (Figure 6-3). IL-2 was thereafter added every three to four days whereas IL-7 and Il-15 were added once a week at the time point of restimulation.
After 4 weekly restimulations with DC, T cells were restimulated every two weeks with autologous PBMC and were weekly investigated for reactivity against autologous Mini-LCL in 24h co-cultures. The supernatants of these co-cultures were investigated for IFN-γ release by IFN-γ ELISA (Figure 6-3). Reactive T cell lines were then cloned by limiting dilution (Figure 6-3) and restimulated every two weeks. for restimulations we used pooled allogeneic irradiated PBMC together with irradiated autologous Mini-LCL. For conditions primed with FMNL1 peptide pools, Mini-LCL were pulsed with peptide. IL-2, IL-7 and IL-15 were added as described above (5.1.8.2). The reactivity of these T cell clones was also tested in 24h co-cultures with appropriate target cells (Figure 6-3).

6.2 Functionality of $CD4^+$ FMNL1-peptide specific T cells

6.2.1 Generation of FMNL1 Pool G-specific T cell lines and clones

Line Ga3 (Pool G primed PBMC, line 3) was tested for cytokine secretion after 4 weekly restimulations and IFN-γ was detected in response to Mini-LCL pulsed with peptide Pool G In contrast, no cytokine secretion was observed in response to an alternative peptide pool (Figure 6-4A).

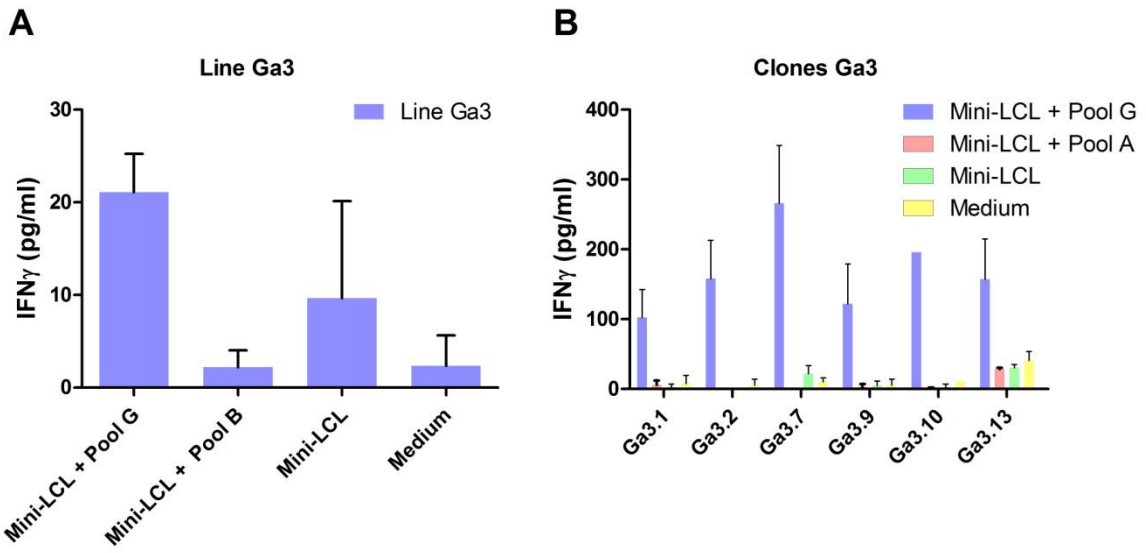

Figure 6-4: T cell clones derived from Line Ga3 recognize target cells pulsed with peptide Pool G

IFN-γ secretion of cells derived from line Ga3 (A) as well as T cell clones originating from this line (B) was analyzed after 24h co-incubation with untreated autologous Mini-LCL or alternatively pulsed with peptide Pool G or an irrelevant peptide pool. Standard deviations (SD) of triplicates are shown.

This T cell line was therefore cloned by limiting dilution. We recovered 64 clones which were mostly reactive in response to target cells pulsed with FMNL1 peptide Pool G. We selected 6 clones, which were also co-incubated with Mini-LCL pulsed with an alternative FMNL1 peptide pool. Clones were not reactive to these cells (Figure 6-4B). Of note, IFN-γ secretion level was enhanced after cloning suggesting that the original T cell line likely contained also other specificities (Figure 6-4).

6.2.2 Epitope identification

Pool G comprised 21 peptides (peptides 122-142) from the FMNL1 peptide pool. We aimed to identify the exact recognized epitope by preparing new overlapping peptide pools (Kern *et al.*, 1999). Each peptide was included in two different peptide pools (Table 6-1).

Pool	I	II	III
a	122	123	124
b	125	126	127
c	128	129	130
d	131	132	133
e	134	135	136
f	137	138	139
g	140	141	142

Table 6-1: Peptide pools for epitope identification

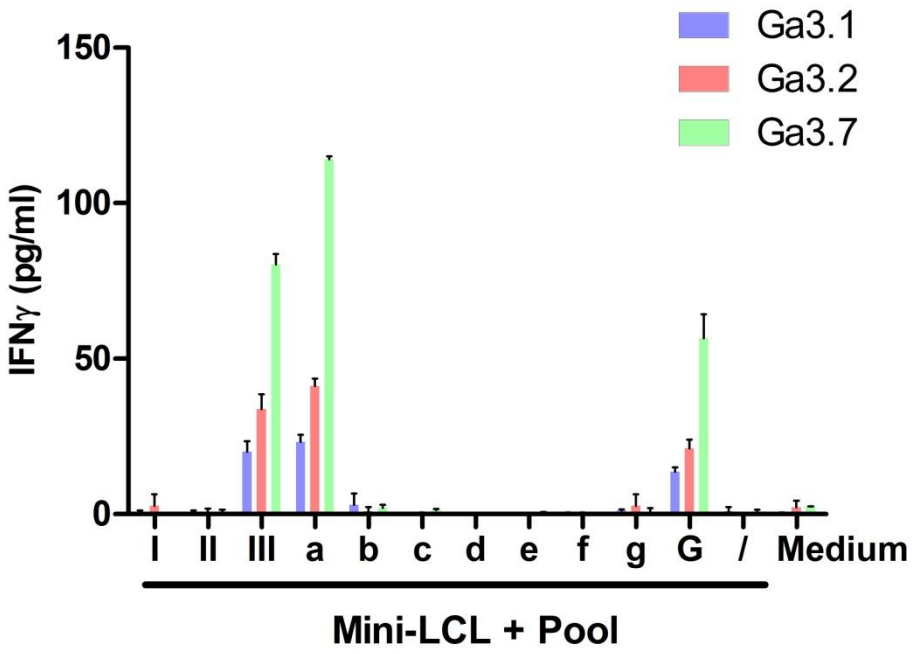

Figure 6-5: Epitope identification of Ga3 clones

Overlapping peptide pools were used for epitope identification by 24h co-incubation of Ga3 T cell clones with autologous Mini-LCL either untreated, pulsed with peptide Pool G as control or with different overlapping peptide pools (I-III, a-g). Supernatants were harvested from 96 well plates and analyzed by IFN-γ ELISA. SD of triplicates are shown.

Mini-LCL were alternatively pulsed with Pool G, Pool I-III or Pool a-g and incubated with T cell clones for 24h. Then, supernatants were harvested and analyzed by IFN-γ ELISA. T cell clones were reactive against Pool G (positive control), Pool a and Pool III (Figure 6-5). This result demonstrated that peptide 124 (sequence: LFFSLFSRFIKAYKKA) was the targeted epitope (Table 6-1).

6.2.3 Functionality of peptide 124-specific T cell clones

We studied the functional avidity of the T cell clones by titration of peptide 124 on Mini-LCL. The half-maximal activity of the T cell clones was varying between the clones but generally very low, at approximately 1 µM (Figure 6-6A).

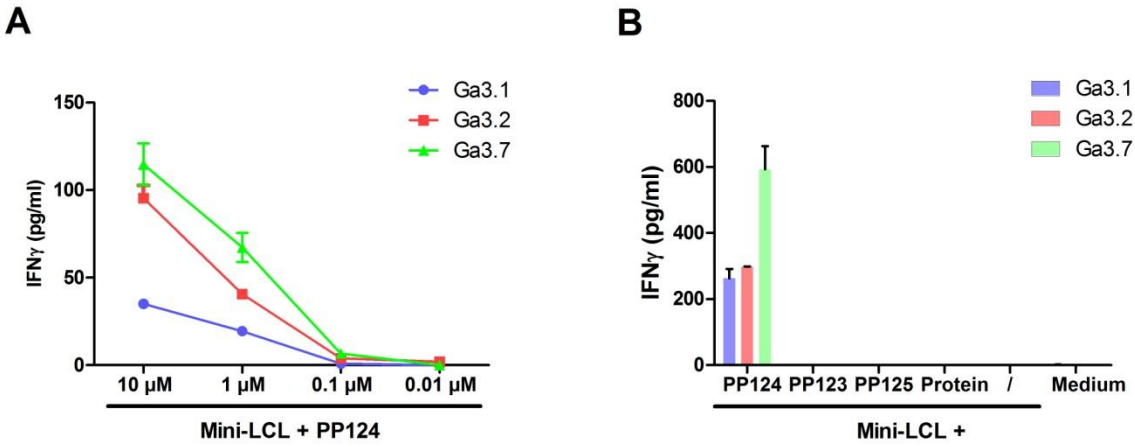

Figure 6-6: Peptide titration and recognition of unpulsed natural target cells
Cytokine secretion of peptide 124 (PP124)-specific T cell clones was analyzed after 24h co-incubation with differentially treated autologous Mini-LCL in 96 well plates. Supernatants were harvested and analyzed by IFN-γ ELISA. (A) Mini-LCL were pulsed with a PP124 peptide in a range of titrated peptide concentrations. (B) Mini-LCL were pulsed either with PP124 or with two overlapping peptides (peptide 123 and 125). Alternatively Mini-LCL were pulsed with protein or non-treated cells before co-culture. SD of triplicates are shown.

Moreover, T cell clones did not recognize natural target cells as unpulsed or FMNL1 protein-pulsed Mini-LCL (Figure 6-6B). Since these T cell clones indeed recognized peptide 124 but no natural target, we focused on T cell clones recovered from priming with FMNL1 protein-pulsed DC.

6.3 Functionality of $CD4^+$ T cell clones primed with protein-pulsed DC

6.3.1 Recovery of T cell lines and clones from different priming procedures

T cell lines secreting low amounts of IFN-γ in response to Mini-LCL could be isolated after several restimulations of PBMC and naïve T cells with FMNL1-pulsed DC and autologous PBMC (Figure 6-7). These T cell lines were cloned by limiting dilution.

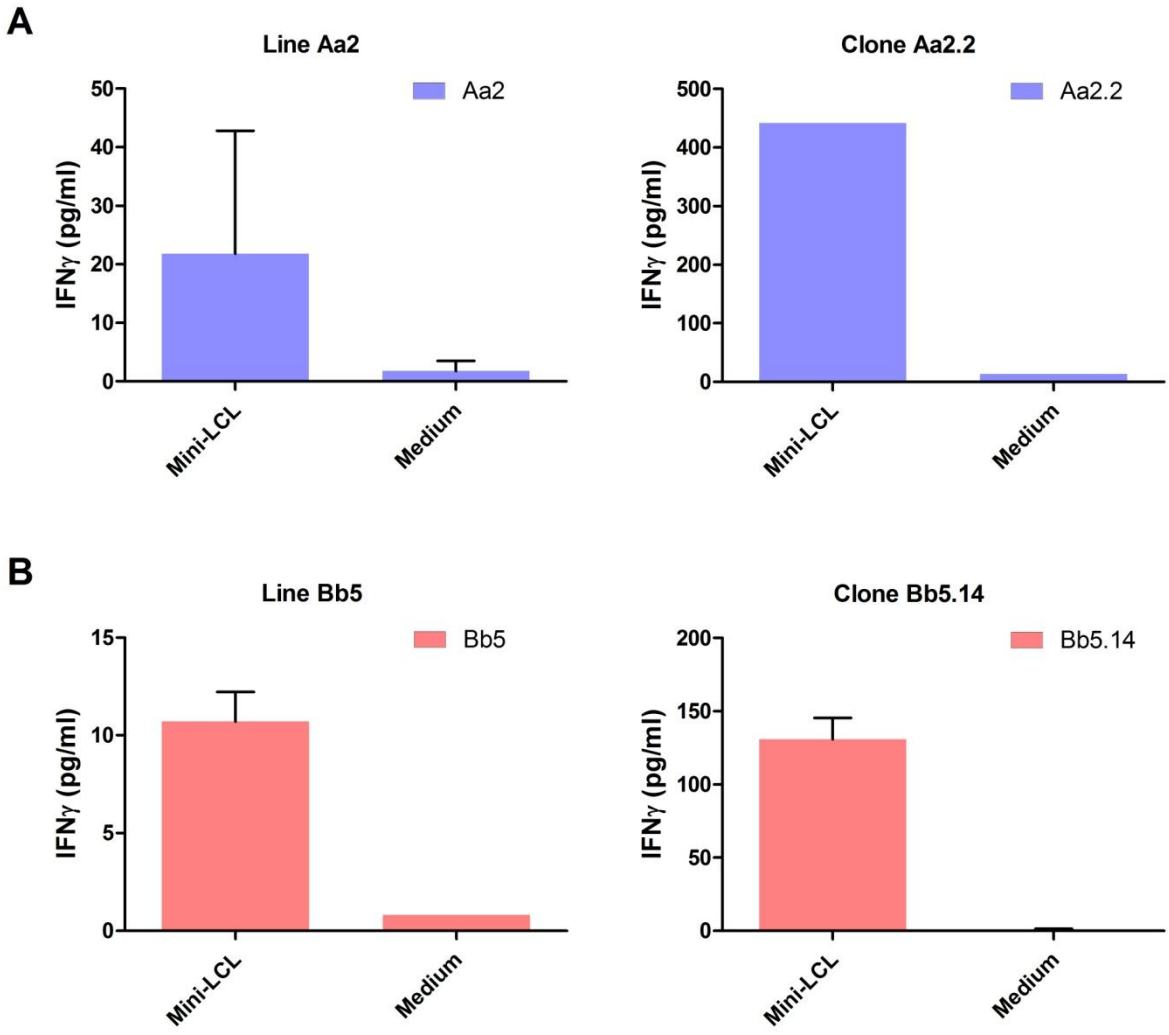

Figure 6-7: Screening of self reactive T cell lines and clones isolated from a healthy donor

Cytokine secretion of isolated T cell lines and clones was analyzed after 24h co-incubation with autologous Mini-LCL in 96 well plates. Supernatants were harvested and analyzed by IFN-γ ELISA. Reactivities of the clones Aa2.2 (derived from non-manipulated PBMC, A) and Bb5.14 (derived from naïve T cells, B) in response to Mini-LCL were analyzed by IFN-γ ELISA (A and B). An E:T ratio of 1:1 was used. SD of duplicates are shown. As growth of clone Aa2.2 was limited, reactivity was tested in this first screening assay only in a unique well (A, right panel). Of note, scales used to indicate the reactivity of both TCR differ significantly.

We obtained two T cell clones recognizing autologous Mini-LCL (Figure 6-7). One of these clones, Aa2.2 (Figure 6-7A) was recovered from PBMC after 4 weekly restimulations with autologous DC and 4 biweekly restimulations with autologous PBMC. Bb5.14 (Figure 6-7B), was isolated from the naïve T cell population, after 4 weekly restimulations with autologous DC and 2 biweekly restimulations with autologous PBMC. Bb5.14 secreted IFN-γ in response to Mini-LCL. Of note, clonal populations were secreting much higher levels of IFN-γ in response to target cells (Figure 6-7).

6.3.2 Recognition pattern of T cell clone Bb5.14

The recognition pattern of T cell clone Bb5.14 in response to hematopoietic cells was investigated.

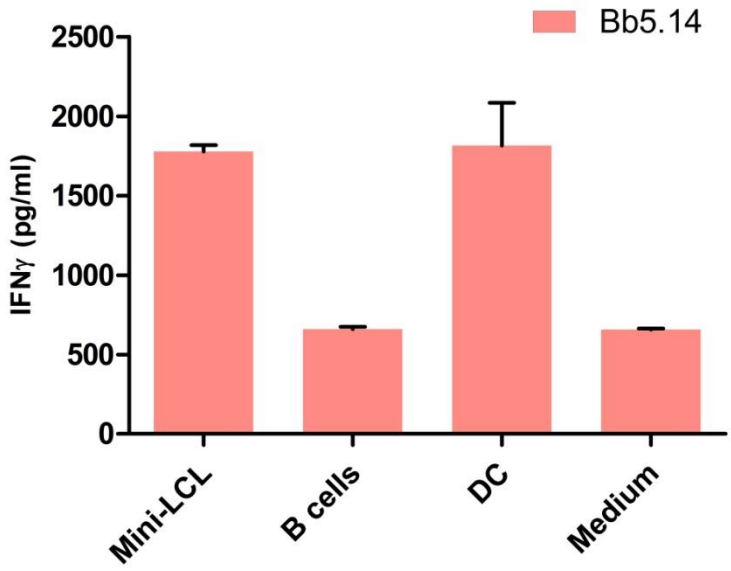

Figure 6-8: Reactivity of T cell clone Bb5.14 in response to Mini-LCL and DC
IFN-γ secretion of Bb5.14 was analyzed after 24h co-incubation with different targets in 96 well plates. Supernatants were harvested and analyzed by IFN-γ ELISA. Autologous Mini-LCL, B cells and DC were used as targets. SD of triplicates are shown.

Bb5.14 recognized autologous Mini-LCL and DC (Figure 6-8). Since Bb5.14 secreted high IFN-γ levels without stimulation (Medium control, Figure 6-8), reactivity towards EBV^- B cells could not be clearly demonstrated (Figure 6-8). For further investigation, the TCR was cloned and transferred into fresh effector cell populations (see 6.4).

6.3.3 Recognition pattern of T cell clone Aa2.2

The reactivity pattern of T cell clone Aa2.2 was investigated more in detail, as this clone was initially well proliferating. Aa2.2 recognized B cells and Mini-LCL, but not DC (Figure 6-9A). Moreover, autologous PBMC were not recognized by Aa2.2 (Figure 6-9A). Detection of B cells and Mini-LCL was significantly enhanced, after pulsing of target cells with FMNL1 protein prior to co-incubation (Figure 6-9A).

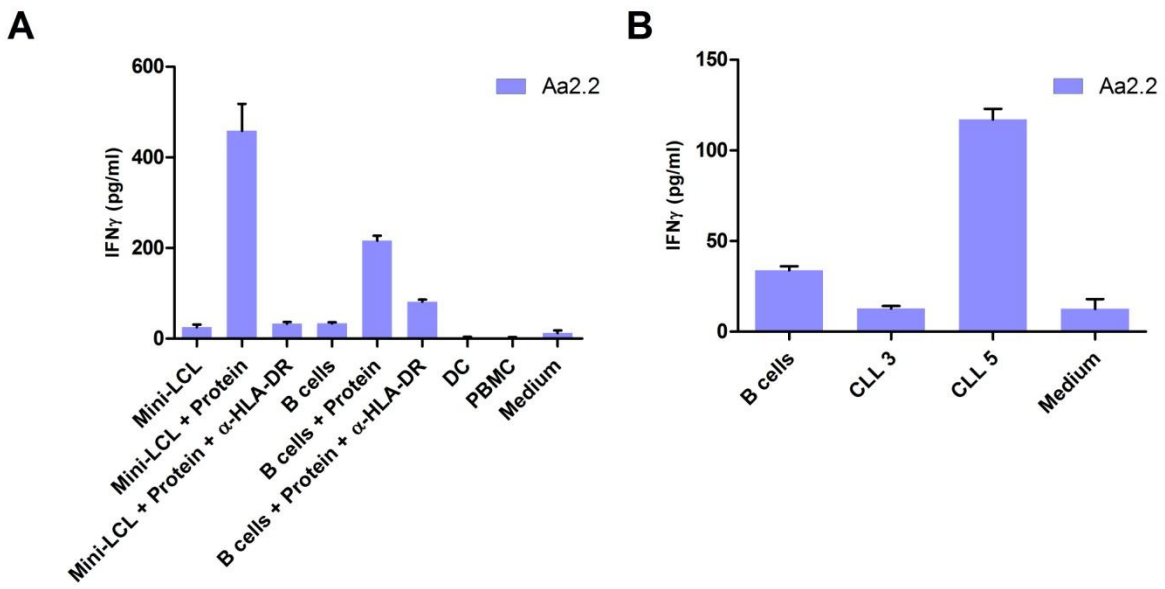

Figure 6-9: T cell clone Aa2.2 recognizes B cells and HLA-matched CLL cells but not DC or PBMC

IFN-γ secretion of Aa2.2 was analyzed after 24h co-incubation with different targets in 96 well plates. Supernatants were harvested and analyzed by IFN-γ ELISA. (A) Autologous Mini-LCL and B cells either untreated or pulsed with FMNL1 protein and alternatively blocked with α-HLA-DR antibody were used as targets. Moreover, we used autologous DC and PBMC as targets. (B) Tumor cell recognition of Aa2.2 was investigated by using CLL samples partially HLA-DR matched with donor I, from which T cell clones were generated: CLL3 (HLA-DR1$^+$) and CLL5 (HLA-DR11$^+$). SD of triplicates are shown.

Recognition of B cells and Mini-LCL could be blocked by addition of HLA-DR antibody (Figure 6-9A). To investigate tumor reactivity of Aa2.2, we used two CLL samples from patients partially HLA-DR matched with donor I in a 24h stimulation assay: HLA-DR1$^+$ CLL3 and HLA-DR11$^+$ CLL5. Interestingly, Aa2.2 recognized only HLA-DR11$^+$ sample CLL5 (Figure 6-9B), suggesting an HLA-DR11 restriction. However, the restriction element recognized by Aa2.2 had to be further analyzed (see 6.4.3).

We attempted to identify the epitope recognized by this T cell clone, which was assumed to be derived from FMNL1 due to the stimulation procedure. Therefore, we adenovirlally transduced B cells with FMNL1 or alternatively GFP as control.

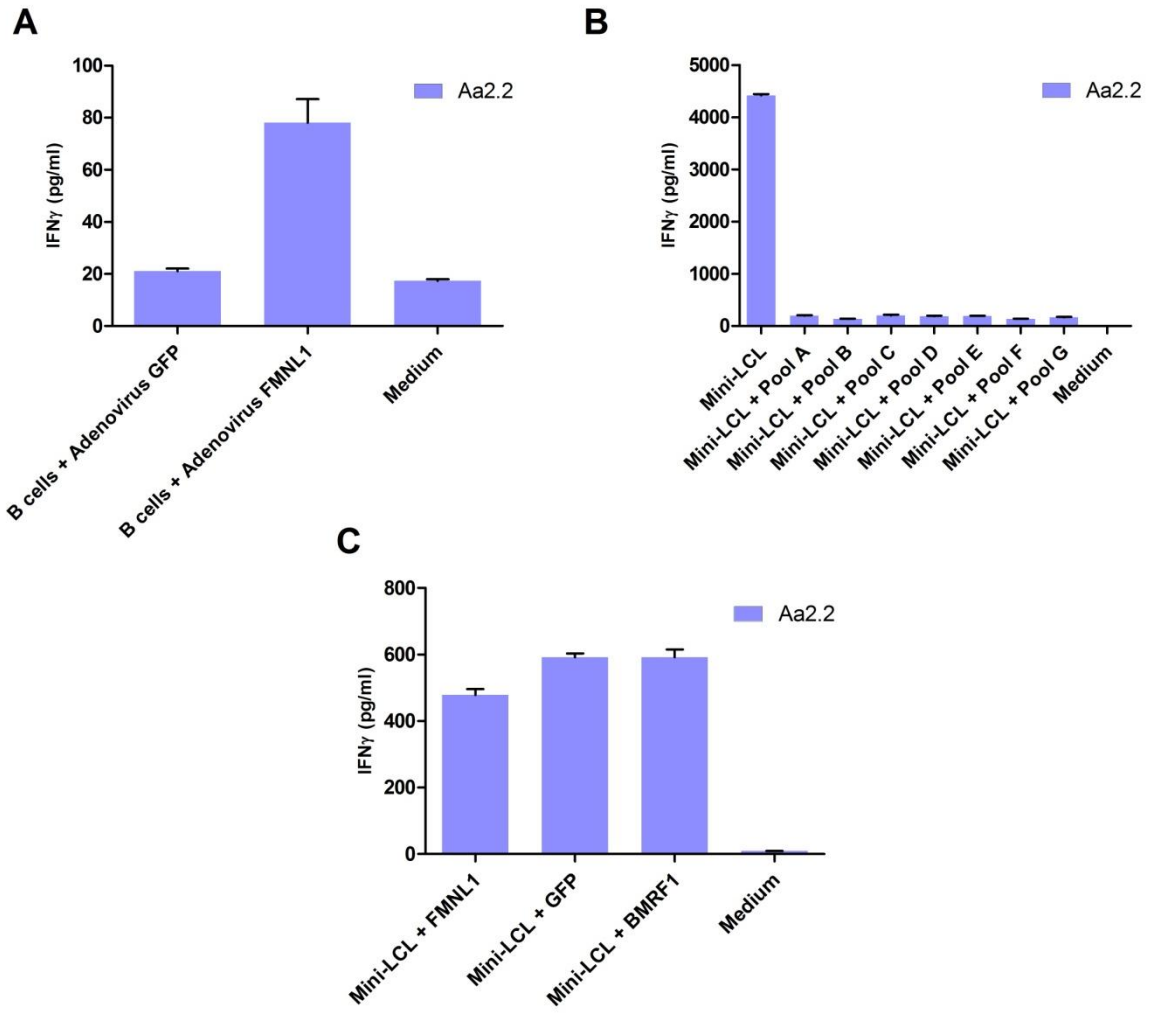

Figure 6-10: Investigation of peptide specificity of T cell clone Aa2.2

IFN-γ secretion of Aa2.2 was analyzed after 24h co-incubation with different targets in 96 well plates to investigate recognition of epitopes from FMNL1. Supernatants were harvested and analyzed by IFN-γ ELISA. (A) B cells adenovirally transduced with FMNL1 or GFP were used as targets. (B) Autologous Mini-LCL were pulsed with 7 peptide pools covering the FMNL1 peptide library or (C) with different proteins purified by the same protocol: FMNL1, GFP or BMRF1 derived from EBV. SD of triplicates are shown.

Interestingly, B cells transduced with FMNL1 adenovirus were considerably better recognized than B cells transduced with GFP (Figure 6-10A). However, pulsing Mini-LCL with different FMNL1 peptide pools did not result in epitope identification, it actually blocked recognition of Mini-LCL (Figure 6-10B). Moreover, further pulsing experiments using two alternative recombinant proteins, GFP and BMRF1 (an EBV protein), purified with the same protocol as FMNL1 showed no difference in IFN-γ secretion compared to FMNL1 (Figure 6-10C), suggesting unspecific stimulation by contaminations of the recombinant proteins (Bendz *et al.*, 2008).

6.3.4 Identification of TCR alpha and beta chains

As original T cell clones became senescent, TCR were identified, cloned and retrovirally transferred into fresh effector cells (see 6.4). Aa2.2 and Bb5.14 both expressed exclusively one α and one β chain. Thus, analysis of the TCR repertoire demonstrated clonality of both T cell clones (Aa2.2 Figure 6-11, Bb5.14 Figure 6-12). Expressed TCR α and β chains are listed in Table 6-2.

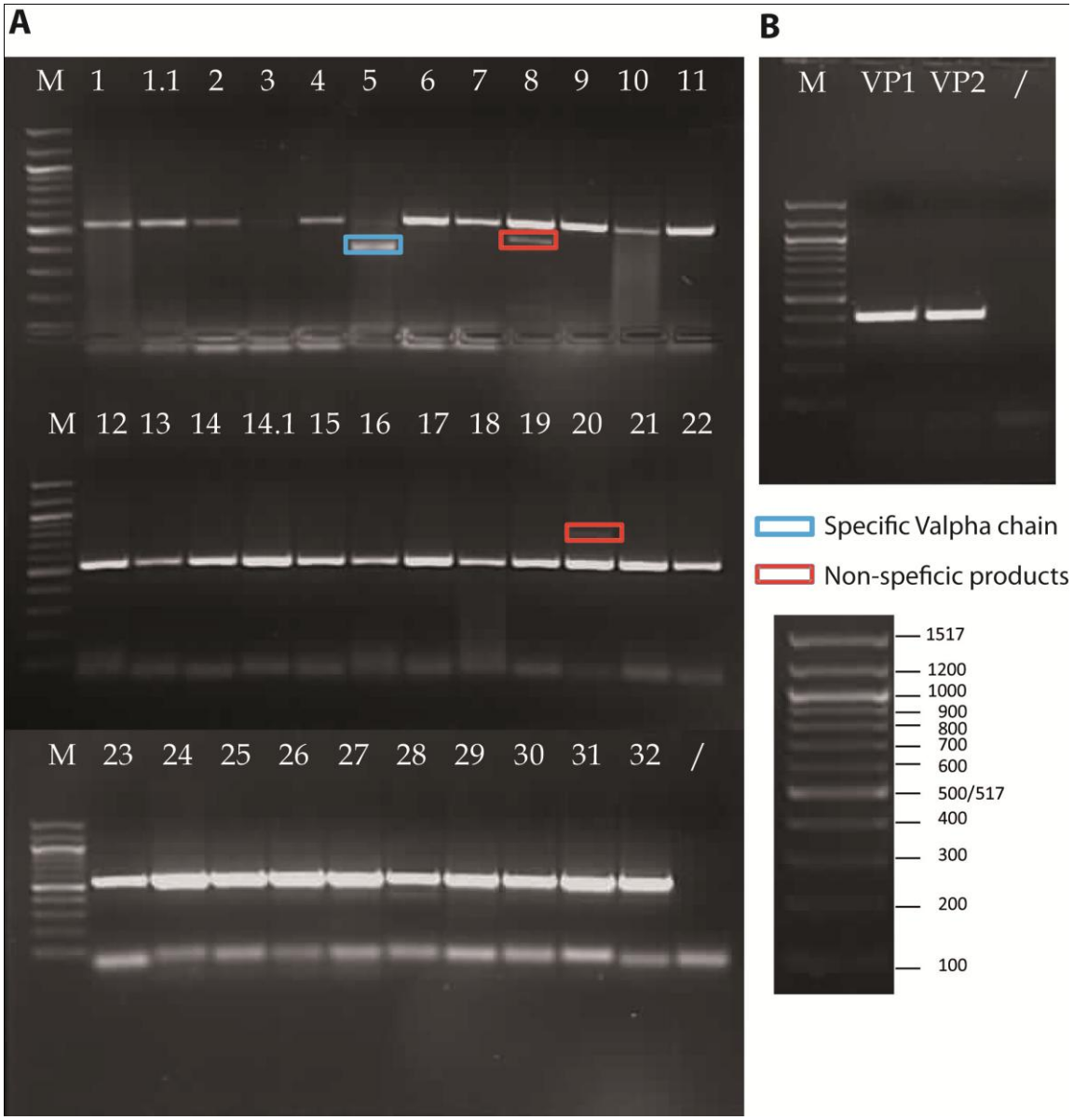

Figure 6-11: TCR α/β repertoire of Aa2.2

RNA was isolated from Aa2.2 and cDNA was synthesized to analyze the TCR α/β repertoire. (A) For TCR alpha chain repertoire one primer pair amplifying the constant TCR alpha chain was used as internal control (product size 550 bp) and one primer pair comprising one specific primer for the variable chains as indicated by the numbers above the lanes (Steinle *et al.*, 1995). Bands present next to the control bands are marked with a blue or red rectangle depending on the specificity of the

product. The blue rectangle represents the specific TCR alpha band whereas the red rectangle marks bands, resulting in non-sense sequences. (B) For the TCR beta chain repertoire, we used degenerated beta chain primer pairs VP1 and VP2, each covering several beta chains (Zhou *et al.*, 2006a). Both primer pairs generated products which were identical in their sequence.

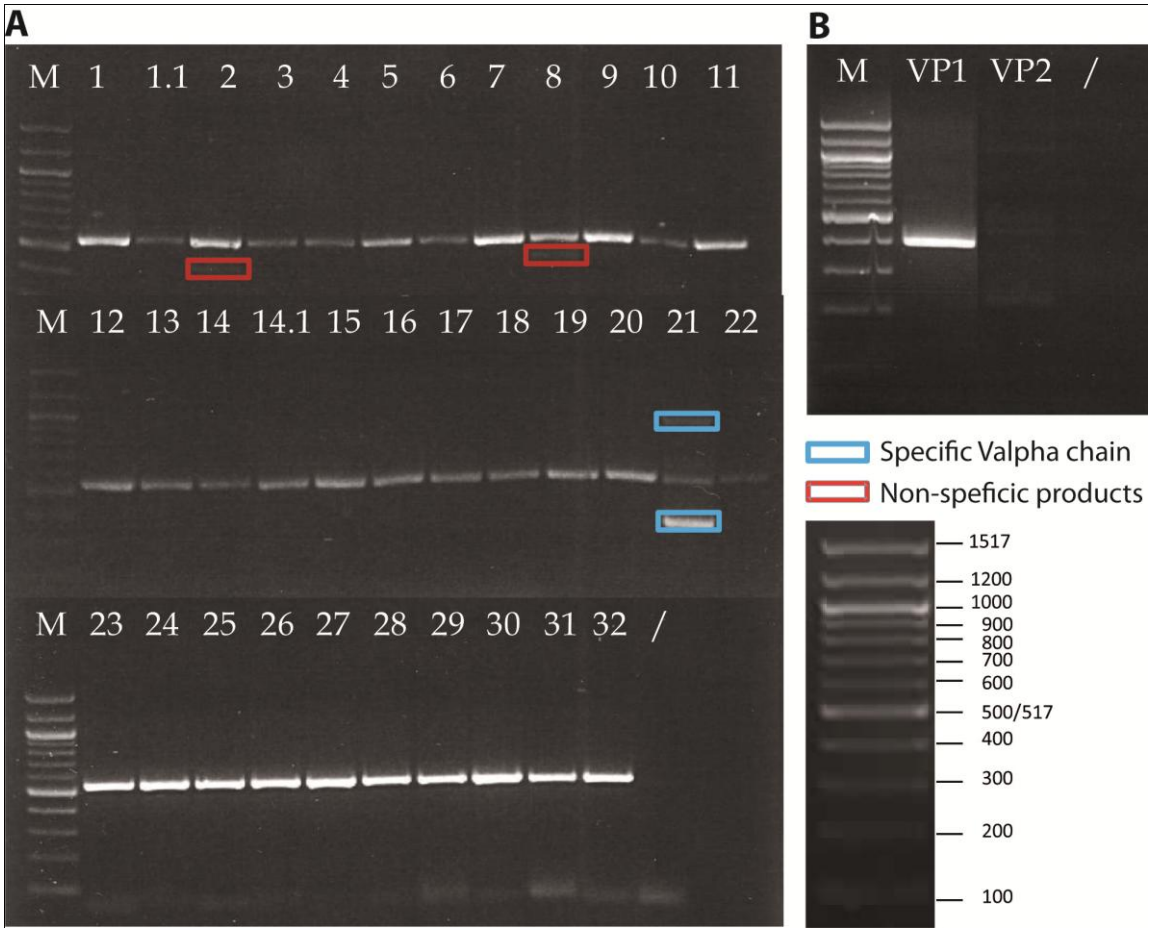

Figure 6-12: TCR α/β repertoire of Bb5.14

RNA was isolated from Bb5.14 and cDNA was synthesized to analyze the TCR α/β repertoire. (A) For TCR alpha chain repertoire one primer pair amplifying the constant TCR alpha chain was used as internal control (product size 550 bp) and one primer pair comprising one specific primer for the variable chains as indicated by the numbers above the lanes (Steinle *et al.*, 1995). Bands present next to the control bands are marked with a blue or red rectangle depending on the specificity of the product. The blue rectangle represents the specific TCR alpha band whereas the red rectangle marks bands, resulting in non-sense sequences. (B) For the TCR beta chain repertoire, we used degenerated beta chain primer pairs VP1 and VP2, each covering several beta chains (Zhou *et al.*, 2006a). Primer pair VP1 generated one specific product identified by sequencing.

Clone	TRAV	TRAJ	TRBV	TRBJ	TRBD
Aa2.2	06*02	04*01	3-1*01	2-2*01	2*02
Bb5.14	29/DV5*01	13*02	6-4*01	1-5*01	1*01

Table 6-2: TCR chains of clones Aa2.2 and Bb5.14

6.4 Further characterization of TCR Aa2.2 and Bb5.14 after TCR-transfer

6.4.1 Establishment of TCR-transfer into fresh autologous and allogeneic effector T cells

To further investigate specificity and tumor reactivity of isolated TCR, we transferred these molecules into fresh T cell populations, such as PBMC, $CD4^+$ or $CD8^+$ T cells. Therefore, we constructed MP71 vectors comprising either α or β chain of TCR Aa2.2 (Aa2.2) or TCR Bb5.14 (Bb5.14). α and β chain were then cotransfected with gag/pol and env bearing vectors in 293T cells. 293T cells produce retroviral particles, subsequently used for infection of T cell populations. However, we were not able to generate transduction rates over 5 % using native TCR chains (data not shown). Therefore, we optimized the TCR by the following two modifications:

- Insertion of a P2A element, originating from the Picorna virus. This allowed us to include both α and β chains into one vector backbone for equimolar expression of both chains (Leisegang *et al.*, 2008).
- Codon optimization, replacing DNA triplets encoding one specific amino acid by codons resulting in improved translation (Scholten *et al.*, 2006).

TCR constructs optimized by these modifications did not result in specific functionality after TCR transfer (data not shown). We therefore investigated the effect of additional modifications:

- Replacement of the constant chains of the TCR by codon optimized mouse constant chains. This modification has been previously demonstrated to result in improved TCR chain pairing, avoiding mispairing with endogenous chains of transduced T cells (Cohen *et al.*, 2006).
- Insertion of an additional disulfide bond between constant chains, which has been shown to improve pairing and enhance expression of the transduced TCR (Cohen *et al.*, 2007; Voss *et al.*, 2010).

These modifications highly enhanced TCR transduction into autologous (Figure 6-13) and allogeneic T cell populations (data not shown).

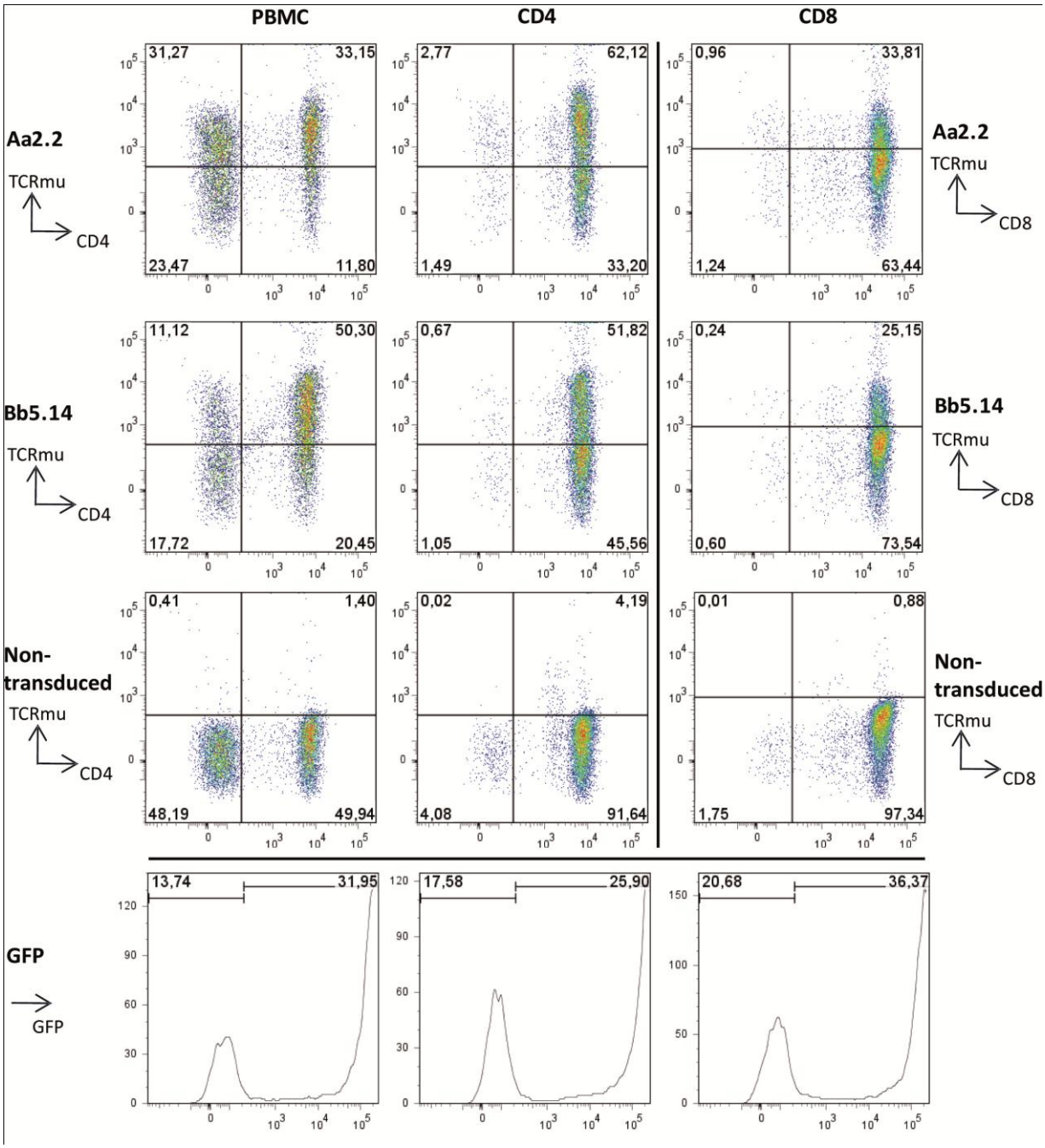

Figure 6-13: Successful transfer of TCR Aa2.2 and Bb5.14 into different effector cell populations

TCR derived from isolated T cell clones were retrovirally transduced in effector T cell populations (PBMC, $CD4^+$, $CD8^+$ T cells) of the same donor. Expression levels of the transferred TCR were analyzed (6 days after transduction) by flow cytometry after staining with anti-human CD4 or CD8 and TCRmu antibody (first and second row). Non-transduced cells were identically stained (third row). GFP-transduced cells were used as mock control (last row).

Transduction rates were investigated by mouse TCR specific antibody (TCRmu) staining. Transduction rates analyzed by α-TCRmu-PE staining were between 18 % and 71 %. In the here shown transduction, 25 % to 64 % of cells were $TCRmu^+$ (Figure 6-13).

6.4.2 Self reactivity of Aa2.2- an Bb5.14- transduced effector cell populations

One important issue which needs to be clarified for a potential clinical setting is the presence of self reactivity of TCR potentially resulting in fratricide of T cells.

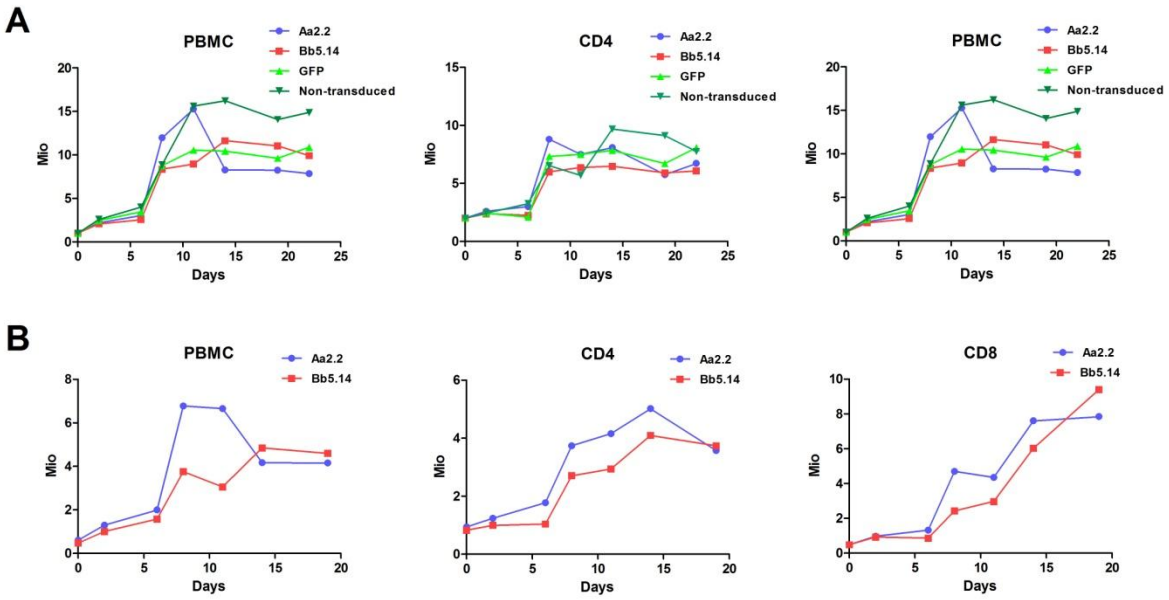

Figure 6-14: No fracticide of TCR-transduced autologous PBMC, CD4$^+$ and CD8$^+$ T cells
(A) Proliferation of cells after retroviral TCR transfer was investigated by vital cell counting of the total cell populations Aa2.2-transduced (●), Bb5.14-transduced (■), GFP-transduced (▲) and Non-transduced (▼). (B) Calculation of proliferation curves of TCRmu$^+$ T cells (Aa2.2-transduced (●) and Bb5.14-transduced (■)).

Therefore, we investigated proliferation of autologous effector populations after TCR transfer in total as well as growth of TCR-transduced T cells (Figure 6-14). No significant differences in proliferation of TCR-transduced, GFP-transduced and non-transduced effector populations were observed in non-purified PBMC, CD4$^+$ or CD8$^+$ T cells (Figure 6-14A). Moreover, TCRmu$^+$ populations, indicating cells transduced with Aa2.2 or Bb5.14, showed no differences in growth in all three transduced effector cell populations (Figure 6-14B).
We additionally investigated if effector cells transduced with these TCR may detect activated T cells, as activated T cells express MHC class II. Effector populations transduced with both TCR did not secrete IFN-γ in response to PBMC or activated PBMC of the same donor (Figure 6-15). Proliferation curves and investigation of self reactivity were also performed with allogeneic TCR-transduced T cells showing similar results. Thus, T cells transduced with Aa2.2 or Bb5.14 do not bear self reactivity against autologous or allogeneic T cells.

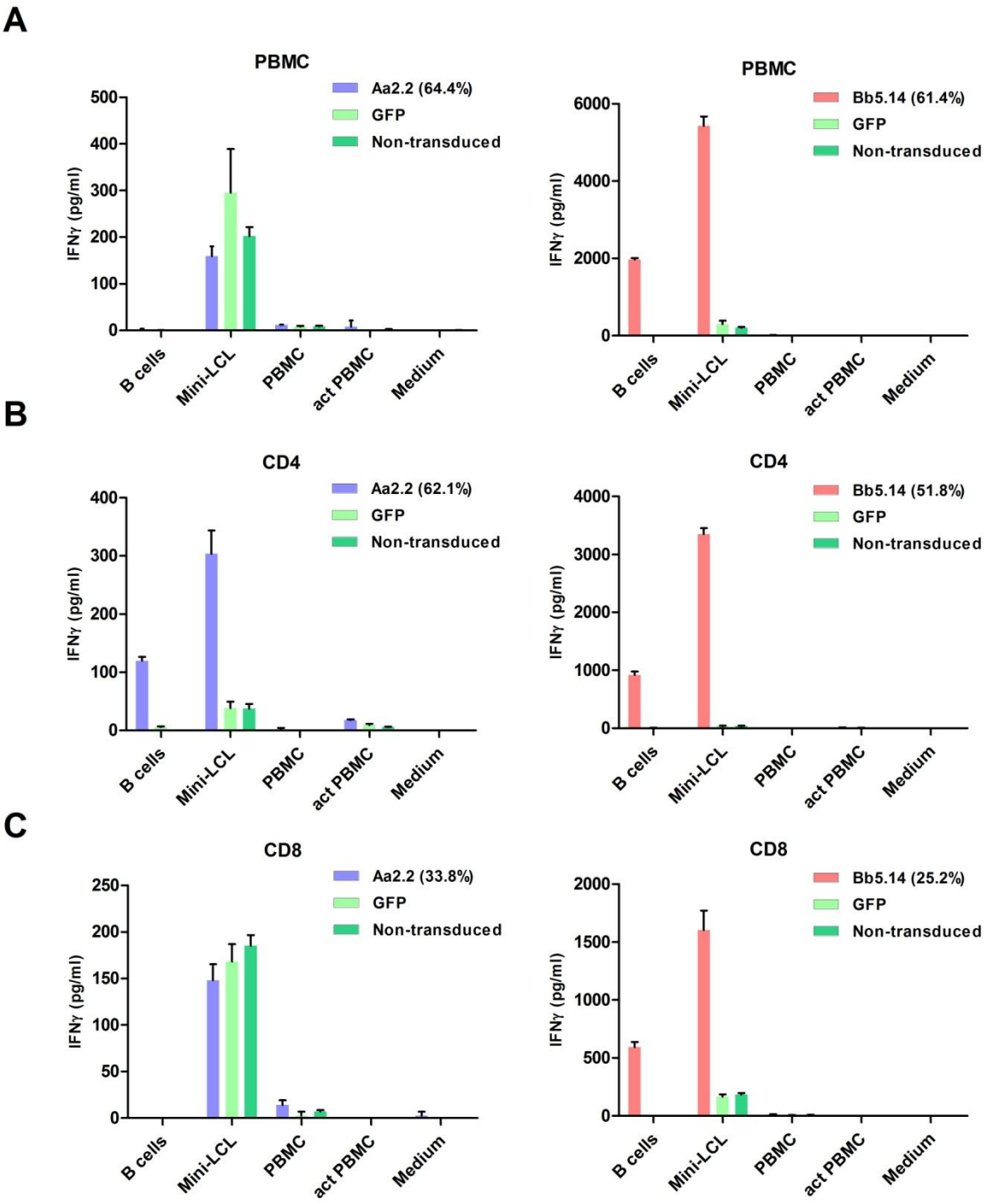

Figure 6-15: Effector cells transduced with Aa2.2 and Bb5.14 are not reactive against autologous T cells

TCR-transduced PBMC (A), $CD4^+$ (B), $CD8^+$ (C) T cells were co-incubated with diverse target cells (E:T ratio = 5:1) such as autologous B cells, Mini-LCL, PBMC and PBMC activated with IL-2 and Okt-3 (act PBMC) for 24h. Percentages of $TCRmu^+$ cells at the time point of this assay (6 days after transduction) are indicated between brackets in the graphs. GFP-transduced and non-transduced effector cell populations were used as controls. SD of triplicates are shown. Of note, scales used to indicate the reactivity of both TCR differ significantly.

6.4.3 Target cell recognition patterns of Aa2.2- and Bb5.14-transduced T cells

To further characterize both TCR, TCR-transduced T cells were incubated in 24h stimulation assays with a variety of target cells. Data shown in Figure 6-16 and Figure 6-17 were performed with autologous TCR-transduced $CD4^+$ T cells. Similar results were observed in experiments with other autologous ($CD8^+$, PBMC) as well as allogeneic effector populations (data not shown).

Effector cells transduced with Aa2.2 detected exclusively B cell derived cell lines (Figure 6-16A and B), whereas Bb5.14-transduced cells secreted IFN-γ in response to different professional antigen presenting cells such as DC and macrophages specifically differentiated by cytokines into type 1 or 2 macrophages (M1 and M2) (Figure 6-16A). Of note, reactivity of Bb5.14-transduced T cells was much stronger compared to Aa2.2-transduced T cells (Figure 6-16A). Recognition of B cells by both TCR could be inhibited by treatment of target cells with HLA-DR blocking antibody whereas blocking with an MHC class I specific antibody was not effective (Figure 6-16B). To define the restriction element of both TCR, we used EBV-negative Burkitt BJAB cells transfected with HLA-DRB1*01, HLA-DRB1*11 or GFP as target cells (Figure 6-16C). Aa2.2-transduced T cells secreted over background IFN-γ in response to BJAB cells transfected with HLA-DRB1*11, whereas Bb5.14 was clearly restricted to the genotype HLA-DRB1*01 (Figure 6-16C).

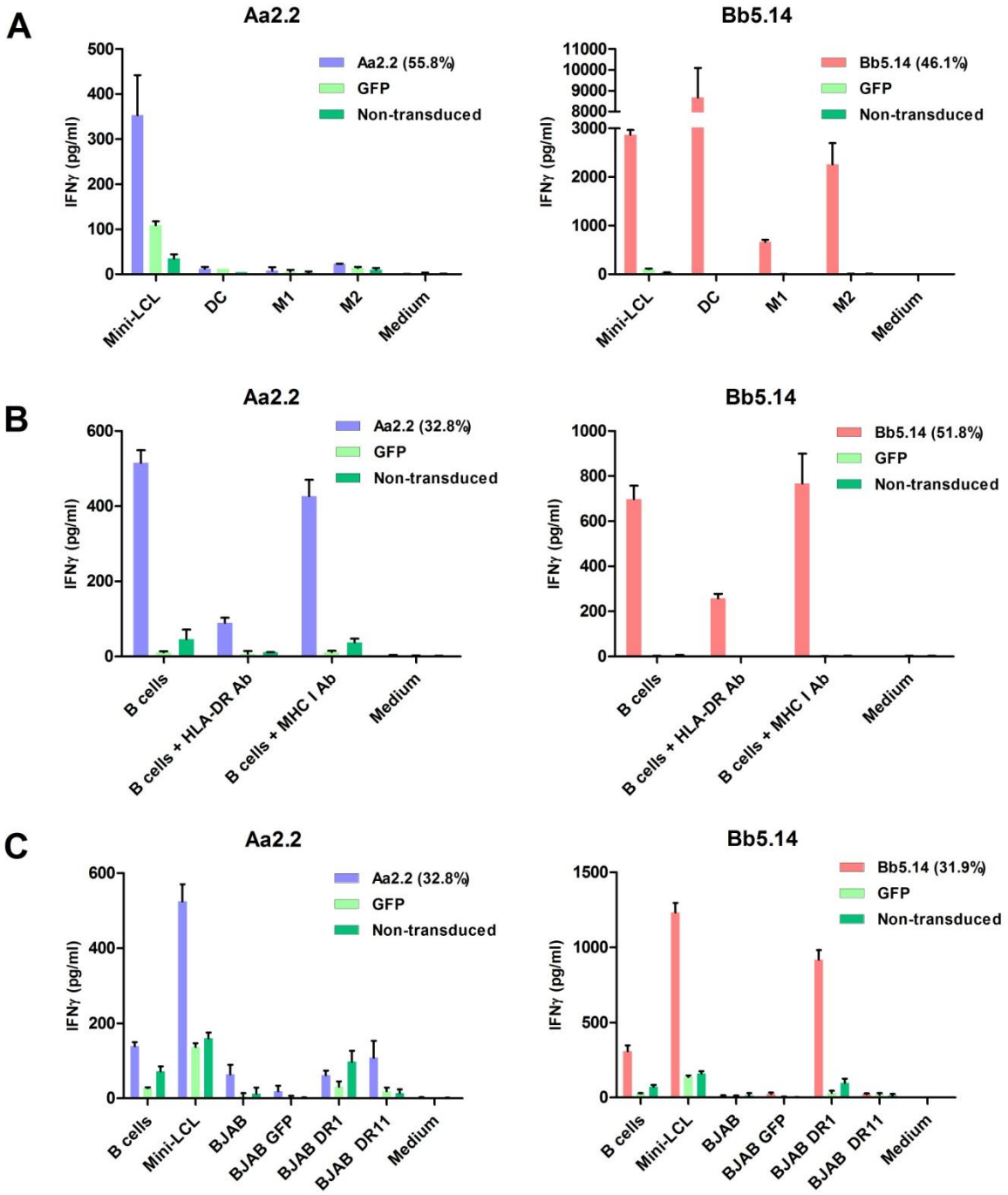

Figure 6-16: Aa2.2- and Bb5.14-transduced T cells reveal significant differences in functionality and reactivity patterns and recognize distinct MHC class II restriction elements

Both TCR were investigated after transfer into autologous $CD4^+$ T cells and co-culture with different target cells (E:T ratio = 5:1). Supernatants of co-cultures were removed after 24h and analyzed by IFN-γ ELISA. Percentages of $TCRmu^+$ cells at the time of the experiments (8 and 12 days after transduction) are shown in each graph. GFP-transduced and non-transduced T cells were used as controls. SD of triplicates are shown. (A) Different professional antigen presenting cells as Mini-LCL, DC and macrophages differentiated into type M1 or M2 were used as targets. (B) Anti-HLA-DR and anti-MHC I antibodies were used for determination of MHC restriction. (C) The distinct restriction element of the TCR was determined by Burkitt lymphoma BJAB cells transfected with donor-specific HLA-DRB1*01, HLA-DRB1*11 or GFP. Of note, scales used to indicate the reactivity of both TCR may differ significantly.

Investigation of several HLA-matched LCL confirmed these results (Figure 6-17A). T cells transduced with Aa2.2 recognized most HLA-DRB1*11 expressing LCL beyond background whereas Bb5.14-transduced T cells demonstrated strong reactivity when co-cultured with HLA-DRB1*01 expressing LCL (Figure 6-17A). Most importantly, T cells transduced with both TCR, Aa2.2 or Bb5.14, recognized CLL samples from partially HLA-matched patients (Figure 6-17B), rendering these TCR interesting for potential clinical application.

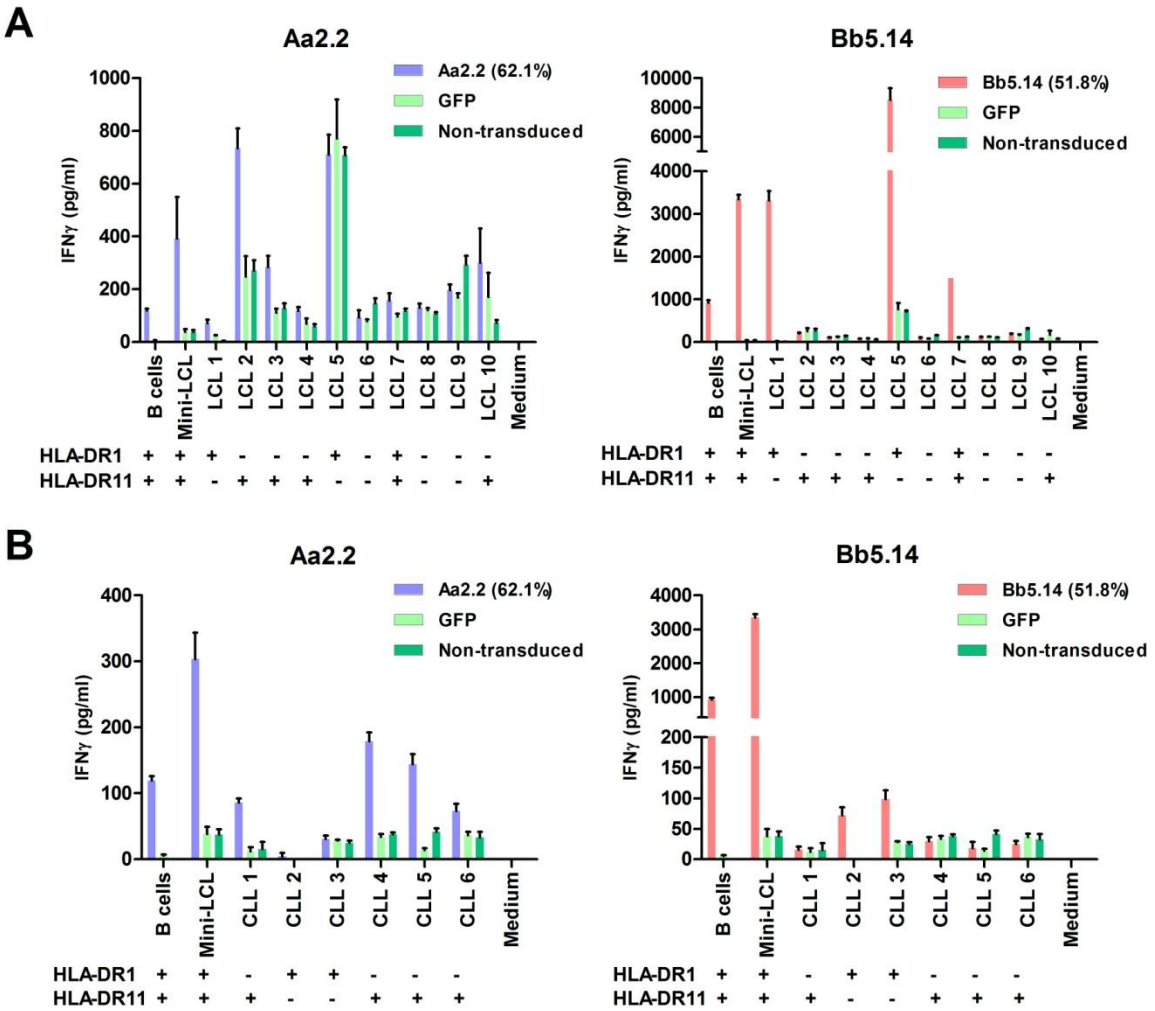

Figure 6-17: T cells transduced with Aa2.2 and Bb5.14 recognize matched LCL and CLL samples

Both TCR were examined after transfer into autologous $CD4^+$ T cells and co-culture with different target cells (E:T ratio = 5:1). Supernatants of co-cultures were removed after 24h and analyzed by IFN-γ ELISA. Percentages of $TCRmu^+$ cells at the time of the experiments (6 days after transduction) are shown in each graph. GFP-transduced and non-transduced T cells were used as controls. SD of triplicates are shown. (A) TCR-transduced effector cells were incubated with HLA-DR matched LCL. (B) Tumor reactivity was investigated by co-incubation with HLA-DR matched, CD40L-activated primary CLL cells. Of note, scales used to indicate the reactivity of both TCR may differ significantly.

6.5 Treatment of target cells with LPS mediates oppositional effects on regulation of TCR-transduced effector populations depending on the transgenic TCR

6.5.1 Protein and LPS treatment of target cells induces oppositional effects on both TCR

As not only FMNL1 protein, but also alternative proteins led to enhancement of IFN-γ levels secreted by clone Aa2.2 (Figure 6-10), we further investigated this finding in TCR-transduced T cells. We suspected that the protein could be contaminated with LPS, a cell wall component of gram$^-$ bacteria, since the protein was dialysed under non-sterile conditions (Bendz *et al.*, 2008). LPS-detection by TLR4 provokes stimulation of the immune system and could thus possibly influence T cell respose. Therefore, we pulsed Mini-LCL (data not shown), B cells and C1R cells transfected with HLA-DRB1*0101, HLA-DRB1*1101 or GFP either with FMNL1 or LPS and incubated so treated cells with autologous TCR-transduced PBMC (data not shown) and CD4$^+$ T cells (Figure 6-18).

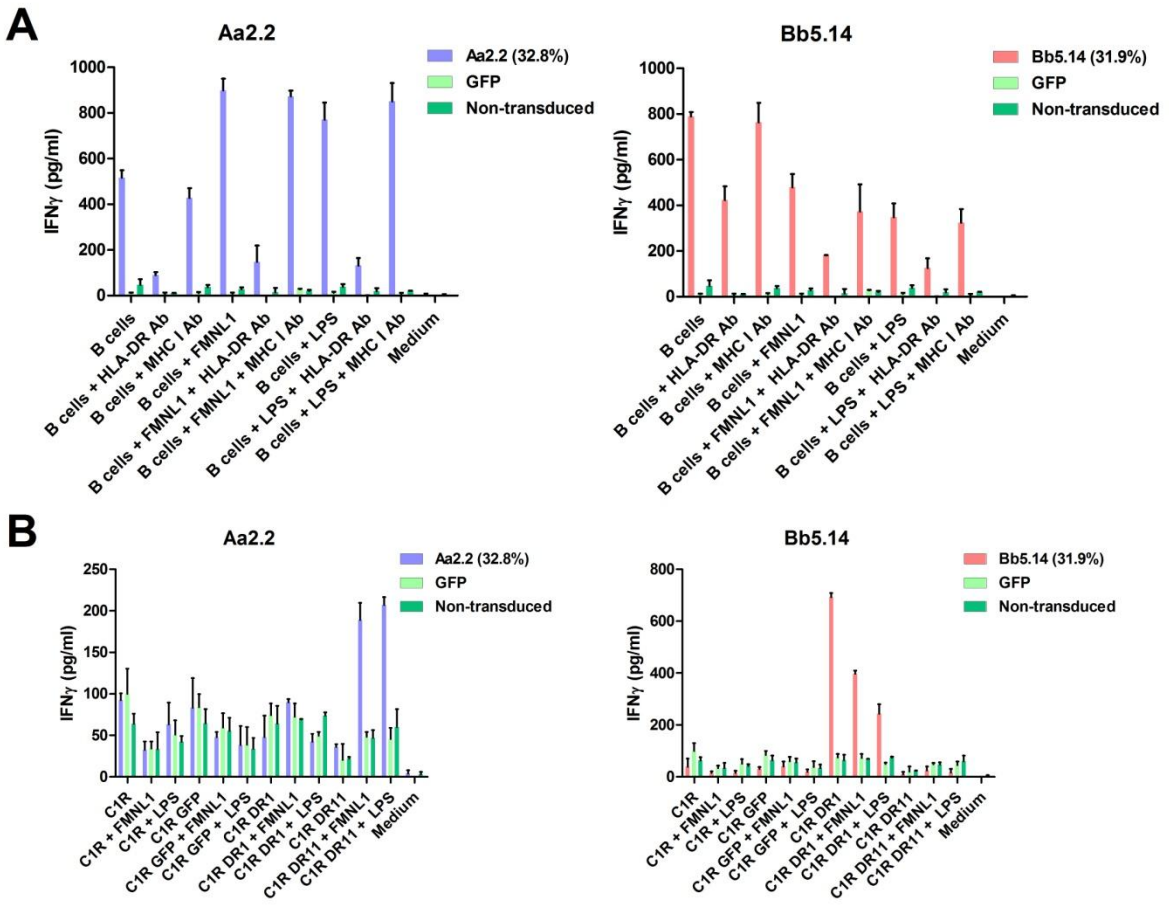

Figure 6-18: Modulation of B cell recognition by protein as well as LPS treatment of B cells

B cells were pulsed with recombinant FMNL1 protein or alternatively with LPS to compare the impact of these treatment modalities on the functionality of both TCR. Supernatants of mixed cell cultures (E:T ratio = 5:1) were collected after 24h and IFN-γ concentration was determined by IFN-γ ELISA. Percentages of $TCRmu^+$ cells at the time point of the assays (5 days after transduction) are indicated in each graph. GFP-transduced and non-transduced effector cell populations were used as controls. SD of triplicates are shown. (A) TCR-transduced $CD4^+$ T cells were stimulated with autologous B cells or B cells treated with FMNL1 or LPS. Target cells were alternatively blocked with α-HLA-DR or α-MHC I antibodies. (B) C1R cells or C1R transfected with HLA-DRB1*01, HLA-DRB1*11 or GFP were used as targets. Alternatively these cells were previously pulsed with FMNL1 or LPS. Of note, scales used to indicate the reactivity of both TCR may differ significantly.

Treatment of target cells with recombinant FMNL1 protein as well as LPS, showed the same effect on the TCR-transduced T cells. Interestingly, this effect was distinct for both TCR: reactivity of Aa2.2-transduced T cells was enhanced when target cells were pulsed with protein or LPS whereas reactivity of Bb5.14-transduced T cells was clearly reduced when target cells were pretreated in this way (Figure 6-18). IFN-γ secretion could be blocked by HLA-DR antibody, but not by MHC I blockade (Figure 6-18A). Moreover, this effect was dependent on expression of the adequate HLA-DR molecule on target cells (Figure 6-18B).

6.5.2 Different TLR agonists provoke opposed regulation of TCR Aa2.2 and Bb5.14

As previously described, LPS is a cell wall component of gram$^-$ bacteria detected by TLR4. LPS elicited differential regulation of T cell responses depending on the transgenic TCR. We therefore pulsed B cells with TLR3 ligand Poly (I:C), TLR5 agonist Flagellin or CpG ligand TLR9 in addition to LPS. B cells were then incubated for 24h with autologous TCR-transduced PBMC, CD4$^+$ or CD8$^+$ cells (Figure 6-19). Similar results were observed in experiments with allogeneic effector populations (data not shown).

All four TLR ligands had the same distinct effect on T cells transduced with Aa2.2 or Bb5.14. Reactivity of Aa2.2-transduced effector populations was significantly enhanced when B cells were pulsed with TLR ligands, whereas Bb5.14-transduced T cells secreted lower levels of IFN-γ in response to TLR ligand pulsed B cells (Figure 6-19). IFN-γ secretion could be blocked by treatment with HLA-DR antibody in PBMC (Figure 6-19, first row), CD4$^+$ and CD8$^+$ T cells (data not shown). This confirmed dependence of the observed effect on specific TCR activation and signalling.

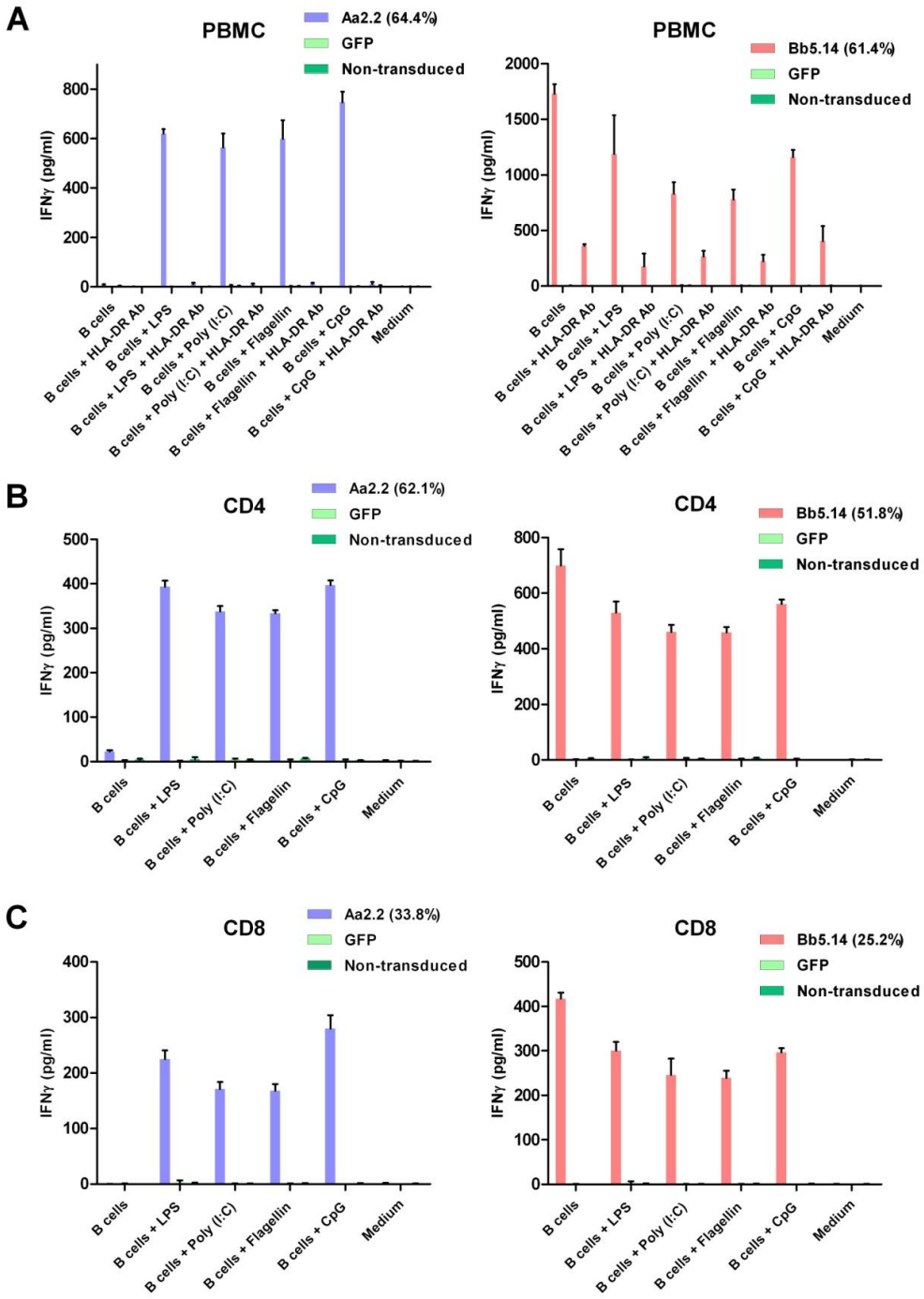

Figure 6-19: Modulation of the T cell response of TCR-transduced effector cells by TLR-ligand treatment of target cells

The effect of TLR ligand treatment of target B cells on the functionality of TCR-transduced autologous PBMC, $CD4^+$ or $CD8^+$ T cells was examined. Supernatants of mixed cell cultures (E:T ratio = 5:1) were collected after 24h and IFN-γ concentration was determined by IFN-γ ELISA. Percentages of $TCRmu^+$ cells at the time point of the assays (5 days after transduction) are indicated in each graph. GFP-transduced and non-transduced effector cell populations were used as controls. SD of triplicates are shown. B cells were treated with different TLR ligands for 2h followed by intensive washing. B cells and B cells previously pulsed with different TLR ligands were incubated with (A) PBMC, (B) $CD4^+$, (C) $CD8^+$ T cells. In (A), each of these conditions was additionally treated with HLA-DR antibody. Of note, scales used to indicate the reactivity of both TCR differ significantly.

Moreover we pulsed T cells with TLR ligands prior co-culture with B cells, in order to examine whether the effect was induced by TLR dependent modulation of the B cells, or by a direct effect on T cells (Figure 6-20). Reactivity of Aa2.2-transduced T cells in response to B cells was not enhanced when T cells were pretreated with TLR ligands (Figure 6-20, left column). IFN-γ secretion by Bb5.14-transduced T cells was slightly reduced when PBMC or $CD8^+$ T cells were pretreated with TLR ligand (Figure 6-20, right column upper and lower panel). This was less evident for $CD4^+$ T cells (Figure 6-20, right column, central panel). T cells did not unspecifically secrete IFN-γ in response to TLR ligand treatment without TCR stimulation (Figure 6-20). Thus, TLR ligands have a distinct impact on TCR-dependent cytokine secretion depending on characteristics of the specific TCR. Modulation of TCR-induced effector functions are at least in case of Aa2.2 mediated by an indirect effect of TLR ligands on antigen presenting cells.

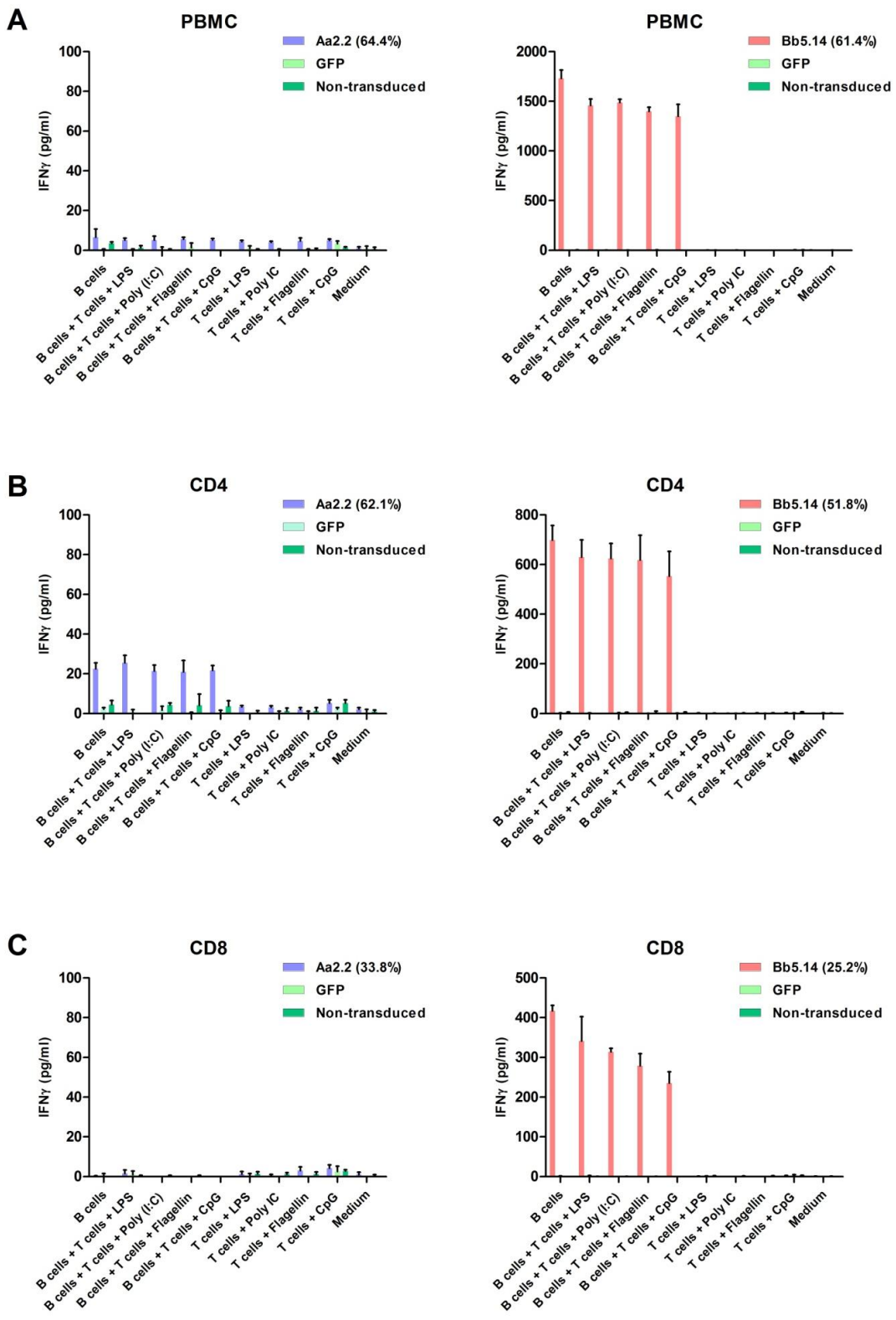

Figure 6-20: Effect of TLR-ligand treatment of TCR-transduced T cells on the specific T cell response

(A) PBMC, (B) $CD4^+$, (C) $CD8^+$ T cells were treated with different TLR ligands for 2h and were incubated after intensive washing with B cells or alternatively alone. Supernatants of mixed cell cultures (E:T ratio = 5:1) were collected after 24h and IFN-γ concentration was determined by IFN-γ ELISA. Percentages of $TCRmu^+$ cells at the time point of the assays (5 days after transduction) are indicated in each graph. GFP-transduced and non-transduced effector cell populations were used as controls.

SD of triplicates are shown. Of note, scales used to indicate the reactivity of both TCR differ significantly.

6.6 Investigation of multifunctionality of distinct TCR after transduction in different effector cell populations

6.6.1 TCR-transduced effector cells reveal a mixed Th lineage cytokine pattern modulated by TLR ligands, dependent on the transgenic TCR and the target cell type

Cytokine secretion patterns are very important for specific functions of $CD4^+$ T cells and play a critical role in the tumor environment (O'Shea and Paul, 2010; Wan and Flavell, 2009). Therefore, we investigated cytokine secretion of TCR-transduced effector cells more in detail. T cells transduced with Aa2.2 secreted IL-5, IL-10, IL-13, IL-17 and IFN-γ in response to B cells only after TLR ligand pulsing of target cells (Figure 6-21A). T cells transduced with TCR Bb5.14 showed a similar cytokine secretion pattern but additionally secreted IL-2, IL-4 and TNF-α in response to B cells (Figure 6-21A). Cytokine levels secreted by Bb5.14-transduced T cells were reduced when target cells were pulsed with TLR ligands, and secretion of IL-2 and TNF-α could not be detected anymore (Figure 6-21A). Hence, multiplex analysis confirmed the observations made after analysis of the supernatants by IFN-γ ELISA with respect to modification by treatment of target cells with TLR ligands (Figure 6-19). Both TCR showed a high IFN-γ/IL-10 ratio in response to B cells (Figure 6-21A). In contrast, the IFN-γ/IL-10 ratio was reversed when T cells were incubated with Mini-LCL, LCL or CLL samples (Figure 6-21B and C) and IL-17 secretion disappeared entirely (Figure 6-21B and C). Hence, the constitution of these mixed Th1 / Th2 cytokine patterns is not only dependent on the transferred TCR, but also on target cells.

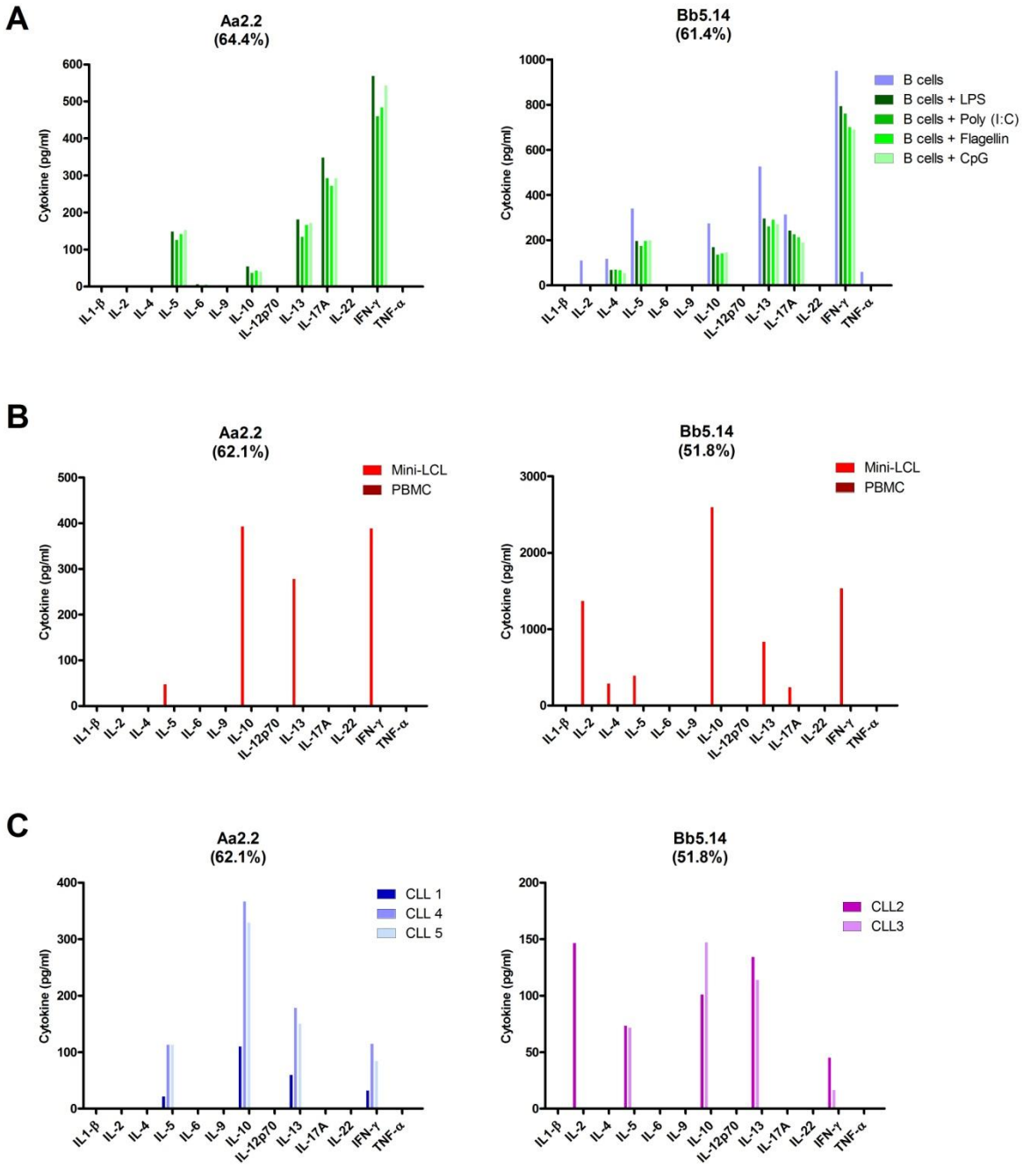

Figure 6-21: TCR-transduced effector cells reveal a mixed Th lineage cytokine pattern modulated by TLR ligands, dependent on the transgenic TCR and the target cell type

TCR-transduced effector cells were analyzed for the secretion of different cytokines in response to selected target cells (E:T ratio = 5:1). Supernatants collected from 24h stimulation assays were investigated by Flow Cytomix. Percentages of TCRmu$^+$ cells at the time point of the assays (5 and 6 days after transduction) are indicated in each graph. GFP-transduced effector cell populations were used as controls (data not shown). (A) B cells and B cells treated with different TLR ligands were used as targets for stimulation of TCR-transduced autologous PBMC. (B) Mini-LCL and autologous PBMC as well as (C) HLA-DR-matched CLL samples were co-incubated with autologous TCR-transduced CD4$^+$ T cells. Of note, scales used to indicate the reactivity of both TCR may differ significantly.

6.6.2 Cytotoxitic effect of both TCR towards target cells

It has previously been shown that $CD4^+$ T cells are able to mediate cytotoxicity towards target cells (van de Berg *et al.*, 2008). This function may be helpful for clinical application of adoptive T cell therapy (Quezada *et al.*, 2010). Therefore we investigated the ability of Aa2.2- and Bb5.14-transduced T cells to degranulate (Figure 6-22).

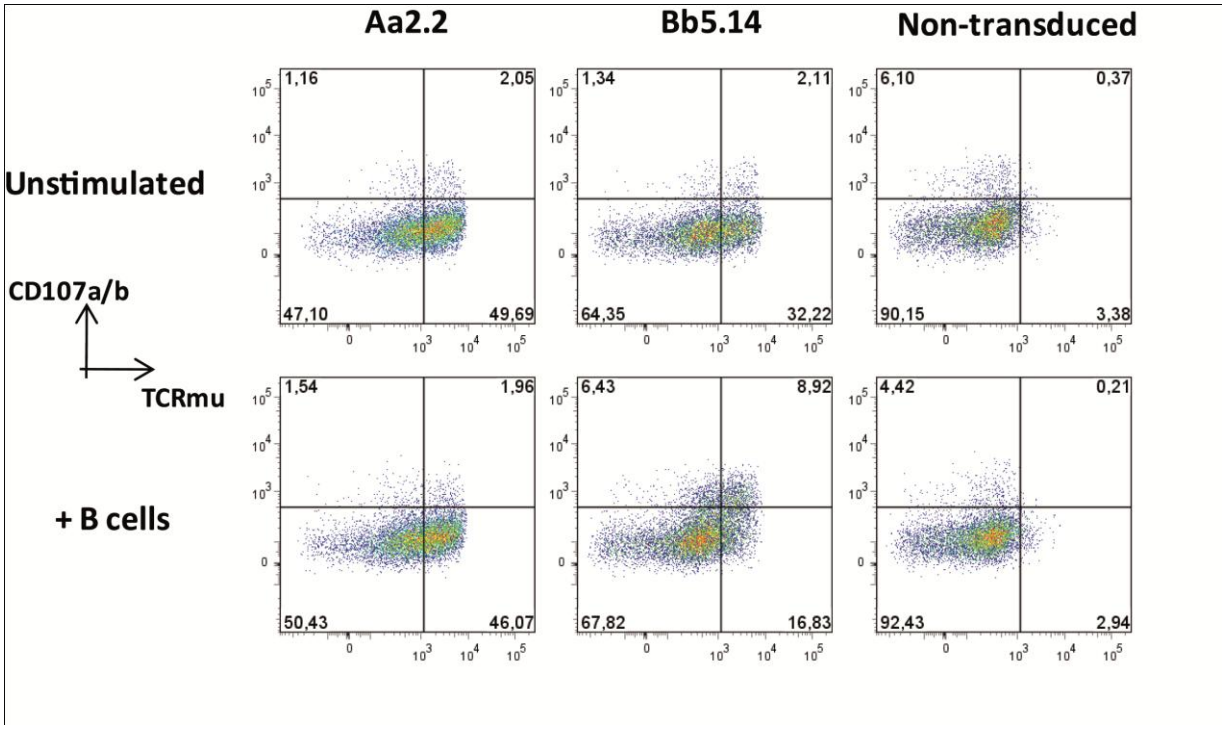

Figure 6-22: Degranulation of Bb5.14-transduced T cells in response to B cells

Allogeneic TCR-transduced PBMC were co-incubated 8 days after transduction with Far Red stained B cells. Brefeldin A was used for inhibition of secretion and Fitc-labelled CD107a/b antibodies for detection of degranulation. After 4h, cells were stained with surface markers followed by analysis by flow cytometry. The $CD8^-$ population is shown. B cells were excluded from analysis by gating following Far Red staining.

B cells were used as target cells in a 4h stimulation assay with allogeneic TCR-transduced PBMC. The $CD8^-$ population of Bb5.14-transduced PBMC degranulated in response to B cells, conversely Aa2.2-transduced T cells showed no degranulating population (Figure 6-22).

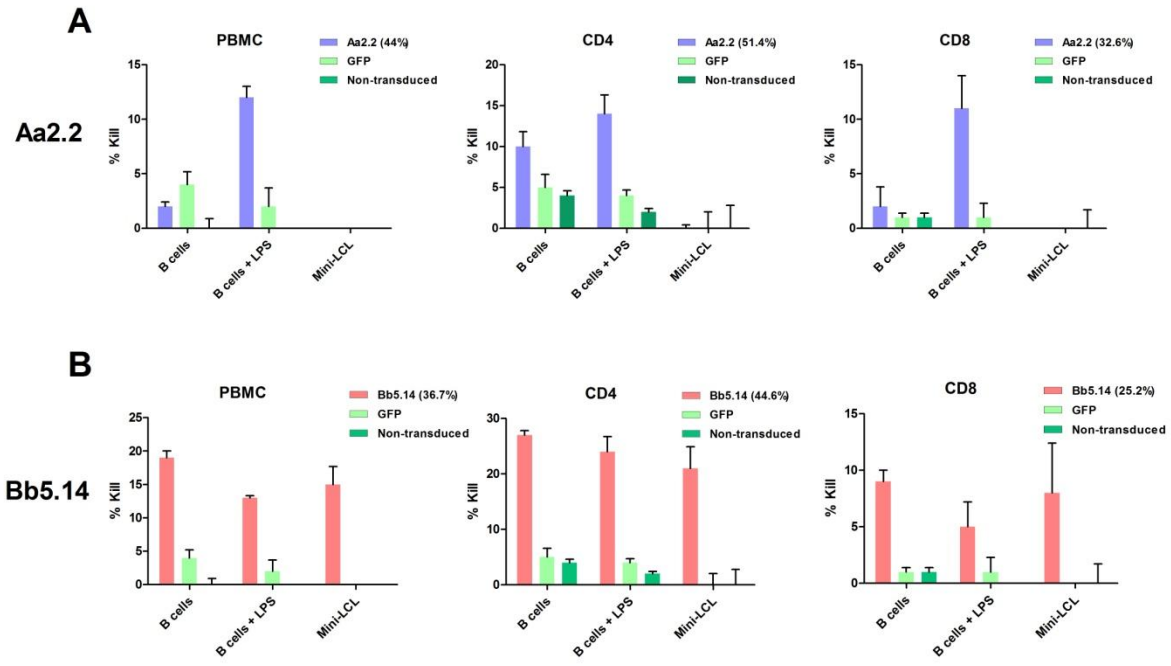

Figure 6-23: Cytotoxicity of Aa2.2- and Bb5.14-transduced PBMC, CD4$^+$ and CD8$^+$ T cells dependent on target cells and modulated by TLR ligands

Cytotoxicity of TCR-transduced T cell populations was analyzed by chromium-release assay. Target cells (B cells, B cells + LPS, Mini-LCL) were co-cultured with TCR-transduced, GFP-transduced or non-transduced effector cell populations (E:T ratio = 15:1). After 4h, supernatants were analyzed using a γ-counter. Percentages of TCRmu$^+$ cells at the time point of the assays (10 days after transduction) are indicated in each graph. SD of triplicates are shown.

Moreover, we further investigated cytotoxicity of TCR-transduced PBMC, CD4$^+$ and CD8$^+$ T cells in response to autologous B cells, LPS-pulsed B cells and Mini-LCL in a chromium release assay (Figure 6-23). Autologous Aa2.2-transduced PBMC and CD8$^+$ T cells killed B cells exclusively when pretreated with the TLR ligand LPS (Figure 6-23A). Aa2.2-transduced CD4$^+$ T cells were actually additionally slightly cytotoxic in response to untreated B cells (Figure 6-23A). Bb5.14-transduced T cells killed all tested target cells (B cells, LPS-pulsed B cells and Mini-LCL; Figure 6-23B). In the case of PBMC and CD8$^+$ T cells, we observed slight inhibition of killing activity when B cells were pulsed with LPS prior to the assay (Figure 6-23B). Thus, different T cell effector populations, including CD4$^+$ T cells, were able to mediate cytotoxicity after TCR-transduction. Moreover, cytotoxicity was again differentially influenced by TLR-stimulation of targeted B cells.

6.6.3 Antigen-induced proliferation of T cells transduced with both TCR

Antigen-specific proliferation is another criterion for multifunctionality of T cells. We therefore cultured CFSE-stained TCR-transduced T cells with B cells or B cells treated with LPS.

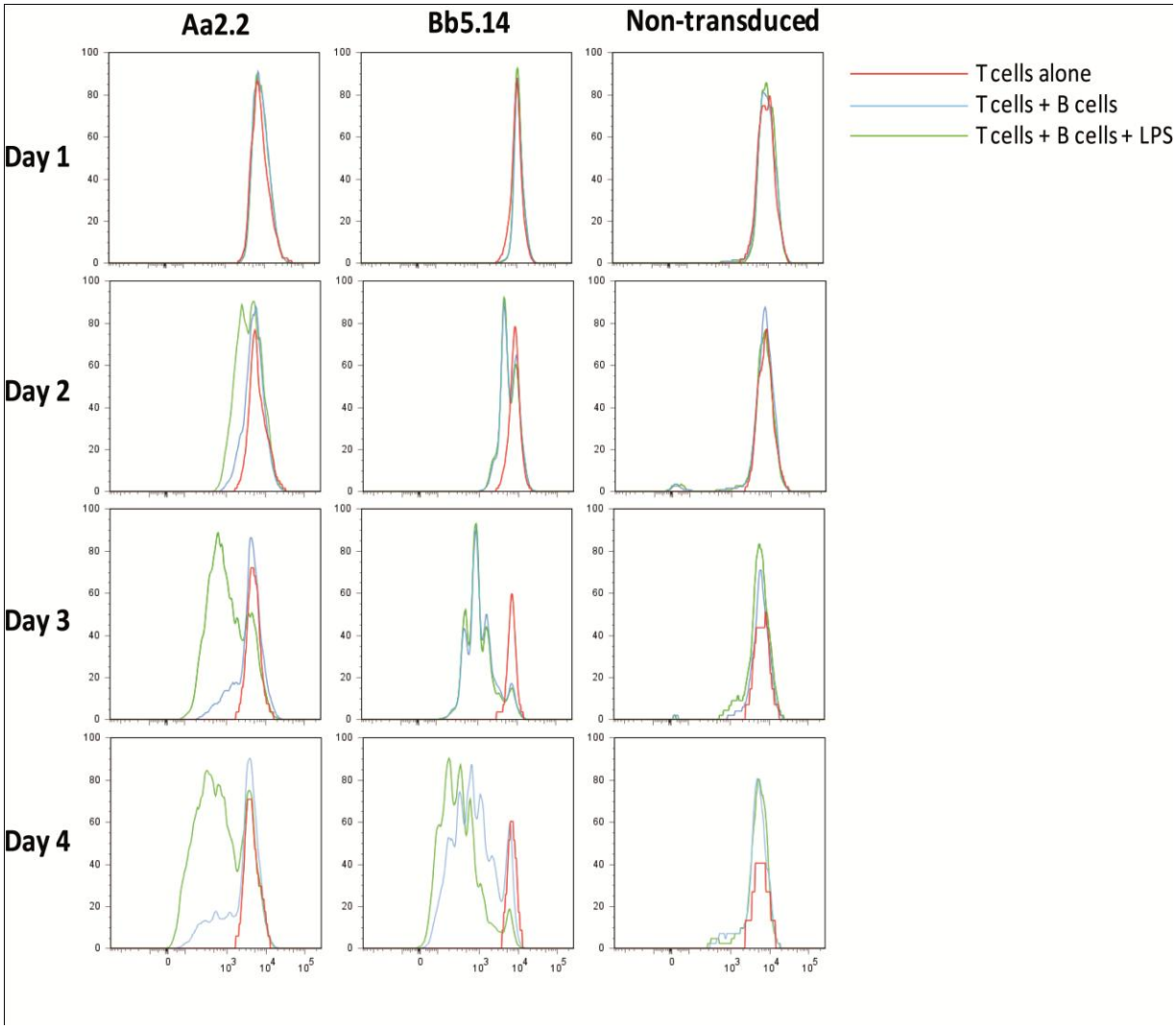

Figure 6-24: Specific proliferation of TCR-transduced T cells is enhanced by the TLR ligand LPS
TCR-transduced T cell populations (PBMC, $CD4^+$ and $CD8^+$ T cells) were stained with CFSE and co-cultured for 4 days with Far Red-stained, irradiated B cells or Far Red-stained, irradiated LPS-pulsed B cells. Alternatively, T cells were cultured in medium as control. T cells were daily analyzed by flow cytometry. Data shown in this graph derive from experiments performed with TCR-transduced $CD4^+$ T cells (22 days after transduction) after exclusion of B cells by gating. The percentage of $TCRmu^+$ cells was 62.9% in Aa2.2- and 65.6% in Bb5.14-transduced cells. The red line represents T cells incubated alone, the blue line indicates T cells incubated with B cells and the green line shows proliferation of T cells incubated with B cells previously treated with LPS.

Aa2.2-transduced $CD4^+$ T cells showed only low proliferation in response to B cells (Figure 6-24). However, when B cells were pulsed with LPS, proliferation of Aa2.2-transduced T cell populations was highly enhanced (Figure 6-24). Bb5.14-transduced $CD4^+$ T cells proliferated

well in response to B cells (Figure 6-24). However, in contrast to cytokine secretion and cytotoxicity, proliferation of Bb5.14-transduced effector cells was even slightly enhanced when target cells were treated with LPS (Figure 6-24). Other autologous TCR-transduced effector cell populations such as PBMC and $CD8^+$ T cells showed similar results (data not shown).

6.7 Investigation of the distinct mechanisms responsible for functional modulation of both TCR by TLR treatment of target cells

6.7.1 Expression of costimulatory molecules on the surface of B cells

We aimed to elucidate mechanisms involved in the modulation response cell functions after stimulation of TCR-transduced T cells with TLR ligand treated targets. TLR ligand treatment of B cells mediated distinct modulation of the T cell response depending on the transduced TCR (Figure 6-18, Figure 6-19, Figure 6-20, Figure 6-21, Figure 6-23). In contrast, treatment of Aa2.2-transduced T cells themselves with TLR ligands did not lead to enhancement of IFN-γ secretion in response to B cells (Figure 6-20). Bb5.14-transduced T cells treated with TLR agonists were only marginally inhibited in their reactivity towards B cells (Figure 6-20). Since these data suggested in case of Aa2.2 that modulation of the target cells may be responsible for functional alteration of T cells, we investigated the expression pattern of costimulatory molecules and HLA-DR on the surface of B cells.

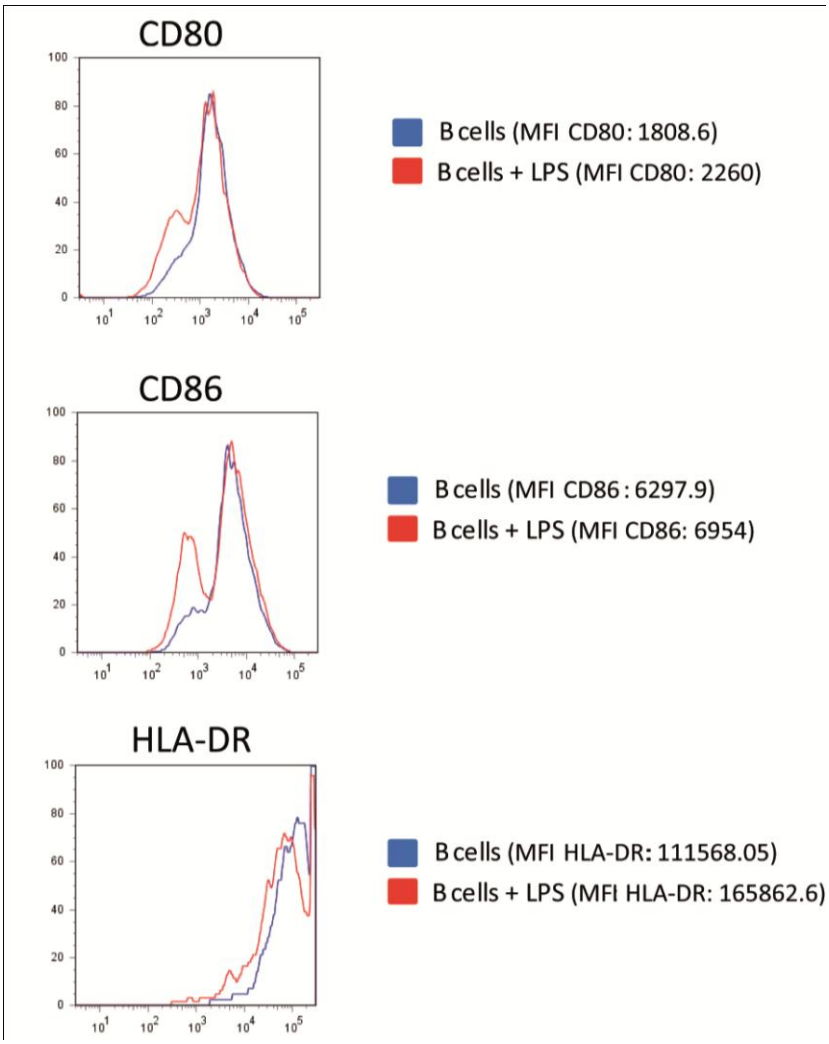

Figure 6-25: Moderate increase of expression of costimulatory molecules on B cells by LPS treatment

B cells or alternatively B cells treated with LPS were stained with α-CD80, α-CD86 or α-HLA-DR antibody to investigate the influence of LPS on expression of these molecules. Mean fluorescence intensities of the investigated molecules are indicated on the right side of the histogram between brackets.

CD40-activated B cells expressed CD80, CD86 and HLA-DR without further stimulation, however, when B cells were treated with LPS, the mean fluorescence intensity was slightly enhanced (Figure 6-25). These data suggest that treatment with LPS lead to a slight up regulation of the number of expressed costimulatory molecules and HLA-DR on the surface of these cells.

6.7.2 Cytokine secretion of B cells with and without TLR agonist treatment

B cells are able to secrete different cytokines (Abken *et al.*, 1992; Benjamin *et al.*, 1994). Therefore, we investigated cytokine secretion of CD40L activated B cells with and without TLR agonist treatment.

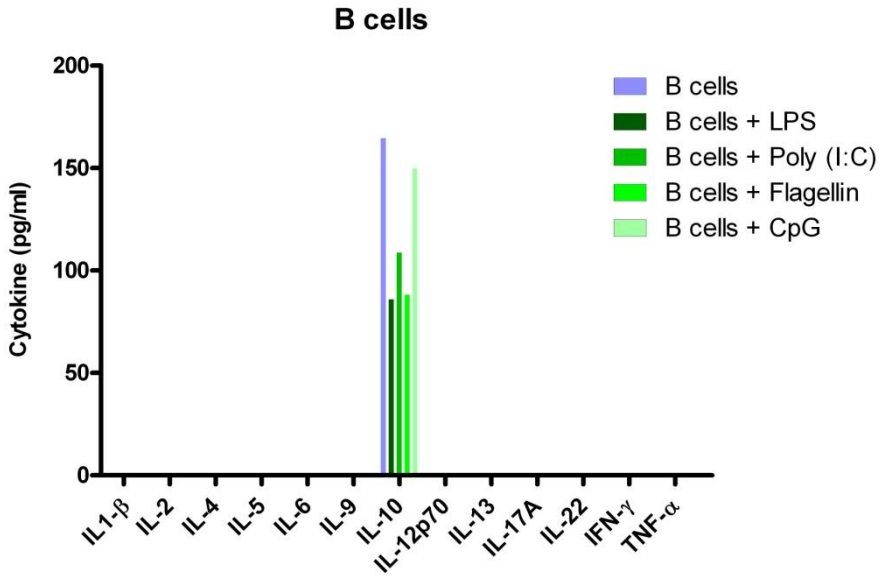

Figure 6-26: B cells secrete low levels of IL-10 with and without TLR agonist treatment
B cells and B cells previously treated with TLR ligands were analyzed for the secretion of different cytokines. Supernatants collected from a 24h incubation assay were investigated by Flow Cytomix.

B cells secreted low amounts of IL-10 and IL-10 levels were not significantly influenced by TLR agonist treatment (Figure 6-26).

6.7.3 FoxP3 expression of TCR-transduced T cells after stimulation with TLR-treated target cells

We further investigated the percentage of Treg in TCR-transduced T cells without stimulation or after stimulation with B cells and B cells pretreated with LPS in order to investigate if Treg may be involved in modulated functions.

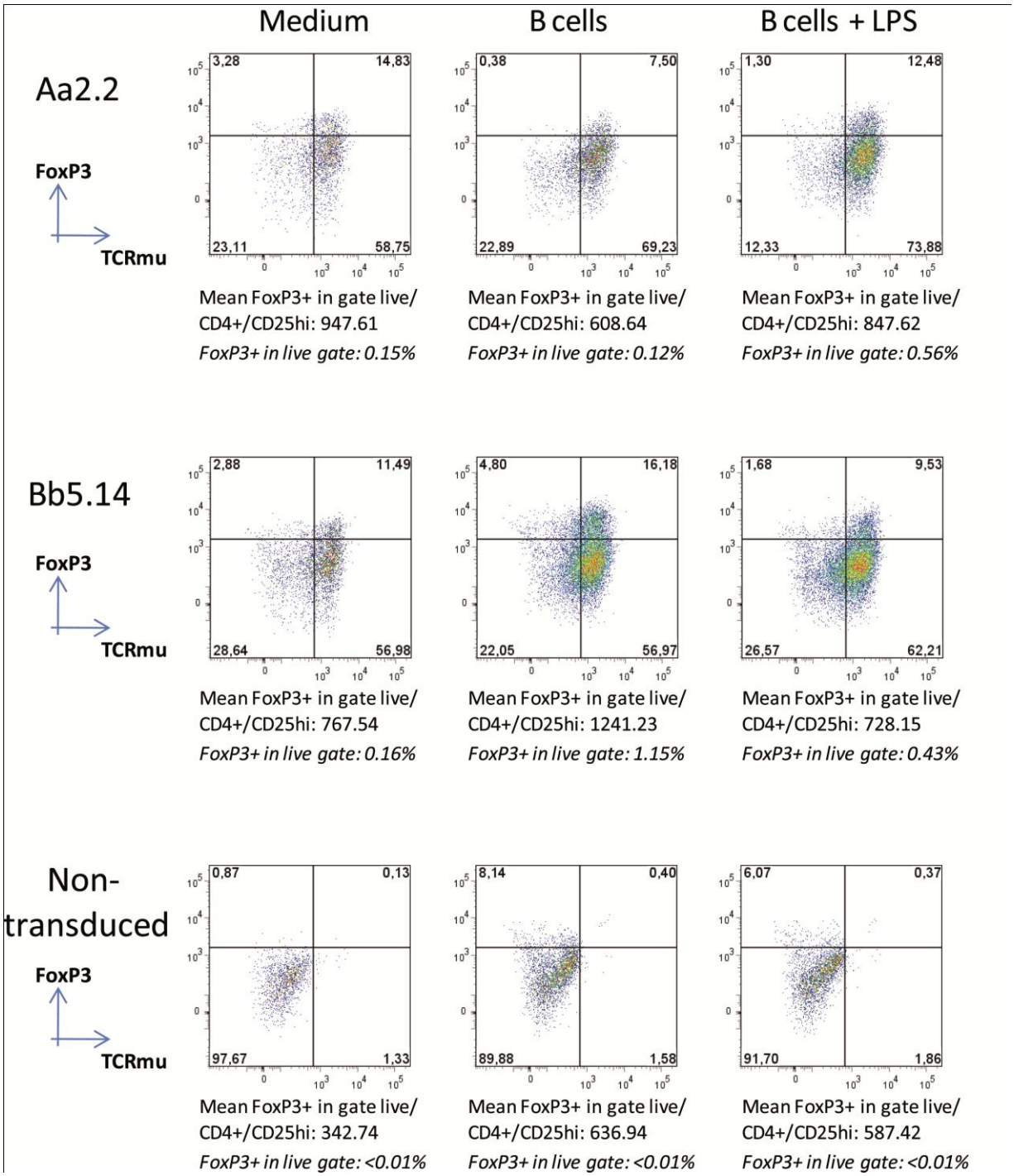

Figure 6-27: Increase of Fox P3 expression in TCR-transduced T cells after stimulation

Autologous TCR-transduced PBMC were stimulated (13 days after transduction) with B cells or LPS-treated B cells for 24h. Cells were then stained and analyzed by flow cytometry. The dot plots show CD4-Pac Blu^+/CD25-PE^{hi} populations. Mean fluorescence intensities of $FoxP3^+$ cells in the CD4-Pac Blu^+/CD25-PE^{hi} population are indicated below the dot plots. In addition, the percentage of $FoxP3^+$ cells in the live gate are indicated below the dot plots.

Interestingly, the percentage of $CD4^+$ $CD25^{hi}$ $FoxP3^+$ T cells, corresponding to the Treg population, was mirroring the effect induced by TLR ligand-treated B cells on cytokine

secretion and cytotoxicity of TCR-transduced T cells (Figure 6-27). The Treg population in Aa2.2-transduced T cells increased after stimulation with LPS-pulsed B cells (Figure 6-27). Incubation of Aa2.2-transduced T cells with untreated B cells did not lead to a notable change of the fraction of Treg population compared to unstimulated TCR-transduced T cells (Figure 6-27). In contrast, the percentage of $CD4^+$ $CD25^{hi}$ $FoxP3^+$ Bb5.14-transduced T cells was enhanced by stimulation with B cells or B cells pulsed with LPS, compared to the unstimulated population (Figure 6-27). However, when B cells without TLR ligand treatment were used as targets, the portion of Treg in Bb5.14-transduced T cells was most prominent (Figure 6-27).

6.7.4 CTLA-4 expression of TCR-transduced T cells after stimulation with TLR-treated cells

CTLA-4 has been described, to play a major role in peripheral T cell tolerance and to be involved in regulation of Treg (Fife and Bluestone, 2008; Rudd, 2008). Therefore, we investigated CTLA-4 expression of TCR-transduced T cells after specific stimulation with B cells or B cells pulsed with LPS.

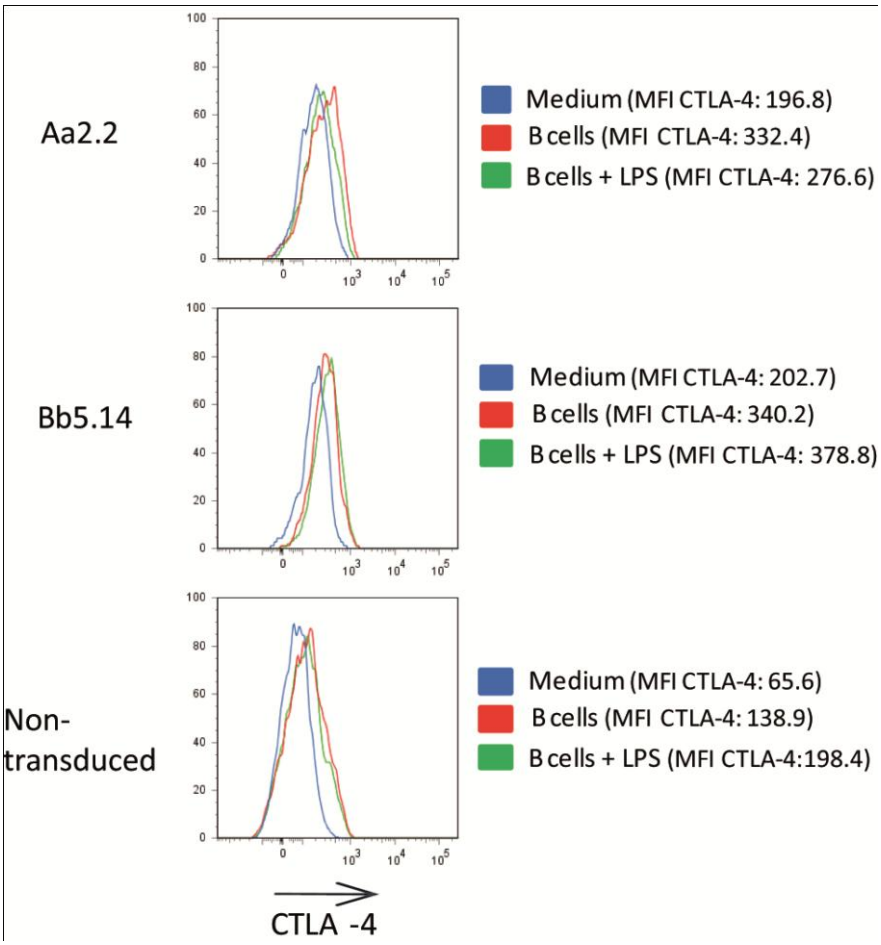

Figure 6-28: CTLA-4 expression on TCR-transduced cells after stimulation with B cells with or without prior treatment with LPS

Autologous TCR-transduced PBMC were stimulated with B cells or LPS-pulsed B cells for 24h. Cells were then stained and analyzed by flow cytometry. Histograms represent 7-AAD-/CD4+/TCRmu+ populations for TCR-transduced populations. In case of non-transduced PBMC, histograms show 7-AAD-/CD4+ populations. Mean fluorescence intensities of CTLA-4 are indicated on the right side of the histogram between brackets.

Here we observed that in contrast to cytokine secretion, cytotoxicity and FoxP3, CTLA-4 expression was slightly down regulated Aa2.2-transduced T cells were stimulated with B cells treated with LPS compared to untreated B cells. Accordingly, CTLA-4 expression was minimally up-regulated after stimulation with LPS-pulsed B cells compared to untreated B cells (Figure 6-28).

7 DISCUSSION

Adoptive T cell therapy is a powerful new approach for the treatment of malignancies (Dudley *et al.*, 2002a). The effectivity of this approach may be improved by transferring T lymphocytes redirected with TCR specific to tumor cells (Morgan *et al.*, 2006; Sadovnikova and Stauss, 1996). However this approach also harbours the danger of side effects, which have to be considered (Johnson *et al.*, 2009). This approach may be especially useful in hematological malignancies, since bone marrow transplanted patients could receive redirected tumor specific T lymphocytes from the donors instead of conventional donor lymphocyte infusions currently transfused to patients with bad prognosis (Kolb *et al.*, 2004).

Presently, most studies focus on the use of $CD8^+$ T cells and only few publications focus on the application of $CD4^+$ T cells in cancer therapy (Bos and Sherman, 2010; Perez-Diez *et al.*, 2007; Xie *et al.*, 2010). Hunder et al. reported a successful transfer of autologous NY-ESO-specific $CD4^+$ T cells to a patient with metastatic melanoma (Hunder *et al.*, 2008). This report demonstrates that $CD4^+$ T cells may be a powerful tool in cancer treatment.

Here, we report the successful isolation of $CD4^+$ T cells from the autologous system with specificity for cells of the hematopoietic lineage and tumor tissue. Diverse effector cell populations were successfully redirected with TCR originating from these T cell clones and showed cytokine secretion, cytotoxicity and proliferation in response to target cells. Moreover, we observed differential regulation of the function of both TCR when TLR ligands were used as additional stimulus with target cells.

7.1 Establishment of prerequisites for generation of tumor reactive self-restricted $CD4^+$ T cells

As FMNL1 has been shown to be an attractive tumor-associated antigen (Krackhardt *et al.*, 2002b; Schuster *et al.*, 2007), we aimed to prime T cells with DC pretreated with recombinant FMNL1 protein or with FMNL1 peptide pools. In contrast to FMNL1 protein, the FMNL1 peptide library was acquired from a company. To obtain recombinant FMNL1 protein, one strategy would have been production in bacterial systems as *E. coli* for example. Expression in such prokaryotic systems may, however, harbour incorrect post-translational modifications which would need to be overcome by specific biotechnological strategies (Sahdev *et al.*, 2008). Moreover, FMNL1 is a 140 kilodalton (kDa) protein, which is too large for bacterial expression (Demain and Vaishnav, 2009). We therefore chose expression of FMNL1 in 293T

cells to bypass such difficulties. However, the yield of recovered protein was certainly lower than normally achieved in bacterial systems (Demain and Vaishnav, 2009).

For purification of FMNL1, a His-Tag sequence was attached to the C-terminus of the original FMNL1 sequence. This recombinant protein could then be purified from the 293T cells through binding of Ni-NTA agarose beads to the His sequence of the recombinant protein (see 5.2.1). We observed additional bands to the FMNL1 band on the Silver stained gel. However, most additional bands were also visible in the lane loaded with lysis buffer, suggesting unspecific background in the gels purchased from Invitrogen. When we analyzed these bands in the mass spectrometry (matrix-assisted laser desorption/ionization; MALDI), the sequences detected in the bands and the corresponding control bands cut from the lane with lysis buffer alone were mostly similar and corresponded to contaminations with skin components, which occurred during sample preparation. As FMNL1 was detected in the relevant 140 kDa band, we used this purified protein for T cell priming.

7.2 T cell clones specific for FMNL1-PP124 – a naturally presented epitope?

We primed PBMC with DC pulsed with FMNL1 peptide pools in an autologous setting as described in 6.1.3. We recovered many clones from line 3 of the PBMC priming condition with FMNL1 peptide Pool G pulsed DC. These clones recognized PP124 from Pool G with the peptide sequence LFFSLFSRFIKAYKKA. However, clones had only low functional avidity and did not recognize unpulsed Mini-LCL. The low half-maximal activity of approximately 1 µM could be a reason for the absence of Mini-LCL recognition, since the physiological density of one specific peptide on the surface of an antigen presenting cell is considerably lower than after peptide pulsing. To overcome this limitation, one possibility would be to stimulate T cells with this specific peptide in an allorestricted setting. This approach improves the recovery of T cells with much higher functional avidity by circumventing negative selection of high avidity T cells in thymus as it is the case in the autologous system. This approach has been established for $CD8^+$ T cells by Sadovnikova and Stauss (Sadovnikova and Stauss, 1996). Investigation of the PP124 sequence by the SYFPEITHI algorithm, predicting peptide binding motifs on HLA-molecules, confirmed probable binding of PP124 to HLA-DRB1*0101 and to HLA-DRB1*1101. However, the analysis of the PP124 sequence LFFSLFSRFIKAYKKA with Paproc, an algorithm predicting proteasomal cleavage sites, revealed several proteasomal cleavage sites within the central part of this peptide, at positions 9, 10 and 12. Thus, these results of both algorithms regarding this peptide may suggest that PP124 binds to both HLA-

DR molecules expressed by the donor, but that based on cellular proteasomal mechanisms its natural occurrence is unlikely. Therefore, we decided not to follow an allogeneic priming procedure focusing on the use of this peptide sequence.

7.3 Recovery of self and tumor reactive MHC II-restricted T cell clones

We focussed on the analysis of T cell lines and clones derived from priming procedures comprising DC pulsed with FMNL1 protein. We recovered two highly interesting T cell clones, one original from untouched PBMC stimulated with DC matured with cytokine cocktail A (IFN-γ, TNF-α, CD40L; Aa2.2) and one original from the naïve T cell population stimulated with DC matured with cytokine cocktail B (IL-6, TNF-α, IL-1β; Bb5.14). Both clones recognized Mini-LCL in screening assays and represented monoclonal populations, expressing both exclusively one TCR alpha and beta chain.

Both clones, Aa2.2 and Bb5.14, were reactive against autologous antigen presenting cells. Thus, we were able to generate $CD4^+$ self reactive T cell clones from a healthy host. The presence of self reactive T cells in a healthy individual has already been shown using autologous macrophages as stimulating cells (Hausman *et al.*, 1981). This phenomenon has been named autologous-mixed lymphocyte reaction and has been shown to be reduced in immunodeficiency diseases (Gupta and Damle, 1983). We identified and characterized for the first time TCR of T cell clones conveying such self-reactivity. Moreover, we showed that both T cell clones recognized distinct autologous cell populations. Clone Bb5.14, isolated from the naïve T cell population, revealed recognition of autologous DC and autologous B cells, whereas clone Aa2.2, isolated from PBMC, exclusively recognized autologous B cells. Hence, both T cell clones recognize distinct peptides. Clone Aa2.2 additionally recognized one HLA-DR matched CLL sample, demonstrating tumor reactivity. Interestingly, Aa2.2 secreted more IFN-γ in response to B cells transduced with FMNL1 than transduced with the control protein GFP. However, pulsing of Mini-LCL with FMNL1 peptide pools did not lead to epitope identification. The failure of epitope identification using the FMNL1 peptide library could have two reasons. First, the recognized epitope could derive from another antigen than FMNL1. Secondly, assuming that FMNL1 is the targeted antigen, the recognized peptide could be longer than the proposed 16-mers and could comprise a sequence extended over 2 to 3 peptides proposed by the peptide library. Using B cells pulsed with different recombinant proteins as targets, we surprisingly observed enhanced IFN-γ secretion, independently of the used protein. This finding questioned again, if FMNL1 actually was the targeted antigen. However, we could not further investigate the finding at this point, since primary T cell clones

are not long-living. Hence, we needed to transfer specificities of these T cell clones into fresh T cells to further investigate the characteristics and recognition patterns of both TCR.

7.4 Transfer of TCR Aa2.2 and Bb5.14 into fresh effector cells allowed further characterization of their recognition patterns

We cloned TCR alpha and beta chains of clones Aa2.2 and Bb5.14 into MP71, a retroviral vector. TCR transfer was first ineffective using two separate vectors for alpha and beta chains of the targeted TCR. We therefore inserted a P2A element from the Picorna virus to ensure expression of both TCR chains in one transduced cell and of equimolar expression of both TCR chains (Leisegang *et al.*, 2008). Moreover, codon optimization was performed to improve translation of TCR chains (Scholten *et al.*, 2006). However, these modifications did not lead to sufficient TCR expression in transduced T cells. This was probably due to mispairing of the transduced TCR alpha and beta chains with endogenous TCR chains of the transduced cells. TCR mispairing leads to the assembly of unstable chimeric TCR molecules with unknown specificity. These difficulties were overcome with two further modifications of the targeted TCR chains. Firstly, the human constant chains were replaced by murine constant chains, lowering the danger of mispairing of introduced alpha and beta chains with endogenous chains (Cohen *et al.*, 2006). Secondly, an additional disulfide bond was inserted in the constant region of the TCR to additionally improve expression (Cohen *et al.*, 2007; Voss *et al.*, 2010). We were then able to transfer both TCR into fresh allogeneic and autologous PBMC as well as into autologous $CD4^+$ and $CD8^+$ T cells.

TCR-transduced T cells were then investigated in detail regarding the self reactivity and recognition of a panel of cells from the hematopoietic lineage and malignant cells. The proliferation curves and co-cultures of Aa2.2- and Bb5.14-transduced T cells with autologous PBMC and activated PBMC revealed no sign of T cell fratricide. This was an important finding with regard to a potential future clinical application. The recognition of peptides presented by activated T cells could lead to an uncontrollable immune response and to mutual elimination of the transferred T cells. Aa2.2-transduced T cells restrictedly recognized autologous B cells and Mini-LCL, whereas Bb5.14-transduced T cells additionally recognized autologous DC, M1 and M2 macrophages. Both TCR were HLA-DR restricted, since target recognition could be blocked by an anti-HLA-DR antibody. The exact restriction element could then be identified by co-incubation of TCR-transduced T cells with transfectants of the EBV^- Burkitt cell line BJAB. BJAB transfectants expressed HLA-DRB1*0101, HLA-DRB1*1101 or alternatively GFP as control. Accordingly, TCR Aa2.2 is DRB1*1101 and TCR Bb5.14 DRB1*0101 is restricted.

These restriction elements could be confirmed by stimulation experiments with partially matched LCL and CLL samples. Aa2.2-transduced T cells recognized most DRB1*1101 expressing LCL and CLL samples and in contrast, Bb5.14-transduced T cells recognized DRB1*0101 expressing LCL and CLL samples. However, IFN-γ secretion levels observed after stimulation with CLL samples was quiet low. This could be due to various tumor escape mechanisms, such as MHC II down regulation, loss of costimulatory molecules or secretion of inhibitory cytokines (Khong and Restifo, 2002). Restriction elements of both TCR were identified and both TCR showed tumor reactivity. Taken together, these data demonstrate that both TCR could become potential candidates for the treatment of leukemia and lymphoma by adoptive T cell therapy.

7.5 Modulation of TCR-transduced T cell functionality by various TLR ligands

We observed in earlier experiments using the original T cell clone Aa2.2, that the reactivity against Mini-LCL was enhanced when target cells were pulsed with protein, independent of the protein used for pulsing. Clone Aa2.2 secreted more IFN-γ when Mini-LCL were pulsed with recombinant FMNL1 as well as when Mini-LCL were pulsed with two other recombinant proteins (GFP or BMRF1) purified in the same way as FMNL1. Since these proteins were purified and dialysed under non-sterile conditions, we suspected contamination of the proteins with bacterial components. Bacterial cell wall contaminations are usually an important issue when proteins are expressed in prokaryotic systems (Bausinger *et al.*, 2002; Bendz *et al.*, 2008; Wallin *et al.*, 2002). To examine whether the stimulatory effect of FMNL1 was potentially mediated by the protein itself or a contamination, we pulsed Mini-LCL with FMNL1 or LPS in the same experiment. Interestingly, FMNL1 and LPS had the same stimulatory effect on Aa2.2-transduced T cells. In contrast, both agents had the contrary effect on Bb5.14-transduced T cells; Bb5.14-transduced T cells were less reactive Mini-LCL pulsed with FMNL1 or LPS in comparison to untreated Mini-LCL. This highly interesting finding did not only confirm that the effect provided by protein pulsing was due to bacterial contamination, it surprisingly demonstrated also an opposite effect of LPS on two distinct TCR.

To further investigate this finding, we used three alternative TLR stimulants other than LPS: Poly (I:C), Flagellin and CpG. All four TLR ligands showed the same antidromic effects on T cells transduced with both TCR. Aa2.2-transduced T cells were activated by B cells pulsed with TLR ligands. In contrast, Bb5.14-transduced T cells, which were highly reactive towards

B cells, were inhibited in their function when B cells were treated with TLR agonists prior to co-incubation. The T cell response of both TCR towards B cells treated with TLR-agonists could be inhibited by addition of HLA-DR antibody. These data demonstrate that TLR ligands mediate distinct effects on T cell activation which are dependent on the expressed TCR.

Importantly, the treatment of T cells with TLR ligands without addition of target cells did not induce cytokine secretion of TCR-transduced or control T cell populations. Aa2.2-transduced T cells pulsed with TLR ligands prior to co-culture did not have an effect on the IFN-γ secretion in reponse to autologous B cells. In the case of Bb5.14-transduced T cells, TLR ligand treatment prior to co-incubation with B cells resulted in a marginal down regulation of IFN-γ secretion. These data suggest that the strength of the T cell response is influenced by the condition of the B cells, at least in the case of Aa2.2. For Bb5.14, a direct effect of TLR agonists on TLR in T cells is not obviated. It has been shown that TLR3, 4 and 5 targeted by the applied agonists Poly (I:C), LPS and Flagellin are expressed in T cells on mRNA and protein level (Zarember and Godowski, 2002). However, TLR9 targeted by CpG could be detected only on mRNA level (Zarember and Godowski, 2002). Others report moreover, that TLR 9 expression is restricted to B cells and plasmacytoid DC (Iwasaki and Medzhitov, 2004; Krieg, 2007). CpG-pulsed Bb5.14-transduced T cells reduced IFN-γ secretion most apparently among applied TLR ligands. This finding and the absence of TLR9 ligand described in the literature makes a direct effect of TLR agonists on T cells less probable. All TLR stimulated in these experiments with TLR agonists have been shown to be expressed in B cells and to enhance the immunoglobulin secretion of plasma cells in peripheral blood (Dorner *et al.*, 2009). However, especially TLR4 expression in B cells has been reported to vary depending on the condition of B cells (Booth *et al.*, 2011; Zarember and Godowski, 2002).

Thus, if the immune modulation would be provided by B cells, how would B cells influence T cell reactivity? One possibility may be represented by up regulation of costimulatory molecules permitting a more effective activation in the case of the “weaker” TCR Aa2.2 whereas inducing anergy in the case of the “stronger” TCR Bb5.14. Another possible explanation would be that B cells vary their cytokine secretion pattern, sustaining or inhibiting thereby the T cell response. An alternative explanation for the distinct effect of TLR ligands on B cell recognition could be the modulation of the panel of presented peptides. First experiments addressing this question have been performed (see 6.7) and are further discussed in 7.7.

7.6 Multifunctionality of TCR-transduced effector cells

Multifunctionality of T cells is required for an efficient immune response. Therefore, we investigated cytokine secretion patterns, cytotoxicity and antigen-induced proliferation of TCR-transduced effector populations. Here we focused also on the effect of TLR agonists on the quality of the T cell response.

Cytokines secreted by $CD4^+$ T cells are crucial for the definition of their role in the anti-tumor response. $CD4^+$ T cells can either induce an effective immune response against cancer (Hunder *et al.*, 2008; Muranski *et al.*, 2008) or impede the adaptive anti-tumor response by secreting IL-10 (Kim *et al.*, 2000; Strauss *et al.*, 2007). IL-10 has been described as Th2 cytokine and is the key cytokine secreted by Treg (Wan and Flavell, 2009). Indeed, it has also been shown that IL-10 is also secreted by Th1 cells which control thereby their reactivity (O'Garra and Vieira, 2007).

In the supernatants investigated by multi-cytokine analysis, we observed that Aa2.2-transduced T cells did not secrete any cytokine when B cells were not treated with TLR ligand. Cytokine pattern secreted after incubation with TLR agonists treated B cells was similar to the pattern of Bb5.14-transduced T cells: we observed IL-5, IL-10, IL-13, IL-17 and IFN-γ secretion. Bb5.14-transduced T cells additionally secreted IL-2, IL-4 and TNF-α. However, secreted cytokine levels were generally enhanced and IL-2 and TNF-α were only secreted when B cells were untreated before co-incubation with Bb5.14-transduced T cells. These data suggest that both activated TCR secrete similar mixed Th cytokine patterns, with IFN-γ and for Aa2.2-transduced T cells also IL-17 as major cytokines in response to B cells. Interestingly, this cytokine pattern is quiet similar to the one discovered by Kyte et al comprising IL-5, IL-10, IL13 and IFN-γ in a clinincal vaccination study (Kyte *et al.*, 2009). They observed positive correlation of vaccine efficacy to a high IFN-γ / IL-10 ratio secreted by reactive T cells. Since IFN-γ was the major cytokine secreted by T cells transduced with both TCR, the IL-10, IL-5 and IL-13 secretion we observed after incubation of B cells or B cells pulsed with TLR agonists with TCR-transduced T cells would possibly not impede an anti-tumor response. However, we observed a contrary IFN-γ / IL-10 ratio when TCR-transduced T cells were incubated with Mini-LCL or CLL samples. This would in contrast potentially reduce an effective anti-tumor response. It is not clear whether the detected IL-10 was secreted by T cells or B cells, since B cells or B cells treated with TLR agonists incubated alone secreted low levels of IL-10. This finding is in line with earlier publications describing IL-10 secretion by B cells (Benjamin *et al.*, 1994). The origin of IL-10 will have to be further analysed by intracellular cytokine staining. Given cytokine secretion patterns of tumor cells

and $CD4^+$ T cell subsets (Gor *et al.*, 2003; O'Shea and Paul, 2010), the establishment of a successful $CD4^+$ T cell transfer in a clinical setting harbors complex challenges.

Another aspect of T cell multifunctionality is cytotoxicity in response to target cells. It has been shown that $CD4^+$ T cells can eradicate a tumor by direct tumor cell cytotoxicity broadening the well described helper function (Quezada *et al.*, 2010). We observed degranulation of Bb5.14-transduced T cells co-cultured with B cells. Similarly to some other experiments in which T cells transduced with Aa2.2 were generally not very active, these cells showed no degranulation in response to untreated B cells. To further investigate cytotoxic activity of T cells transduced with both TCR, we performed a chromium-release assay with PBMC, $CD4^+$ and $CD8^+$ T cells. This assay revealed that both TCR were able to mediate cytotoxicity. All three effector populations transduced with Aa2.2 only killed B cells pretreated with LPS. In contrast, Bb5.14-transduced T cell populations killed all three proposed target cells: B cells, B cells treated with LPS and Mini-LCL. Interestingly, TCR-transduced $CD8^+$ T cells were also able to kill target cells, showing that activation of both MHC II-restricted TCR was not dependent on CD4 expression. This independence of MHC II-restricted TCR on CD4 expression for MHC II-ligand complex binding has already been shown by Xiong et al (Xiong *et al.*, 2001). This is in contrast with reports concerning activity and stability of MHC I-restricted TCR which have been described to be mostly dependent on CD8 expression (Choi *et al.*, 2003; Johnson *et al.*, 2009). In addition, we observed the same differential effect of LPS treatment on both TCR as already observed for cytokine secretion. This finding emphasises the opposite effect of TLR ligands on T cell functionality depending on the transduced TCR. As already mentioned above, the mechanism underlying this effect will have to be clarified and first findings are discussed in 7.7.

As a last factor of multifunctionality, we investigated antigen-induced proliferation of TCR-transduced T cells. The ability of T cells to proliferate in response to antigen-induced activation could have an impact on the effectivity of an adoptive T cell transfer. To date, one major problem of adoptive T cell transfer is the *in vivo* persistence of transferred tumor-specific T cells (Dudley *et al.*, 2002b; Yee *et al.*, 2002). T cells transduced with both TCR were stimulated to proliferate by B cells and B cells transduced with LPS. For Aa2.2-transduced T cell populations, we observed a strong enhancement of proliferation when B cells were pulsed with LPS as it was the case for other functions (cytokine secretion and cytotoxicity). Surprisingly, in the case of Bb5.14-transduced T cells LPS had the same stimulating effect on T cell proliferation as on Aa2.2-transduced T cells. We observed this effect similarly for PBMC, $CD4^+$ and $CD8^+$ T cells. This surprising finding enforces one of the above described possible mechanisms presuming anergy induction of Bb5.14-transduced T cells by TLR ligand treated B cells. Schneider et al. described that CTLA-4 induces T cell anergy without inducing cell death by activation of phosphatidylinositol 3-kinase and protein

kinase B (Schneider *et al.*, 2008). TCR and CD28 stimulation leads to surface expression of CTLA-4, hindering the development of an uncontrolled immune response. Thus, very strong stimulation leads to CTLA-4 induction and anergy (Rudd *et al.*, 2009). Bb5.14-transduced T cell functionality was reduced by TLR agonist treated target cells while proliferation was enhanced. If this oppositional effect on Bb5.14-transduced T cells is driven by a mechanism involving CTLA-4 will have to be clarified. First experiments addressing this question (see 6.7) are discussed in 7.7.

7.7 Perspective on the mechanisms involved in immune modulation of the specific T cell response by TLR ligands

We observed a differential modulation of TCR-transduced T cells when target cells were treated with TLR ligands prior to co-incubation. Aa2.2-transduced T cells were more reactive towards target cells pulsed with TLR agonists, whereas the function of Bb5.14-transduced T cells was inhibited when target cells were treated likewise. At present, we cannot exclude a direct effect of TLR agonists on T cells, at least for Bb5.14. We observed a slight inhibition of reactivity when Bb5.14-transduced T cells were pulsed with TLR ligands prior to incubation with target cells. It has been reported that TLR 9 expression is restricted to B cells and plasmacytoid DC (Iwasaki and Medzhitov, 2004; Krieg, 2007). This would suggest that the observed reduction of IFN-γ secretion of Bb5.14-transduced CpG-pulsed T cells after incubation with B cells, would be driven by binding of remaining CpG in the culture to TLR9 in B cells.

One possible starting point of the immune modulation of T cells could be an alteration of cytokine secretion patterns of target cells and of the density of costimulatory molecules on the surface of target cells. As discussed in 7.6, we did not observe an alteration of cytokine secretion after treatment of B cells with TLR3, 4, 5 and 9 agonists. With and without treatment, B cells secreted low levels of IL-10. However, we did not investigate yet whether B cells change cytokine secretion while co-incubated with TCR-transduced T cells. Costimulatory molecule expression on B cells and DC and expression of Th1 supporting cytokines by DC have been shown to be enhanced by TLR treatment (Kanzler *et al.*, 2007; Krieg, 2006). Therefore, we compared expression of constitutive costimulatory molecules CD80 and CD86 and HLA-DR expression level on B cells with TLR agonist treated B cells. However, in our experiments, TLR4 agonist LPS induced only a low increase of the density of these molecules on the B cell surface. This aspect will have to be further investigated with other TLR ligands, such as CpG.

We further investigated potential mechanisms involved in the differential reactivity of T cells evoked by TLR ligand treatment in combination with specific TCR stimulation. TLR ligands have been described as potential adjuvants in cancer immunotherapies (Amos *et al.*, 2011; Nava-Parada *et al.*, 2007; Tormo *et al.*, 2006). However, after promising preclinical studies, clinical trials were partially disappointing (Appay *et al.*, 2006; Kuball *et al.*, 2010; Weber *et al.*, 2009). One reason for therapeutic failure could be that TLR ligands can not only mediate a Th1 response, but can also promote Treg (Conroy *et al.*, 2008). Treg have been described to coexpress CD4, CD25 and FoxP3 (Sakaguchi, 2005). We therefore investigated the percentage of $CD4^+$ $CD25^{hi}$ $FoxP3^+$ cells in TCR-transduced T cells without stimulation and after stimulation with B cells or B cells pulsed with LPS. When transduced T cells were not stimulated, we observed a marginal increase of Treg compared to non-transduced T cells. Interestingly, the fraction of Treg in TCR-transduced T cells evolved similarly to stimulation. Aa2.2-transduced T cells comprised a higher portion of $CD4^+$ $CD25^{hi}$ $FoxP3^+$ cells after stimulation with B cells pulsed with LPS than after stimulation with B cells or without stimulation. In contrast, the fraction of Treg was higher when Bb5.14-transduced T cells were stimulated with untreated B cells than when LPS treated B cells were used as targets. These data suggest that the application of LPS does not itself lead to an induction of Treg. However, these data imply that stimulation of investigated TCR similarly stimulates the development of a minor Treg population. Since it has been shown that $CD4^+$ T cells are highly modulable in their functionality (O'Shea and Paul, 2010), it will be of interest to analyze function of differentially polarized $CD4^+$ TCR-transduced T cells (Th1, Th2 and Treg) after stimulation with different targets. The influence of target cells, of the transduced TCR and of the initial polarization of $CD4^+$ T cells on the T cell functionality may also be analysed at a clonal level, because the induction of Treg could impede successful clinical application (Appay *et al.*, 2006; Zhou *et al.*, 2006b).

Another molecule possibly influencing differentially T cell reactivity after TLR agonist treatment is CTLA-4. The role of this molecule is to control the T cell response by antagonizing the costimulatory molecule CD28 (Rudd *et al.*, 2009). Moreover, CTLA-4 has been described to be expressed at high levels in Treg and is essential for functionality of Treg (Friedline *et al.*, 2009). We stained for surface CTLA-4 after stimulation of TCR-transduced T cells with B cells or B cells treated with LPS prior co-incubation. We observed only marginal variations in CTLA-4 expression in preliminary experiments. Aa2.2-transduced T cells expressed less CTLA-4 when B cells were pulsed with LPS, whereas Bb5.14-transduced T cells contrarily expressed more CTLA-4 in response to similarly treated B cells. As described above, Bb5.14-transduced T cells were not inhibited in their proliferation following stimulation with B cells pulsed with LPS. Thus, overstimulation of these cells could induce CTLA-4 expression leading to anergy, but not to inhibition of proliferation (Schneider *et al.*, 2008).

Whether these minimal changes in CTLA-4 expression play a role in the distinct regulation of TCR activity by TLR ligands needs to be further investigated.

These preliminary data suggest a slight improvement of the costimulatory capacities of B cells treated with TLR ligands. This could be the starting point of both distinct effects of TLR ligands on TCR-transduced T cells, since Aa2.2-transduced T cells may need stronger costimulation to develop full functionality. In contrast Bb5.14-transduced T cells may be overstimulated by stronger costimulation, inducing CTLA-4 expression. However, these first assumptions need to be intensively investigated and other mechanisms such as PD-1 and PD-L1, Fas and FasL expression or cytokine secretion also need to be considered.

8 CONCLUSION

We aimed to generate tumor reactive $CD4^+$ T cells for the treatment of hematological malignancies by adoptive immunotherapy. For this purpose, we applied basically two different priming procedures in an autologous setting. Firstly, we used a peptide library of the TAA FMNL1 for DC pulsing prior to T cell priming. Using this approach, we were able to generate T cells with specificity for a distinct FMNL1 peptide. However, this epitope is most likely not endogenously processed by cellular proteasomes. Secondly, we used DC pulsed with recombinant FMNL1 protein and matured in a distinct way for T cell priming. We were able to recover two self reactive distinct T cell clones from a healthy donor. TCR of these T cell clones were transferred into fresh effector cells. These TCR recognized a different panel of target cells and were multifunctional. Most importantly, these TCR do not convey T cell fratricide and recognize tumor cells rendering these TCR potentially interesting for the treatment of leukemias and lymphoma. To finally reach clinical application the exact recognized epitope of both TCR will have to be clarified and more tumor samples will have to be analyzed. We here moreover describe for the first time that the impact on T cell reactivity provided by TLR agonists is dependent on characteristics of the expressed TCR. This finding may have to be considered when TLR ligands are used as adjuvants for adoptive T cell therapy. Therefore, one future aim will be clarification of the mechanism underlying this immune modulation.

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Publikationen

Distinct functional modulation of human MHC-class-II-restricted TCR with self and tumor reactivity by innate signals.
Weigand LU, Liang X, Schmied S, Mall S, Eppinger E, Klar R, Stötzer OJ, Salat C, Mautner J, Peschel C, Krackhardt AM.
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A Single TCR alpha-Chain with Dominant Peptide Recognition in the Allorestricted HER2/neu-Specific T Cell Repertoire.
Liang X[1], **Weigand LU[1]**, Schuster IG, Eppinger E, van der Griendt JC, Schub A, Leisegang M, Sommermeyer D, Anderl F, Han Y, Ellwart J, Moosmann A, Busch DH, Uckert W, Peschel C, Krackhardt AM.
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Konferenzen

CIMT 2011, Mainz: Poster “Human MHC class II-restricted T cell receptors (TCR) with tumor reactivity are present in the self–restricted T cell repertoire and are differentially modulated by innate immune regulation” (**Weigand LU**, Liang X, Schmied S, Mall S, Eppinger E, Klar R, Mautner J, Peschel C, Krackhardt AM)

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CIMT 2010, Mainz: Poster "Generation of CD4+ T cells with specificity for FMNL1" (**Weigand LU**, Liang X, Schuster IG, Eppinger E, Han Y, Schiemann M, Kremmer E, Moosmann A, Mautner J, Peschel C and Krackhardt AM)

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DGfI Tagung 2007, Heidelberg: Talk "Investigation of allorestricted peptide-specific T cell responses against Her2/neu – Implications for adoptive T cell therapy in solid cancer" (**Weigand LU**[1], Liang X[1], Schuster IG, Anderl F, van der Griendt J, Bernhard H, Ellwart J, Nößner E, Busch DH, Uckert W and Krackhardt AM)
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ERKLÄRUNG

Ich erkläre an Eides statt, dass ich die der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Charakterisierung humaner MHC II-restringierter T-Zellrezeptoren mit Reaktivität gegen B-Zellen und Tumorzellen zur therapeutischen Anwendung im Rahmen eines adoptiven T-Zelltransfers transgener CD4 T-Zellen

in dem Institut für Physiologie
der Technischen Universität München

unter der Anleitung und Betreuung durch

o. Univ. Prof. Dr. Dr. H. H. D. Meyer

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 5 angegebenen Hilfsmittel benutzt habe.

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