The potential (therapeutic) role of BAFF, FLT3L and IL-2 in immune disorders

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Matthias Kreuzaler

aus Freiburg im Breisgau Deutschland

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auf Antrag von: Prof. Antonius Rolink

Prof. Ed Palmer

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Prof. Dr. Jörg Schibler

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Abrevations

aa	amino acid
Ab	antibody
Ag	antigen
AIRE	autoimmune gene regulator
APC	antigen presenting cell
APRIL	a proliferation inducing ligand
bp	base pair
BCMA	B cell maturation antigen
BAFF	B cell activating factor of the TNF family
BAFF-R	BAFF receptor
BCR	B cell receptor
BM	bone marrow
B6	C57/BL6
BDF1	(C57/BL6 x DBA/2)F1
BSA	bovine serum albumine
CAML	calcium modulator and cyclophilin ligand
cAMP	cyclic adenosine monophosphate
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CD	cluster of differentiation
CSR	class switch recombination
DMSO	dimethylsulfoxid
DC	dendritic cell
DL1	delta-like 1

DN	double negative
DNA	deoxyribonucleic acid
DP	double positive
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetate
FCS	fetal calf serum
FLT3	Fms-like tyrosine kinase 3
Foxp3	forkhead box P3
FITC	fluorescein-isothiocyanate
Fol B	follicular B
FLT3	FMS-like tyrosine kinase
FLT3L	FLT3 ligand
GC	germinal center
Ig	immunoglobulin
H chain	heavy chain
kb	kilo bases
L chain	light chain
MALT	mucosal associated lymphoid tissue
min	minutes
MHC	major histocompatibility complex
MZ	marginal zone
NIK	NFκB inducing kinase
NIP	4-Hydroxy-3-nitro-5-iodo-phenylacetyl
OD	optical density
O/N	over night
PCR	polymerase chain reaction

PI	propidium iodide
preBCR	pre-B cell receptor
RAG	recombination activating gene
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcriptase-PCR
SDS	sodium dodecylsulfate
SL chain	surrogate light chain
SLE	systemic lupus erythematosus
T1 (T2, T3)	transitional type 1
TACI	transmembrane activator and CAML interactor
TD	T cell dependent
TdT	terminal deoxynucleotidyl transferase
TG	trans gene
TI	T cell independent
TCD	T cell depletion
TCR	T cell receptor
TEC	thymic epithelial cell
cTEC	cortical TEC
mTEC	medullary TEC
TGF	tumor growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TSA	tissu-specific antigen
TRAF	TNF receptor associated factor

UV	ultra violett
WT	wild type
GvHD	graft versus host disease
HSC	hematopoietic stem cell
IFN	interferon gamma
Ig	immunoglobulin
IL	interleukin
K.O.	knockout
LN	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody
MHC	major histocompatibility complex
N.D.	not determined
NK	natural killer
NKT	NK T cell
NTreg	naturally occurring regulatory T cell
PAMP	pathogen associated molecular pattern
pGE	promiscuous gene expression
PRR	pattern recognition receptor
RNA	ribonucleic acid
SP	single positive
TCD	T cell depletion
TCR	T cell receptor
TEC	thymic epithelial cell
cTEC	cortical TEC

mTEC	medullary TEC
TGF	tumor growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
TSA	tissu-specific antigen

Summary

The TNF family member protein BAFF/BLyS is essential for B cell survival. In humans, increased concentrations of soluble BAFF are found during different pathological conditions, which may be as diverse as autoimmune diseases, B cell malignancies, and primary Ab deficiencies (PAD). We have developed a sensitive ELISA for soluble human BAFF, which allows us to study some parameters that might determine the level of soluble BAFF in circulation. We show that, patients suffering from PAD including severe functional B cell defects, such as BTK-, BAFF-R-, or TACI-deficiencies, were all found to have higher BAFF levels in their blood than asplenic individuals, patients having undergone anti-CD20 B cell depletion, chronic lymphocytic leukemia patients, or healthy blood donors. In a comparable manner, transgenic mice constitutively expressing soluble human BAFF were found to have higher concentrations of circulating human BAFF in the absence of B cells. Therefore, our data strongly suggest that the steady-state concentration of BAFF mainly depends on the number of B cells present as well as on the expression of BAFF-binding receptors. Because most patients with PAD have high levels of circulating BAFF, the increase in BAFF concentrations cannot compensate for the defects in B cell development and function.

In a second study, we showed that treatment of mice with the fms-like tyrosine kinase ligand (FLT3L) as well as with an IL-2/αIL-2 mAb complex (JES6-1A12, S4B6) led to an expansion of the Treg compartment. We show that this increased number of NTregs is due to proliferation of pre-existing NTregs, likely due to favored interactions with the increased number of DCs. The increase of NTregs in the IL-2/αIL-2 complex treated mice is due to a direct effect of IL-2 signaling. Thus the lifespan and availability of the IL-2 molecules is prolonged due to the complex with the mAb and therefore stronger/longer signals via the IL-2R can be achieved. We investigated the potential of FLT3L and IL-2/αIL-2 pretreatment of mice and could show that administration of FLT3L could prevent death induced by an acute GvHD in BDF1 mice. However, when we used a different mouse strain,

(BM1xBM12)F1, no protection could be observed, even though the Treg cells increase in these mice was similar to the one in BDF1 mice. By depleting the NK cell compartment of acute GvHD mice, we could show that NTregs themselves are not protective in this system.

In a third study, the potential use of FLT3L and IL-2/αIL-2 complex (JES6-1A12) pre-treated recipients of solid allografts were investigated. To test whether Flt3L treatment was effective in prolonging allograft survival we used the transplant model where the tail skin from a BM12 mouse was transferred to the trunk of B6 mice. An IL-2/αIL-2 complex pre-treatment, previously shown to be highly effective in a pancreatic islet allograft transplant model, was used as a control. All PBS treated B6 mice rejected the graft within 12 days, the IL-2/αIL-2 treated mice kept their allografts for a maximum of 70 days +/- 5 days. In the FLT3L treated group, 12,5% of the mice rejected the graft with similar kinetics as the PBS treated mice. 62,5% of the mice showed a delayed rejection of the graft by 4-14 days. Only 25% of recipient mice had a graft survival similar to the IL-2/αIL-2 treated mice.

Reducing the numbers of Tregs in FLT3L treated mice by a αCD25 mAb injection resulted in a graft survival time similar to that observed in PBS treated mice suggesting that the FLT3L induced prolonged graft survival was due to increased numbers of Tregs.

Taken together these findings indicate that FLT3L treatment could be a possible prophylactic therapy for preventing solid organ rejection.

1. Haematopoiesis

Vertebrates developed throughout evolution an immune system with different levels of complexity involving various cell types with different degrees of specialization.

The first steps of haematopoiesis take place in the bone marrow and all cells of the blood except T cells are generated there. T cell development takes place in the thymus, an organ composed of an inner cortex and an outer medulla. The exact identity and homogeneity of thymus-settling cells are still unclear however it is not an haematopoietic stem cells (HSCs). HSCs are characterized by their ability to self-renew. They can give rise to all mature blood cell types. These unique properties of pluripotency and self-renewal capacity have been used for many years in medicine during bone marrow transplantations in order to reconstitute a life-long complete haematopoietic system in immunodeficient patients. **(1)**

As it can be seen in **Figure 1**in the mouse, haematopoiesis starts already at the embryonic stage. It takes place in the yolk sac at day E7,5.

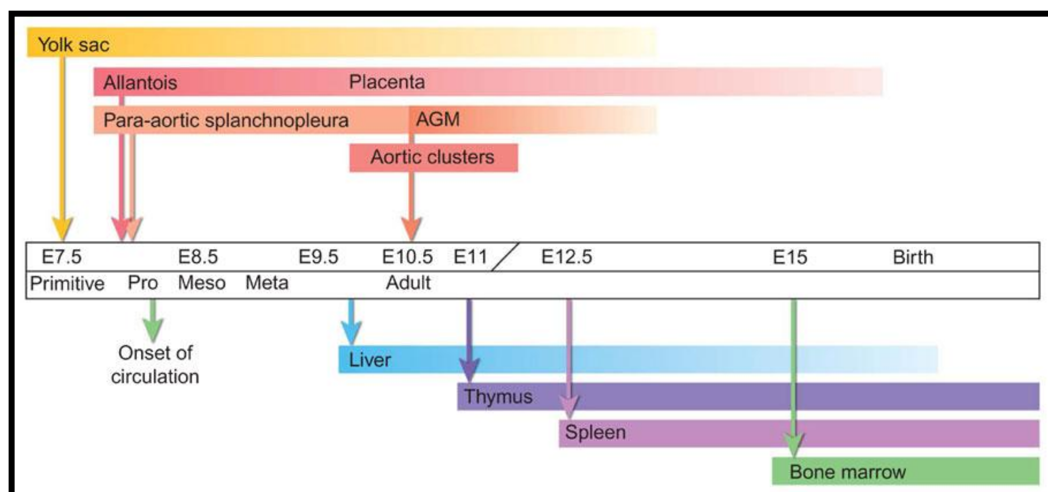


Figure 1 Haematopoiesis. *Arrows above indicate the onset of specific hematopoietic cell generation and/or appearance; arrows below indicate the earliest time of colonisation of the secondary hematopoietic territories. Adapted from Dzierzak and Speck, 2008.*

At day E10 HSC migrate to the foetal liver, where further development takes place. After that, thymus and spleen are colonized and at day E15 the BM becomes the main site for the haemotopoietic development. Every day 10^{11}-10^{12} blood cells are produced here.

Within the HSC population there are cells with and other cells without self-renewal capacity. HSC without self renewal capacity express the cytokine receptor fms-related tyrosine kinase 3 (FLT3), whereas cells that are lacking FLT3 expression have lost this ability. These cells are then referred to as multipotent progenitors (MPP).

Three different lineages can arise from HSCs that are defined as: Lineage negative (lin^-), Stem-cell antigen 1 positive ($Sca1^+$) and $cKit^{hi}$ (CD117) or LSKs. One is the erythroid cell lineage which are red blood cells and megakaryocytes. These cells arise from megakaryocyte/erythroid progenitors (MEPs), which themselves originate from MPPs.

Second are the myeloid lineage which also derive from MPPs and give rise to granulocytes, megakaryocytes and macrophages.

The third lineage goes towards the lymphoid cells like B and T lymphocytes, which are the key players of the adaptive immunity. These cells develop from Common lymphoid progenitors (CLP) which are characterized by low expression of cKit, high levels of interleukin 7 α-chain (IL-7Rα) and FLT3.

Along their differentiation towards committed precursors, HSCs express various key transcription factors such as Pax5 or Ikaros **(Figure 2)**. These transcription factors are essential, as mutations or deletions of these genes result in a complete or partial block in cell lineage development. These transcription factors are used to define different developmental stages of haematopoiesis in the way that they define the commitment of a cell to a distinct cell lineage. The different developmental stages can also be characterized by expression of specific receptors for cytokines, such as the receptor for IL-7 or the growth factors colony stimulating factor (CSF). Receptor expression is tightly regulated since it conditions the responsiveness of a stage-specific cell to receive a signal that allows it to further differentiate. Many of the ligands for these receptors, cytokines as well as growth

factors, are provided by the microenvironment and more specifically by stromal cells that support HSC growth and differentiation. Hence, the HSC's fate is thought to be determined by the microenvironment, which provides the growth factors necessary for cell survival.

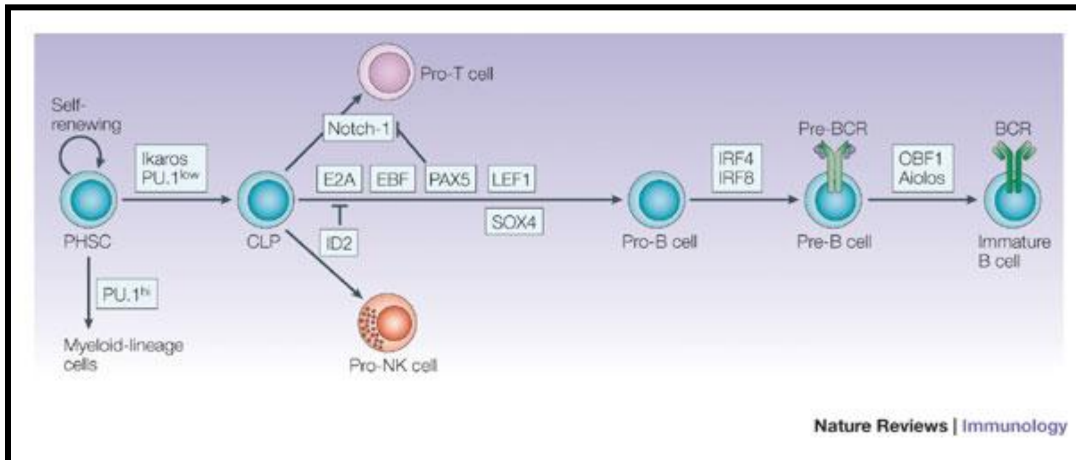
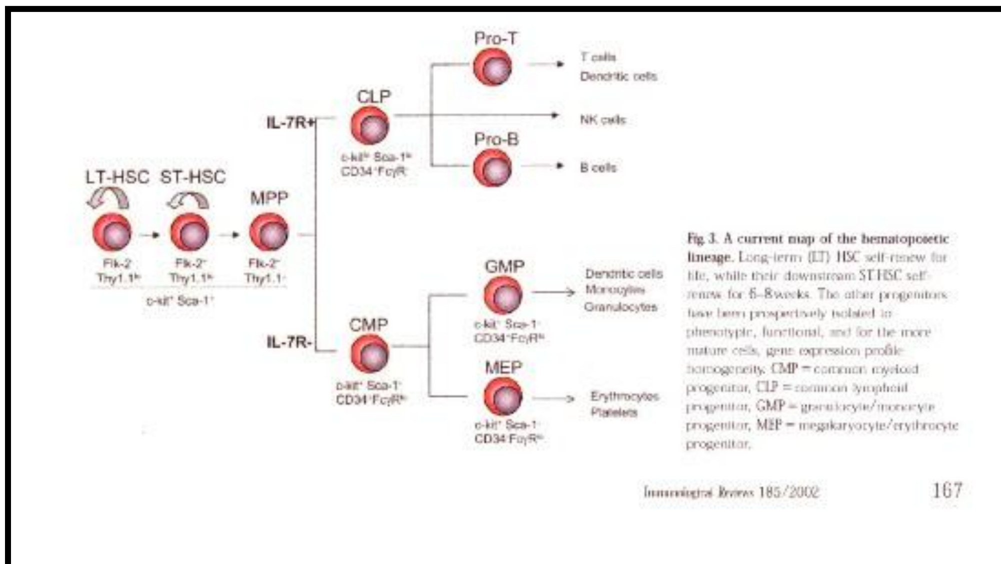


Figure 2: The genetic control of B-cell specification and commitment in the bone marrow.*This diagram shows a scheme of the genetic switch that regulates early B-cell specification. For simplicity, only some of the differentiation stages are shown. The developmental steps that are affected by the mutation of specific transcription factors are indicated. In some cases, the simultaneous mutation of two factors is required for a developmental phenotype to be observed. ID2 (inhibitor of DNA binding 2)-deficient mice have impaired natural killer (NK)-cell development and increased E2A activity, which correlates with an increased frequency of class switching to IgE. BCR, B-cell receptor; EBF, early B-cell factor; CLP, common lymphoid progenitor; IRF, interferon-regulatory factor; LEF1, lymphoid-enhancer-binding factor 1; OBF1, OCT (octamer-binding transcription factor)-binding factor 1; PAX5, paired box protein 5; PHSC, pluripotent haematopoietic stem cell; SOX4, sex-determining region Y (SRY) box 4 (adapted from Matthias and Rolink).*

Different models exist to describe haematopoiesis. Originally it was thought that HSC differentiation was rather linear, with a progressive loss of lineage potential corresponding with increased differentiation. However recently it has been shown that committed progenitors may actually maintain the potential to differentiate into various lineages until late in development. For example, proB cells from Pax-/- mice can develop into myeloid, NK and T cells in vivo and in vitro. **(187)**

Among the different models the Weissman model is widely accepted. It is based on cell surface phenotypes. **(Figure 3) (186)**



<u>Figure 3 Weissman Model:</u> *<u>Conceptual hematopoietic trees in adult mice:</u> Indicated cell populations can be purified based on the cell surface phenotype. Multipotent progenitors (MPPs), at least at the population level, can differentiate into all types of hematopoietic cells, but have no detectable self-renewal potential in vivo. Megakaryocyte progenitors have recently been identified. Pro T cells are present in the thymus (Adapted from Kondo et al., 2003.).*

The Weissman model describes the differentiation from HSCs to differentiated lymphoid, granulocytes and myeloid cells. This model is based on different surface markers which define the cell stages. The early developmental steps, which take place in the BM start off with the multipotent long-termed hematopoietic stem cells (LT-HSCs). LT-HSCs are the pluripotent progenitors for all cells of the hematopoietic system. These cells have self-renewal capacity. LT-HSCs are characterized as Sca^{hig} $FLT3^{-}$ $CD34^{-}$ $VCAM^{+}$ Lin^{-}. **(2-4)**

LT-HSCs develop into short-term HSC (ST-HSC), which is followed by the multipotent progenitor MPP stage. MPPs are defined as $Sca1^{high}$ $c\text{-}kit^{high}$ $FLT3^{hig/low}$ $CD34^{+}$ $VCAM1^{-/+}$ Lin^{-}.

From the MPPs the cells develop either into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). These cells have no self-renewal capacity anymore, and are at least in this model dedicated to go towards lymphoid or myeloid lineage. The CMPs are developing into granulocytes-macrophage progenitors (GMPs) and Megakaryocyte-erythroide progrnitor (MEPs). These cells eventually develop towards Dendritic cells, Monocytes and Granulocytes (GMP) or to Erythrocytes and Platelets (MEP).

The CLPs develop towards Pro-T or Pro-B cells and eventually go towards T cells, DC, NK cells or B cells.

Even though all the mentioned cells can be indeed isolated, it seems more and more clear that this model should be re-considered. There are increasing amount of data, which demonstrate that there is no exclusive decision regarding myeloid or lymphoid fate but rather a progressive loss of differentiation potential influenced by cells intrinsic and extrinsic

2. T cell development

2.1 Early steps in T cell development-The transit of T cell precursors from the BM to the Thymus

T cells are very important for the organism since they have a central role in the immune response. Just like the B cell system -and every other cell system in the body- the T cell development relies on the hematopoietic stem cell system (HSC) with its self renewal capacity. As described earlier, these cells reside in the BM and throughout life give rise to blood cells.

The first steps of haematopoiesis for all cells take place in the BM. All cells, except for the T cells which develop in the thymus, fully develop here. They all develop from common precursors, which have self renewal capacity and are called haemotopoietic stem cells (HSCs). These cells can give rise to all blood cell types.

In the thymus the T cell development takes place. The function of a T lymphocyte is to develop specialized immunity against intracellular microbes like viruses or intracellular bacteria. They also provide help to B cells for the generation of antigen specific antibodies.

The thymus **(Figure 4)** is located in the upper anterior thorax above the heart and consists of different tissue structures. A capsule surrounding the outer cortex, which containes a dense collection of thymocytes, and an inner medulla where selected mature cells exit into the

bloodstream. The thymus shows three major cell types of distinct developmental origin. Mesenchymal cells and thymic epithelial cells, which form the stroma, and BM derived T-Lymphocytes and dendritic cells.

Mesenchymal cells are important for the thymic development. They influence the initial stage of thymic formation by regulating thymic epithelial cell differentiation and proliferation through direct interaction and production of fibroblast growth factor (FGF). Also, they support T cell precursor survival and early maturation by secreting IL-7.

Thymic epithelial cells (TEC) are stromal cells of endodermal origin. They constitute a complex network and through that provide a optimal microenvirement for development of thymocytes, macrophages and DCs. There is a very strong interaction between TECs and thymocytes which in the end leads to thymocyte maturation. This all happens by providing cytokines essential for thymocyte proliferation. It is important to know that the rate of T cells produced is not constant throughout the life of an organism. The highest efficiency of the thymus occurs during the early years of the organism. In later years the efficiency of the thymus drastically slows down. This process, called thymus involution, results in very low T cell production in older individuals. The TEC´s can -at least in the mature thymus- be subdivided in medullary and cortical (mTEC, cTEC). The function of TECs is to mediate central tolerance together with BM derived thymic DCs (BMdDCs).

Uncommited lymphoid precursors from the BM enter the thymic cortex via blood vessels at the corico-medullary junction. Immature thymocytes are double negative (DN) for the T cell markers CD4 and CD8. DN thymocytes are a small population within the total thymocytes, constituting only 5%. The thymic precursors in the cortex undergo intense proliferation and differentiation. They migrate from the cortex and become double-positive (DP) for the surface markers CD4 and CD8. They go to the medulla, where a minority of the thymocytes become single positive (SP) for either CD4 or CD8. **(Figure 5)**

Along the way, the thymocyte cells interact with various cell types, like cTEC, macrophages in the cortex and mTECs, thymic DCs and macrophages in the medulla.

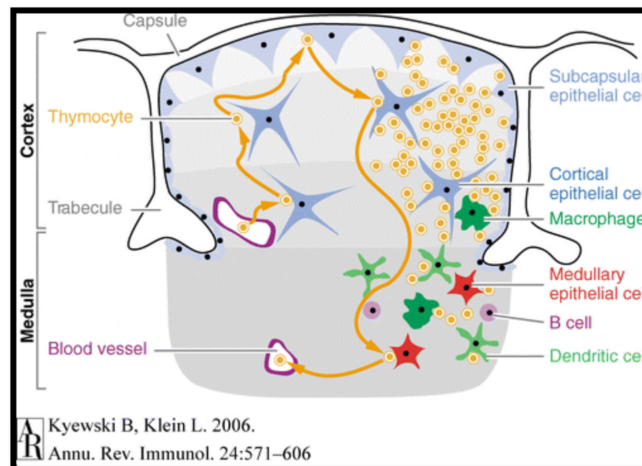


Figure 4: Cellular composition of the thymus. *The major cell types and the sequential cell-cell interactions along the migratory route of developing thymocytes are depicted here. The different APCs are color-coded. mTECs (red), play an essential role in the self-tolerance induction toward tissue-restricted self-antigens. Shaded areas depict functionally distinct stratified microenvironments as recently proposed* ***(Petrie, 2003)****. (Adapted from Kyewski, 2006.)*

During the commute to the medulla, the thymocytes rearrange their genes for the T cell receptor (TCR). First the β-chain gene is rearranged followed by the α-chain gene.

The DP thymocytes undergo positive selection in the cortex. During this step, cells that recognize self-MHC molecules are selected. This is followed by negative selection, which eliminates cells recognizing self-antigens with high affinity. Both selection processes will be discussed later in detail.

95% of thymocytes will die by apoptosis in the thymus throughout the maturation process. This is due to several possible reasons. For example, unability to produce a functional TCR, the cells did not receive a survival signal after positive selection, or inefficient rearrangement of their TCR genes or they received an apoptotic signal during negative selection. After the selection processes, the thymocytes down-regulate one of their co-receptors CD8 or CD4. They become single positive (SP).

α/ β TCR $CD4^{+}$ T cells represent about 12% of the adult thymocte population. These cells are MHCII-restricted and have a helper activity function. α/ β TCR $CD8^{+}$ cells are MHCI-restricted and have

cytolytic activity. Once these two mature populations reach the periphery, they are called $CD4^+$ helper (Th) and $CD8^+$ cytotoxic (CTL) T lymphocytes.

2.2 T cell development in the thymus-Notch, IL7 and SCF are required for the development

As mentioned before MLP´s give rise to B cells as well as T cells. MLP´s are considered the most probable physiological originator of the early T cell progenitors. In the thymus there are no HSC´s, which is why there is a constant need for BM progenitors to colonize the thymus in order to maintain a constant role of T cell development.

A very important factor for T cell commitment is Notch1 and the Notch receptor signaling as in $Notch1^{-/-}$ mice, the thymus is colonized by B cell precursors. **(111)** The earliest thymus-settling progenitors (TSP), which retains B cell potential and loses it upon Notch signaling, are the double negative (DN) population. The DN population can be divided in four different subpopulations according to their expression of CD25 and CD44. These populations are DN1, DN2, DN3 and DN4 cells. These 4 subpopulations correspond to four successive developmental stages.

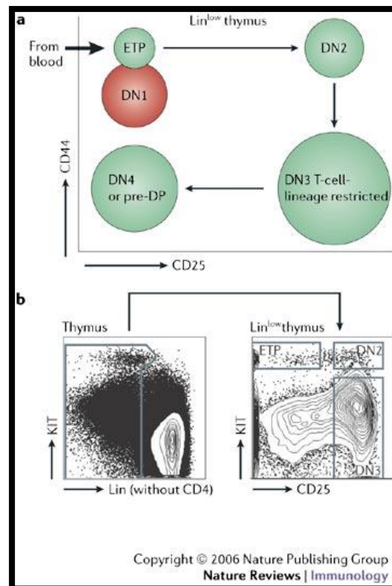


Figure 5: Immature thymocyte progenitor subsets.

a. *Shows the subsets of double-negative (DN) thymocytes, based on their expression of CD25 and CD44 and their sequential development through the DN1 ($CD25^{-}CD44^{+}$), DN2 ($CD25^{+}CD44^{+}$) and DN3 ($CD25^{+}CD44^{-}$) stages. $CD25^{-}CD44^{-}$ cells, which are also called DN4 cells, express Cd4 and Cd8 mRNA and are termed pre-double-positive (pre-DP). Early T-cell progenitors (ETPs), which are c-$kit^{hi}CD25^{-}CD44^{hi}$, are efficient T-cell progenitors that are transcriptionally equivalent to DN1 cells.*

b. *Defining ETPs. C57Bl/6 total thymocytes ($25x10^{6}$ cells) were stained with antibodies specific for c-kit and CD25, as well as a cocktail of antibodies specific for the following lineage (Lin) markers: NK1.1, T-cell-receptor -chain (TCR), TCR, macrophage receptor 1 (MAC1), B220, TER119 (also known as Ly76), CD3, CD8, CD8, CD11c and CD19, but not CD4 because some ETPs express of CD4 on the cell surface. Gating for lineage markers is adjusted so as not to exclude c-kit^{hi} cells that are expressing low levels of Lin (left panel). ETPs are defined as Lin^{low}c-$kit^{hi}CD25^{-}$ (right panel), (Adapted from Bhandoola and Sambandam, 2006).*

The T cell progenitors (Figure 8) enter the thymus via the blood as Lin^{low} $ckit^{high}$ $Cd25^{-}$ cells. They develop into DN1 cells. DN1 cells are defined as $CD25^{-}$ $CD44^{+}$. The cells then acquire CD25 and become DN2 cells. At this stage they start to rearrange the β chain locus. In the next step the cells down-regulate CD44 and c-kit. They are now $CD25^{+}$ $CD44^{-}$ DN3 thymocytes. At this point the DN3 go into an arrest until they productively rearrange the β chain locus of the TCR. This checkpoint is called β selection. The TCR β chain pairs with a surrogate preTα-chain, forming the pre-TCR. The pre-TCR causes the cell to go into cell cycle. The cells are now defined as the DN4 cells and are $CD3^{low}$ $CD25^{-}$. They finish the β rearrangement and undergo proliferation and acquire CD4 and CD8. After

proliferation is done, DP cells decrease in size and start to rearrange their α locus gene segments. After that, the DP lymphocytes express low levels of α/ β TCR as well as CD3. These cells will undergo positive and negative selection.

It could be shown that DN1 and DN2 cells require Notch signaling with Notch dependent factors like IL-7 and c-kit respectively. Both cell types are not yet committed to the T cell lineage. DN3 and DN4 cells loose c-kit expression and are unable to differentiate into anything else but T cells. DN3 still require Notch signaling to continue T cell development, but their growth is independent of IL-7 and c-kit.

2.3 <u>Selection of MHC-restricted α/β TCR</u>

Due to the antigen receptor recombination, a very high number of TCR´s with a guaranted Ag recognition is achieved. However, just like in the BCR system, the TCR has to be tested for Ag recognition properties. Affinity and specifity are tested. What is most important for thymocytes is that they have to be MHC-restricted, specific for a foreign Ag and tolerant to self-Ag. This is why during the development of thymocytes peptide-MHC complexes interact with the TCR. Cells with a TCR that interacts weakly with the self-peptide-MHC of stromal cells will survive. This process is refered to as positive selection. Lymphocytes that do not receive a positive signal die by neglect. If a receptor binds strongly to the self-peptide-MHC complex the cell undergoes apoptosis and are negatively selected.

2.3.1 Positive selection

All emerging DP thymocytes have passed the β selection, and start to rearrange the TCR α-locus. If a DP thymocyte has rearranged its α, the α/ β TCR is "tested" for pairing and for its binding capacities toward MHC. If a α/ β TCR binds to the MHC, the expression of recombination machinery is down regulated. **(114)**

Even though α and β chains have inherent propertiey to bind MHC, TCR-MHC matching is quite rare due to the high degree of polymorphism of MHC molecules. **(115)**

This is the reason to why most of DP thymocytes maintain an elevated level of RAG expression. Each thymocyte can undergo multiple alpha chain gene recombinations at the same allele. The recombination of thymocytes are limited only to the life span of a DP thymocytes, which is roughly 4 days. Two transcription factors are important to transmit the Bcl-X_L dependent survival of DP thymocytes. They are known as RORγ and TCF1. **(116) (117)**

If a cell does not receive a signal from their TCR due to peptide MHC binding within 4 days, RORγ and TCF1 won´t induce Bcl-X_L expression. The cell will not receive a survival signal and will die by neglect. It is important to know that due to the incomplete allelic exclusion of the α locus, it is possible, that one lymphocyte carries two different rearranged α chains. Due to this, the chance of being positively selected is increased. **(118)** Aproximately 1/3 of the mature T cell population has two α chains expressed on their surface. The peptide presented during positive selection on MHC are not just any peptide, but low affinity self-peptide ligands with structural homology to a possible foreign antigen peptide. **(119)** All physical components of a TCR like α,β chain, CD3γ and CD3ε have to be in place for a successful positive selection. However, transcription factors like E proteins and Schnurri-2 are important to maintain a successful positive selection. **(120)** The positive selection takes place within the thymic microenvirement formed by cTECs, which are key cellular components of positive selection. **(121)**

This could be shown by BM chimeric experiments from mice with distinct MHC haplotypes. The 3D architecture is of great importance since a monolayer culture of cTECs is unable to complete positive selection. **(122)** cTECs do not just present the selection ligand on their MHC, they also provide the specialized accessory interactions that are necessary for positive selection. During positive selection the expression of either CD4 or Cd8 is determined. This happens due to the TCR specifity for MHCI or MHCII. It is widely accepted that for positive selection the TCR as well as the co-receptors are important.

2.3.2 Negative selection

During positive selection, cells with to low affinity towards MHC die by neglect. However, cells which are potentially autoreactive and have a high affinity towards self have to be eliminated. This happens during negative selection. Only cells with intermediate affinity to self-peptide-MHC should be active. Where the negative selection takes place is a controversial discussion in the field. Some experiments indicate it happens in the cortex **(123)**, while others suggest it takes place in the medulla. **(124)**

Experiments with high affinity self antigen expressing TCR transgenic mice showed atrophy of the cortex along with a high rate of clonal deletion among DP thymocytes. However, this is not a physiological system. The very high expression of transgene TCR on the DP thymocytes might introduce a bias towards negative selection in such mice. **(125)**

Also, mice with MHCII expression restricted to the cortex show an increased mature autoreactive CD4 T cell population. This proves that some negative selection does occure outside of the cortex. **(126)** Also, cTEC´s of the cortex do not express the co-stimulatory molecules CD80 and CD86 on their surface. These molecules are thought to play a role during clonal deletion. **(127)** In the medulla, two types of APC are present and play a major role during negative selection. These cells are mTECs and BM derived DC´s (BMdDCs). mTECs are crucial for negative selection, since they are responsible for

AIRE (autoimmune regulator transcription factor) dependent promiscuous gene expression (PGE) of various tissue specific self antigens. **(128)**

In PGE, these cells have the ability to express and present nearly the entire peripheral self peptide repertoire. The transcription factor AIRE is important for the negative selection. How important it is proves the fact that $AIRE^{-/-}$ patients develop a multi organ autoimmune syndrome, known as APECED. **(129)**

mTECs are efficient in antigen presentation and are sufficient to mediate negative selection of CD4 autoreactive T cells. **(130)** The DC´s in the thymus are important for the central tolerance. They are able to acquire self-antigen from mTEC´s by the mechanism of cross-presentation and induce clonal deletion of CD4 and CD8 T cells, thus responsible for induction of central tolerance. **(131)**

Wheather a TCR signal by itself can induce negative selection, or if a second signal is necessary is still not fully understood. It should be noted that Ab against the TCR added in vitro cannot promote clonal deletion. However, if APC´s are added negative selection can be restored. **(132)**

It was proposed that co-stimulatory molecules like B 7-1 and B 7-2 provide the second signal to DP thymocytes, inducing apoptosis. **(133)** αCD28 mABs show a similar effect on DP thymocytes in vitro. Also the use of αCD43 or/and αCD5 mABs have been shown to induce the death of $CD4^{+}$ $CD8^{-}$ $CD24^{high}$ cells. **(134)** However, CD28-/- mice have a normal thymopoiesis. **(135)**

CD40L deficiency in thymocytes leads to an impaired negative selection. However this block is incomplete and it might only be a delayed negative selection. **(136)** LIGHT, a TNF receptor family member which binds to lymphotoxin β receptor, has likewise been proposed to provide a second signal for negative selection. However the corresponding KO mouse models show no deficiency in thymopoiesis. **(188)**. It seems that the co-stimulatory function is so important for negative selection, that not only one, but several molecules are responsible. This could explain the lack of a phenotype in single KO mice (earlier mentioned co-receptor KO mice). High affinity interaction during clonal deletion does not require a co-stimulatory signal. The difference between high and low-affinity

interaction may underlie the differences in negative selection whether an endogenous or exogenous ligand is used. CD4 and CD8 molecules seem to not participate in the clonal deletion. This might be due to the high affinity of the interaction which makes it less dependent on any co-receptor involvement. Some scientists believe that recognition of low affinity peptide MHC complexes mediates positive selection and high affinity interactions lead to clonal deletion. The fact that the same TCR binding results in two different downstream signaling events makes understanding even more difficult. There are two different theories for explaining the thymic selection.

The first theory assumes that the cell can measure the amount of TCR´s that have bound a MHC-peptide. High affinity peptides-MHC will engage more TCR interaction then low peptide-MHC. A certain threshold of occupied TCR´s could trigger negative selection. The second theory states that the duration of the TCR/peptide-MHC interaction decides about the nature of the signal.

In case the signal is only of short duration due to low affinity for peptide MHC, the cell receives only an early TCR signal. This would lead to a low-affinity-mediated positive selection. If the interaction is longer due to high affinity interactions, early and late TCR signals are triggered. This would then lead to clonal deletion.

However, the role of co-stimulatory molecules is not explained in these theories. Also, the intermediate affinity ligand cannot be explained. It could be shown that a certain kinase, namely ERK (extracellular signal regulated kinase), is being activated differently depending on the signals received. If a positive selecting ligand binds, a slow and sustained accumulation of ERK can be seen. If on the other hand negatively selecting ligands bind, a strong but transient burst can be seen. **(137)**

To sum up, the TCR affinity seems to be important for thymocyte fate. DP thymocytes encountering a low affinity peptide MHC complex are positively selected. If they encounter high affinity ligands, they undergo clonal deletion. If no functional TCR, or with no affinity, is present, the cells die by neglect.

2.4 Regulatory T cells development

Although there are several quality controls during T cell development, some auto-reactive T cells can reach the periphery. These cells can cause significant harm to the organism. A very special cell called regulatory T cell or Treg helps to control the proliferation and effector function of such auto-reactive T cells. Two types of Tregs are important for this work. Namely the natural thymus derived $CD4^+$ $CD25^+$ $FoxP3^+$ nTregs and the induced Tregs (iTreg), which stem from $CD4^+$ $Cd25^-$ T cells. Togeather they represent 10-15% of peripheral CD4 T cells.

The question where Tregs originate from has been an intense discussion for years. On the one hand it was believed that Tregs were a product of effector T cell differentiation. Another theory describes the existence of an independent thymic derived Treg lineage. Today it seems that both hypothesis might be correct. There are thymic derived $FoxP3^+$ $CD4^+$ T cells that are called nTregs, however, there are also Tregs generated from effector cells in the periphery (iTregs).

Studies with double transgenic mice co-expressing a specific TCR and its agonist in the thymus showed increased generation of nTregs. **(146) (147)** Considering these results, it was concluded, that the decision whether the Treg lineage is induced depends on the binding of the TCR to its cognate Ag. The interaction at an avidity just below the threshold required for negative selection promotes Treg development. **(148)**

It could be shown that the affinity of the TCR for its antigen determines the T cell developmental outcome; this is called Treg commitment versus negative selection. **(149)** In contrary, another group claimed that T cells are stochastically selected toward Treg lineage. They found, in a similar model of double transgenic mice, no increase in absolute numbers of Tregs, but rather in percentage. They claimed that Tregs are more resistant to induced apoptosis. This preferential survival leads to inflation of the percentage of Tregs within the total thymus. CD4+ CD25- cells are less resistant and are eliminated by negative selection. **(150)**

Lin et al, 2007 could show that the high affinity TCR-AG/MHC interaction initiates the development of Treg cell-like precursors, which upon TCR signaling induces FoxP3 expression. **(151)** However, TCR signaling is not the only signal necessary for Treg lineage commitment. Costimulatory molecules like CD28 and B-7 (B-7 is a ligand for CD28 and is expressed by medullary DC´s) are important for Treg development. CD40 and CTLA-4 are also involved in Treg development. However, mice deficient for all these molecules have reduced numbers of Tregs, but the few Tregs left are functional. This indicates that these factors are not exclusively necessary for Treg development. TGF-β and IL-2 are required for Treg survival in the periphery, but not for their development. **(152, 153, 154, 155)**

The cell in the thymus responsible for Treg selection is most likely the earlier mentioned mTEC. Hence, deficiencies in mTEC key genes, like NF_kB, show an impaired Treg compartment.

Green cells from the FoxP3-gfp KI mouse generated by Rudensky are mostly found in the medulla. **(156)** All costimulatory molecules that might influence Treg development (CD80, CD86, MHCII) are expressed on mTECs. However, it is still unclear if only the medullary compartment is required or if Tregs that are generated in the cortex and the medulla is necessary for further development.

2.4.1 Treg Phenotype and Function

nTregs can protect the organism from autoimmune disease. They control and regulate autoimmunity **(157, 158)** and appear shortly after birth. One marker used nowadays to identify these cells is the alpha chain of the IL-2R, namely CD25. **(156, 158)** Another important factor expressed by Tregs is the transcription factor FoxP3, which is relevant for the function of these cells. **(159)** Phenotypically, Tregs and activated T cells share some factors like CD25 expression, GITR and CTLA-4 expression as well as CD4, CD3 and TCR downregulation. **(160, 161, 162)** Tregs respond very inefficiently to TCR stimulation, plus, they do not produce IL-2 in vitro even though their survival depends on IL-2. This means that these cells are anergic. To overcome this anergy in vitro, αCD28 mAB and IL-2 have to be added to the cultures.

The TCR repertoire on Tregs is thought to be very broad. Their $CD5^{high}$ phenotype protects them from negative selection in the thymus. **(162)** Their function in vivo is to prevent the activation of and to reduce the expansion of activated T cells. **(163)**

They suppress harmful immunological responses to a self AG as well as foreign Ag. It could be shown that $CD4^{+}$ $CD25^{+}$ Tregs can prevent the outbreak of several diseases including experimental autoimmune encephalomyelitis (EAE) or inflammatory bowel disease (IBD). **(164)** Tregs can also mediate tolerance to alloantigens in transplantations. **(165)**

The function of nTregs is a great advantage in autoimmunity, however, can have negative effects in case of lymphopenia-induced proliferation or anti-tumor immunity **(166)**

2.4.2 FoxP3

Foxp3 is a transcription factor belonging to the forkhead/ winged helix family.

This factor carries a zinc finger domain and a leucine zipper motif. It could be shown that the forkhead domain binds to the DNA and the leucine zipper mediates homodimerization. The N-terminal region of this factor mediates suppression by binding to NFAT/AP-1 sites of the 5´ regulatory sequences of the IL-2 promoter. This can explain the anergic phenotype of Tregs. **(167)**

FoxP3 is a mediator for genetic mechanisms that control tolerance. FoxP3 is also an important Treg marker. Mice as well as humans with a mutation in this gene show severe defects that can lead to death.

In general the mechanism on how FoxP3 inhibits the effector function is not absolutely clear. However, it seems that in vivo several cytokines have been shown to play a mojor role in Treg-mediated suppression. For example, IL-10, TGFβ and cAMP seem to be important and necessary for the suppressive effect. cAMP is a second messanger that can inhibit IL-2 production and T cell

proliferation. cAMP is transmitted via a gap junction that forms between Teff and Tregs. **(168)** Other molecules that can regulate Treg functions are IL-2, TNF, CD80 and CD86.

2.4.3 Tregs homeostasis

TGFβ and IL-2 are very important for the maintenance of nTregs in the periphery. **(169)**

$CD4^+$ $CD25^-$ naïve T cells are the source for IL-2. The cognate Ag needs to be present in order to maintain the nTreg population. There are two sources for Tregs. One is the thymus were Tregs develop and then leave to the periphery. The other one is the transformation from $CD4^+$ $CD25^+$ Tregs in the periphery. Phenotype and function are the same in thymic derived nTregs and induced iTregs. **(170)**

One reason why naïve T cells differentiate into regulatory T cells is because of the very specific tolerogenic conditions. This is mediated by a DC subset called tolerogenic DC´s. **(171)**

Also TGFβ induces the regulatory phenotype in naïve T cells **(172)**

2.5. The role of FLT3L on the Treg compartment

FMS-like tyrosine kinase or FLT3 is a type III receptor tyrosine kinase. This family is characterized by an extracellular domain consisting of 5 Ig-like domains and a cytoplasmic domain that is a spilt tyrosine kinase motif. FLT3 is expressed in a broad variety of tissues. It was found in the BM, fetal liver, thymus, spleen gonads, placenta and brain. It is also expressed on early B and T cell progenitors in the BM and the thymus as well as peritoneal macrophages and monocytes. **(173)**

FLT3L is a type I protein that exists in a transmembrane or soluble form. The soluble isoform is produced by cleavage of the membrane bound form or by alternative splicing. Every isoform can

activate FLT3 signaling. FLT3L is ubiquitinously expressed at the mRNA level, however the protein can only be found in stromal fibroblast in the BM and in T cells. **(174)**

FLT3L seems to not have a major effect on the B cell compartment. Hence, FLT3L$^{-/-}$ mice are completely healthy and have a normal mature B cell compartment. The mature B cell numbers were normal in the periphery. However, B220^{+} B cell progenitors were diminished in the BM. Also, FLT3L seems to have an influence on stem cells. In an experiment where repopulation of FLT3$^{-/-}$ BM cells under competitive conditions was tested, these cells failed to reconstitute the hematopoietic system. **(175)**

FLT3L$^{-/-}$ mice have reduced leukocytes in the peripheral blood, spleen, BM and LN. Also DCs and NK numbers were severely reduced. The number of B cell progenitors is decreased as well. It is important to know that FLT3L stimulates the growth of lymphoid and myeloid progenitors in vitro. However cytokines and interleukins are necessary for this. **(176, 177)**

There is no effect of FLT3L on erythroid, mast cells or eosinophils.

FLT3L expands granulocytes, macrophages, Tcells, NK cells and DC´s. If FLT3L is injected into mice, it leads to an expansion of hematopoietic progenitor cells (HPCs) and a stimulation of hematopoiesis. This leads to splenomegaly, BM hyperplasia and enlarged LN and liver. Also, it increases the immature B cell numbers in BM and spleen. However, the biggest effect could be seen in the DC compartment. The DC numbers were extremely high, but the cells were completely functional. **(178)** Moreover, an expansion of NK and Treg cell compartments was observed. Decreasing the number of dendritic cells results in the decrease of Treg cells. This indicates a feedback loop exists between Tregs and DCs emphasizing the role of DCs in establishing and maintenance of peripheral tolerance.**(181)**

2.6. The potential role of IL2/αIL-2 on the Treg compartment

The cytokine IL-2 is produced by activated T cells. It supports the proliferation of effector T cells. The source of IL-2 are naïve T cells, Th1 and some $CD8^+$ cells. If a T cell first encounters their specific Ag in the presence of a co-stimulatory molecule, the cell will go into cell cycle. This will also induce the synthesis of IL-2 as well as the α chain (CD25) of the IL-2R. There are 2 receptors known for IL-2. The high- and the low-affinity receptor. The high affinity IL-2R is a three chain receptor. It consists of α chain (CD25), the β chain (CD122) and the common γ chain (CD132).

Both β chain and γc chain are present on resting T cells and form the low affinity IL-2R. This receptor can induce proliferation but needs a high amount of IL-2 signals.

When activated due to Ag encounter, the α chain is expressed. All three chains form the high affinity receptor, which binds IL-2 very efficient. T cells with a high affinity IL-2R can proliferate much faster and with lower Il-2 concentration then the low affinity IL-2R.

IL-2 is a survival factor and allows the differentiation into effector cells. When a naïve T cell encounters their Ag, the transcription Factors NFAT, AP-1 and NFκB are synthesized. These transcription factors bind and activate the promoter region of the IL-2 gene. Also the co-stimulator CD28 activates IL-2 production. It seems like this happens in three ways.

First, due to a stimulus, PI3K is activated and therefore, AP-1 and NFκB are produced. As a result, this leads to Il-2 mRNA production.

Second, the half life time of IL-2 mRNA is prolonged by the CD28 dependent block of the "instability" sequence of mRNA´s. This leads to a longer translation and due to that more IL-2 production.

Third, the earlier mentioned PI3K activates Akt, a protein kinase, which leads to cell growth and survival, leading to more Il-2 production by activated T cells.

IL-2 stimulation does not just induce T cell proliferation, but also NK cell proliferation.

When IL-2 binds its receptor, three intracellular signaling pathways are activated. Namely MAP kinase pathway, Phosphoinositide 3-kinase (PI3K) pathway and the JAK-STAT pathway. All these signals lead to proliferation of T cells.

IL-2 is a survival and maturation factor for T cells. Sprent el alcould show, that injection of an αIL-2 mAB into mice increased the level of memory phenotype $CD8^+$ cells. These cells would not proliferate under “normal” IL-2 conditions. **(164)** However, these levels are sufficient for the survival of Tregs. This increase in numbers of MP CD8+ cells proves that αIL-2 mAB increases the biological activity of the preexisting IL-2. This is probably due to the formation of IL-2/αIL-2 mAB complexes. When IL-2/αIL-2 mAb complexes are formed in vitro and the complexes are injected into mice, the effect is even stronger. The exact mechanism is still not clear, but it is believed that the half-life of IL-2 is increased through the Ab. Also the duration of IL-2 on the IL-2R might be prolonged leading to an enhanced signal.

Different αIL-2 mAB´s show different effects regarding the cell population. The Ab that was mostly used during this work was the JES-6A12 mAb. This mAb increases Tregs to a great extent, but also NK cells are massively increased.

3. B cell development

B cells develop through several intermediate stages in the bone marrow. These stages can be distinguished by the expression of intracellular or surface markers on/in these B cells.

There are three major types of B lymphocytes known, the B-1, the B-2 and Marginal zone B (MZB) cells. B-1 cells are not as frequent as B-2 cells.

B-1 cells can be mostly found in the peritoneal and pleural cavities. However these cells can also be found in the Spleen and parts of the intestine.

How B-1 B cells develop is still under investigation. Some studies suggest that B-1 cells arise separately from B-2 B cells before they acquire surface BCR expression stage. This 'lineage hypothesis' is supported by the fact that particularly B-1a B cells emerge to a great extent from fetal origin and persist in the adult by their self-renewal capacity **(15)**. Furthermore the identification of a B-1 B cell restricted progenitor with a $Lin^{-}B220^{lo/-}CD19^{+}$ phenotype was published **(16)**. In addition B-1 B cells are independent of BAFF/BAFF-R signals for their generation and survival. This can be explained by their self-renewal capacity **(17)**.

A second hypothesis, the 'induced differentiation hypothesis' claimes that a common progenitor can become either a B-1 or B-2 B cell, depending on the nature and strength of BCR signals at the stage where the B-1 B cells start to express sIgM. Different groups reported the specificity of the BCR influences the formation of B-1a B cells **(18, 28).**

B-2 and MZB cells develop and mature in the bone marrow (BM), a primary lymphoid organ. B-2 B cells are the major group of B cells in mice and humans. After several developmental stages the cells migrate to the spleen where further development takes place. The mature B cells stay either in the spleen or migrate to other secondary lymphoid organs like lymph node (LN), appendix, tonsils or the

Peyer´s patches. Upon activation mature B cells develop into highly specialized effector B cells, secreting antibodies at high rate.

3.1 Early steps in B cell development

The early B cell developmental steps, which take place in the BM start off with the multipotent long-termed hematopoietic stem cells (LT-HSCs).

Via several steps that are described in detail in the chapter haematopoiesis the B cell development proceeds with the common lymphoid progenitor (CLP) **(Figure 6)**.

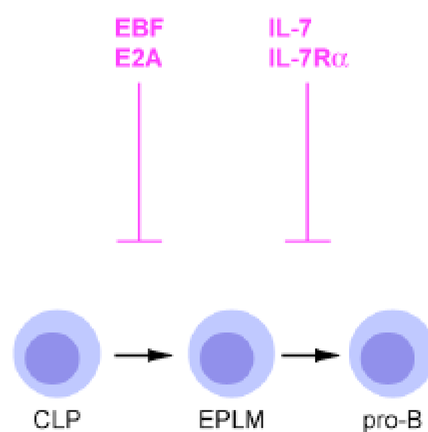


Figure 6: Early stages of B cell development. *B cell development proceeds via several steps to the common lymphoid progenitor (CLP) in the next step the cells develop into the early progenitor with lymphoid and myeloid potential (EPLM). Pro-B cells are the first cells that express CD19, a marker that defines B lineage committment. Adapted from Welner et al., 2008 (8)*

Phenotypicaly this cell is Sca^{low} $c\text{-}kit^{low}$ $FLT3L^{+}$ $CD93^{+}$ $B220^{-}$ $IL7R\alpha^{+}$. The CLP stage is followed by the early progenitor with lymphoid and myeloid potential (EPLM) stage. These cells are defined as Sca^{low} $c\text{-}kit^{low}$ $FLT3L^{+}$ $CD93^{+}$ $B220^{+}$ $IL7R\alpha^{+}$ $CD19^{-}$. EPLM´s are also called pre-pro B cells. After the EPLM´s the pro-B cells are the next precursors in the B cell line. Pro-B cells are defined as Sca^{low} $c\text{-}kit^{low}$ $FLT3L^{+}$ $CD93^{+}$ $B220^{+}$ $IL7R\alpha^{+}$ $CD19^{+}$. CD19 is very important for B cell development since it is part of the co-receptor for the B cell receptor (BCR).

PAX5 is a very important transcription factor. It is a paired homeodomain protein that mediates commitment to the B cell lineage. **(39)**

PAX5 is absolutely necessary for maintaining the B cell fate. **(27, 40)**

It directly activates B cell specific genes like CD19, Igα and represses the expression of other lineage genes, like M-CSFR and Notch-1 (myeloid and T cell lineage). **(41)**

PAX5 also establishes a positive feedback loop by activating the genes E2A and EBF1. A deficiency in Pax5 leads to a block in B cell differentiation downstream of the one seen in $E2A^{-/-}$ and $EBF^{-/-}$ mice. The most mature B cell found in these mice are $CD19^{-}$ and have rearranged D_HJ_H gene segments, while V_H to D_HJ_H rearrangement is impaired. $Pax5^{-/-}$ B cells can, however, differentiate both in vitro and in vivo into all other hematopoietic cells. This includes macrophages, osteoclasts, DCs, ranulocytes, NKs, T-cells and erythrocytes. **(39,42,43)** If PAX5 is down-regulated, it leads to the reprogramming of the B cells and their differentiation into another cell type like macrophages. **(40)**

Another very important factor for the B cell development is IL-7.

$IL\text{-}7^{-/-}$ or $IL\text{-}7R\alpha^{-/-}$ mice have a block in B cell development at the stage of pre-pro B cells. **(59)**

IL-7 is necessary for maintenance of B cell potential in CLP´s. **(60)**

IL-7R signaling is necessary to keep EBF and PAX5 expression above a certain threshold. $IL7R\alpha^{-/-}$ mice have absent Igα, λ5, Vpreβ and RAG1/2 protein levels. **(59)**

$IL\text{-}7^{-/-}$ and $IL7R\alpha^{-/-}$ mice showed another important difference between fetal and adult hematopoiesis with respect to IL-7 dependence. During fetal hematopoiesis, mature B cells develop without IL-7. The cells are either B-1 or MZB. **(61)** This indicates that Fol B development depends more on IL-7R signaling.

At the pro-B cell stage D_H to J_H rearrangement of the IgH chain locus is completed. The rearrangement of IgH chain genes starts already in the EPLM´s with the joining of D_H to J_H elements at the IgH chain locus. For this rearrangement the earlier described RAG proteins are necessary. In particular the RAG-1 and RAG-2 proteins as well as the Tdt enzyme are important. These proteins/enzymes catalyze the insertion of nucleotides at the coding joints which generate the large diversity of B- and T-cell receptors. However it seems like the function of Tdt can be substituted by other factors, since the Tdt KO mice show no phenotype.

Pro-B cells express VpreB and λ5, which together form the co called surrogate light (SL) chain. The next cell in the B cell development is the pre-BI cell, which has the D_HJ_H rearranged. The pre-BI cell continues the heavy chain rearrangement from V_H to D_HJ_H. Also If the rearrangement is in frame, the cells start to express the µH chain. The µH and SL chains form the pre BCR-complex which is expressed on the cell surface. Pre-BI cells that express a functional pre-BCR then develop into large pre-BII cells. These cells are defined as c-kit$^-$ FLT3L$^-$ CD93^{high} B220$^+$ IL7Rα$^+$ CD19$^+$ CD25$^+$. In order to avoid cells with different types of pre-BCR, the genes for Rag-1 and Rag-2 are down regulated. This prevents further rearrangement of the H-chain loci. The genes for Vpreβ, λ5 and TdT are down regulated. Large pre-BII cells undergo cell divisons and become, due to dilution of the long-lived SL chain proteins and the resulting dercease of pre-BCR, resting small pre-BII cells. These cells up-regulate Rag-1 and Rag-2 again and start to rearrange their immunoglobulin light (IgL) chain loci. There are two different light chain loci, κ and λ, whereas there is only one heavy chain loci. The genes for light chain only have V and J elements. The D-element, which is present in the heavy chain loci,

does not exist in the light chain genes. If the L-chain and µH chain pair the cell has a BCR. These cells are then immature B cells. Phenotypically these cells are $CD93^{high}$ $B220^{int}$ $Cd19^{+}$ $CD25^{-}$ IgM^{+}. The levels of surface IgM vary quite strong, however, only cells with high expression of sIgM leave the BM and enter the spleen. **(2)**

In some cases during B cell development, due to the random way of assembling V,D and J Ig segment genes, the cells end up with a non-functional or autoreactive BCR. To prevent negative effects from these BCR´s, several quality control checkpoints exist. Cells that are recognized as not useful are eliminated by negative selection. The first checkpoint can be found at the pre-BI stage. The µH chain has to be able to pair with SL chain and a pre-BCR has to be expressed on the cell surface. Only cells that "pass" this first test can proceed in developing to the large pre-BII cells.

The second checkpoint is between the small pre-BII to immature B cells. At this stage the IgL chain genes are rearranged. The resulting IgL chain has to be able to pair with µH chain. If this is not the case and the cells do not express a functional BCR, these cells undergo another IgL chain gene rearrangement. This mechanism is called receptor editing. **(4)**

Due to this receptor editing the cells get a "second chance" to produce a new, functional IgL chain protein in combination with the already existing µH chain. How often the cells can rearrange depends on the survival time of the pre-BII cells. In order to test the autoreactivety of the BCR, several Tolerance checkpoints are present. At the tolerance checkpoints new complete BCRs are tested for their ability to bind autoantigens. It is assumed, that autoantigens are expressed by stromal cells, in the BM.

The first tolerance checkpoint exists at the immature B cell stage. Here for the first time the complete BCR is expressed. If an immature B cell expresses a BCR with too high affinity to a self-molecule, this receptor undergoes editing. This might lead to an intermediate affinity, resulting in positive selection. If a cell is not able to edit the receptor to a harmless affinity, the cells undergo apoptosis and die. This process is then refered to as clonal deletion.

It was assumed, that most of the newly produced immature B cells express a autoreactive BCR. 25-50% of all B cells underwent receptor editing **(179)**, however, only 10-20% of the immature B cells enter the spleen. **(180)**

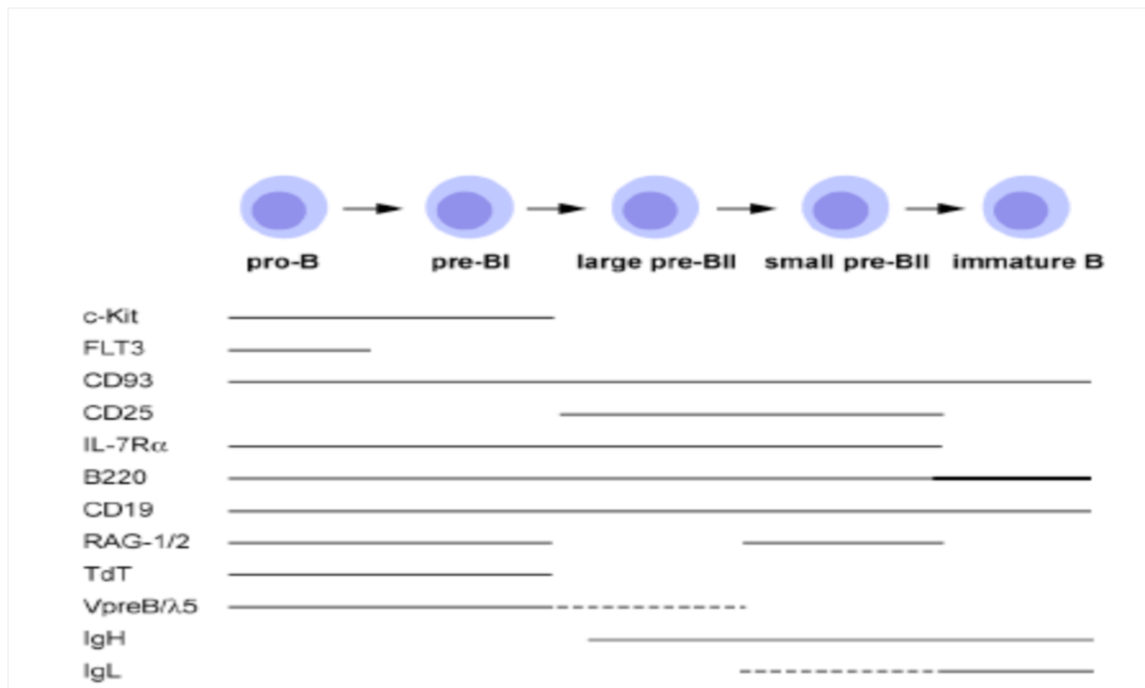


Figure 7: Later stages of B cell development in the bone marrow. *B cell lineage committed pro-B cells develop via several intermediate stages into immature B cells. The immature B cell stage is the first B cell developmental stage where the B cell receptor is expressed. The expression of several surface markers and intracellular proteins is indicated with horizontal lines and the thickness of the lines indicates expression strenght. Factors that cause a complete or partial block at certain developmental stages are shown in pink.*

3.2 Late steps in B cell development

In the previous chapter we discussed early steps in B cell development. All these steps took place in the BM. The precursors had to undergo several steps of negative selection. Cells that survived left the BM and migrated via the blood stream to the spleen **(Figure 7)**.

When newly formed B cells arrive at the spleen they penetrate the marginal zone blood sinus to enter the outer region of the periarteriolar lymphoid sheath (PALS). Immature B cells then undergo two short-lived transitional stages and become long lived mature B cells **(Figure 8)**.

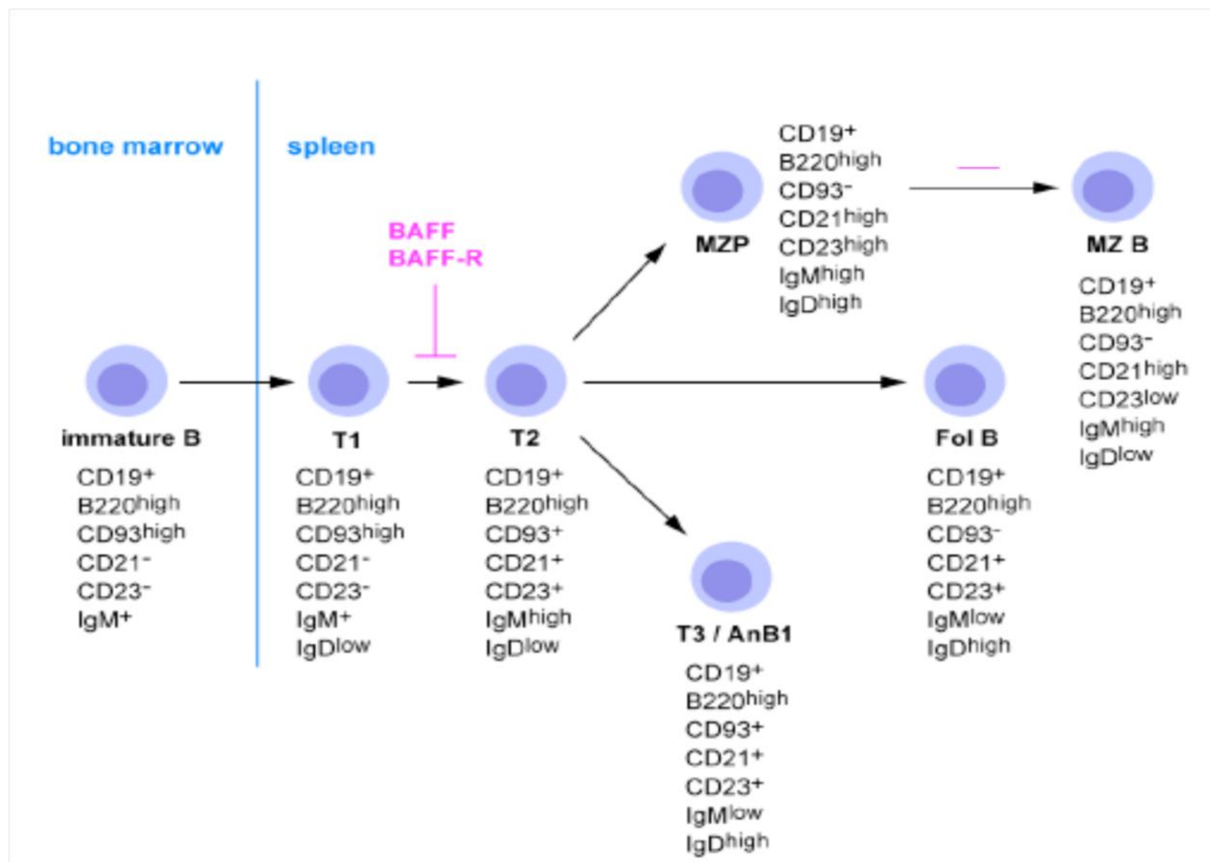


Figure 8: The stages of B cell development in the spleen. *Immature B cells leave the BM and enter the spleen. Here they first develop into short lived transitional B cells, T1 and T2. From the T2 stage, the cells develop either into mature follicular (Fol) B cells or via the marginal zone precursor (MZP) B cells into marginal zone (MZ) B cells. T2 cells that recognize autoantigen are rendered anergic and are now designated anergic population 1 B cells (AnB1 cells) instead of T3 B cells. The expression of several surface markers and intracellular proteins is indicated. Factors that cause a complete or partial block at certain developmental stages are marked in pink.*

Transitional type 1 (T1) cells are the cells of the first developmental stage taking place in the spleen. The T1 B cells are $CD19^{+}$ $B220^{+}$ $CD93^{high}$ $CD21^{-}$ $Cd23^{-}$ IgM^{high} IgD^{low}. This step is followed by transitional type 2 (T2) B cells. These cells are $CD19^{+}$ $B220^{+}$ $CD93^{+}$ $CD21^{+}$ $Cd23^{+}$ IgM^{high} IgD^{high}. T2 B cells show lower expression of CD93 and increased IgD levels. It is important to know that CD21, which is also

called complement receptor 2 (CR2), together with CD19 forms the B cell co-receptor. The ligand for CD21 is the complement cleavage product C3d.

If the B cell co-receptor binds with CD21 to a C3d coated antigen that is captured by the BCR, CD19 is phosphorylated and therefore initiates a signal cascade supporting BCR signaling. **(4)**

CD23 is the low affinity IgE receptor and regulates IgE production. CD23 is expressed as a membrane bound molecule and is later cleaved off by a metalloproteinase called ADAM10 **(5)**. There is also a third type of transitional cell called the T3 B cell. These cells are also $CD93^{+}$ and were therefore considered to belong to the transitional B cells. However, it was recently discovered that the T3 B cells are not able to give rise to mature B cells. Therefore they are not considered precursors of mature B cells. **(6,7)** The T2 B cells develop into mature B cells, namely follicular (Fol) B cells or marginal zone B cells (MZB). T2 B cells develop directly into Fol B cells and differ phenotypically from them by the loss of CD93 expression and the expression levels of IgM and IgD. They are $CD19^{+}$ $B220^{+}$ $CD93^{-}$ $CD21^{+}$ $Cd23^{+}$ IgM^{low} IgD^{high}.

MZB cells also develop from T2 cells however, they develop indirectly. T2 B cells first develop into a marginal zone precursor cell (MZP) and then in the next step develop into MZB cells. The Fol B cells are located inside the Splenic follicles and are by far the largest population of B cells. They circulate between the splenic follicles, the LN and the BM, until they encounter antigen or die.

MZB cells are located in the outer area of the follicles next to the marginal sinus close to the marginal zone macrophages and DC´s. MZP´s are characterized as $CD19^{+}$ $B220^{+}$ $CD93^{-}$ $CD21^{high}$ $Cd23^{high}$ IgM^{high} IgD^{high}. **(29)** MZB´s are $CD19^{+}$ $B220^{+}$ $CD93^{-}$ $CD21^{high}$ $Cd23^{low}$ IgM^{high} IgD^{low}. Unlike the Fol B cell, MZB do not migrate to other organs, but shuttle between the surrounding area of the marginal sinus and the Fol B cell area in the splenic follicles **(8,9)**.

It was long believed that the final steps of B cell development take place in the spleen. However, it was recently found that Fol B cell maturation takes place in both the spleen and the BM. **(10,11)** MZB cells mature only in the spleen. **(12)** When the newly formed B cells leave the BM on their way to the

spleen, they encounter various peripheral antigens, that are “new” to the cells. In case of a strong signal from the BCR, the cell undergoes apoptosis. **(2)** If the signal is not as strong in combination with a high enough occupancy of the BCR, this leads to anergy. **(13)** Anergic B cells have a low IgM expression and a short life span of 4-5 days.

Th T3 B cells, which were thought to be another type of transitional B cell, are believed to be anergic B cells. These cells were renamed to anergic population 1 (An1) B cells. **(14)** This shows that an important tolerance checkpoint for B cells in the Spleen is at the immature transitional B cell stage. 50% of the immature B cells which enter the Spleen are selected into the mature B cell pool. **(2)**

3.3. Antigen dependent development of B cells

Mature B cells need a signal to develop via several steps into activated B cells. There are two possible forms of activated cells: The antibody-secreting cells (ASC), which are plasmablasts and plasma cells. The Plasmablast is the precursor of the plasma cell. The second possible form is the memory B cells. The plasmablast and the plasma cell only differ in their cell-cycle status and the amount of Ab they are able to secrete. Plasmablasts are still dividing and produce low amounts of Ab.

Plasma cells do not divide anymore and produce large amounts of Ab.

Memory B cells on the other hand are long-lived cells that express high affinity BCR´s. During the differentiation steps to plasma cells, the characteristic B cell markers are gradually down-regulated.

Plasma cells are $CD19^-$ $B220^-$ Ig^- $CD21^-$ $CD23^-$ $CD5^-$. Plasma cells and plasmablasts express high levels of CD138. **(19)**

Memory B cells still express CD19, and a subset was found to express increased levels of CD80 and CD73. **(20)**

These cells are not produced at all times as it depends on the location of the encounter with Ag and the elicited immune response. It depends on the Ag whether it will be a T-cell dependent (TD) or T-cell independent (TI) response. A TD response will be induced when proteins are processed and presented on MHCII molecules to $CD4^+$ T-helper (T_H) cells.

TI antigens are subdivided into two categories, Type 1 (TI-1) or Type 2 (TI-2). TI-1 Ag are polyclonal B cell activators (mitogens), like LPS or CpC, that activate B cells via the Toll-like receptors (TLRs) regardless of their BCR specifity.

On the other hand, TI-2 Ag are highly repetitive molecules like polymeric proteins (flagellin) or polysaccharides that activate B cells by engagement and crosslinking of their BCR.

The different types of mature B cells (Fol-B, MZB, B1B) respond differently to TI or TD antigens. During a TD response, mainly Fol B cells are involved, but MZB and B1B cells can also respond. When a Fol B cell encounters an antigen and is stimulated by a T_H cell (through CD40-CD40L), the B cell starts to proliferate. This cell then develops into an ASC directly. These cells are located within the so called extrafollicular region or enter germinal center (GC).

MZB cells respond to a TD antigens differently. They directly differentiate into extrafolicular ASCs and do not participate in a GC reaction. For Fol B cells, the decision of which pathway to take depends on the affinity of the BCR. **(21)** High affinity BCR undergo an extrafollicular plasma cell differentiation. Cells with a weaker affinity BCR tend to go toward a GC reaction. In an extrafollicular region, the activated B cells undergo rapid proliferation. The cells differentiate into plasmablasts and eventually to plasma cells.

Plasma cells formed in extrafollicular regions express a low affinity Ab, mainly of the IgM class, and have a short life span of about 3 days.

GC´s are structures that develop in the folicules of peripheral lymphoid tissues, like spleen, LN, PP or tonsils. **(22)** Germinal centers consist of a dark zone comprised almost exclusively of densely packed

proliferating B cells, the so called centroblasts, and a lighter one, which is made up of non-dividing centrocytes, follicular dendritic cells (FDCs), T cells and macropahges. If an activated Fol B cell enters a GC, they first develop into centroblasts. Centroblasts are fast proliferating cells that are prone to apoptosis. At this stage somatic hypermutation (SHM) take splace in the variable regions of IgH and IgL. The SHM process is initiated by a very important enzyme called activation-induced cytidine deaminas (AID). It catalyses the targeted deamination of deoxycytidine residues. This results in U:G mismatch, which is then repaired by DNA repair mechanisms. This leads to a nucleotide exchange and occasionally to an amino-acid substitution.

The differentiation then continues into centrocytes. They migrate to the light zone of GC. Here cells with a mutated BCR with high affinity for the antigen are selected. Cells with low affinity BCR for the antigen die by apoptosis. Intermediate affinity BCR´s can develop back into centroblasts and undergo a second round of SHM. Due to this process, high numbers of centrocytes with high affinity receptors are formed. This process is called affinity maturation of antibodies. Essential for this process is the induced mutation by SHM and the subsequent selection of cells with a mutated BCR.

Now in the positively selected centrocytes a process called class-switch recombination (CSR) take place. AID expression is crucial for this process. The B cells switch from expression of IgM and IgD to one of the other classes. These new Igs now have different effector function. Which of these Ig is expressed depends on the cytokines that are secreted by follicular T_H cells. **(23)**

How the centrocytes further develop seems to depend on the affinity of the mutated BCR. If a cell has a high affinity BCR, they tend to develop via plasmablasts into plasma cells. **(24)** If on the other hand the cells have a broader affinity spectrum, they go towards memory B cells. **(25)**

Plasma cells which are formed during GC reaction secrete high-affinity antibodies and have an increased life span compared to plasma cells formed in extracellular foci. The antibody producing plasma cells stay in the GC, move to the inflamed tissue or to the BM. The plasma cells then develop into long-lived plasma cells.

Memory B cells move to the Spleen, LN and BM. In case of a re-encountering of the specific antigen, the previously formed memory B cells can rapidly develop into plasma cells.

In a TI immune response, mainly MZ and B-1 B cells are involved. Here no GCs are formed and only short-lived plasma cells develop. The cells originate from extrafollicular regions. MZ and B-1 B cells can develop into short lived plasma cells when they are activated via Toll-like receptors by a TI-1 antigen.

It was claimed that TI responses also form memory B cells. **(26)** MZ and B-1 B cells have a more innate-like B cell phenotype, similar to activated Fol B cells. Through the expression of CD80 and CD86, both cell types can be easily activated and respond rapidly to an antigenic stimuli. **(27)** The characteristics (fast encounter with antigen and location) of MZB cells enables these cells to act between the immediate innate immune response and the delayed TD GC response. B-1 cells can be activated and produce natural IgM in the serum without the stimulus of exogenous antigen. Hence, these cells are a first line of defense.

3.4. BAFF and its receptors, BAFFR, TACI and BCMA

3.4.1 Structure and expression of BAFF

BAFF or B cell activating factor has several names. It is also called Blys (B lymphocyte stimulator), TALL-1 (TNF- and ApoL-related leucocyte expressed ligand 1), THANK (TNF homologue that activates apoptosis, NF-B and JNK) and zTNF4. **(62-66)** BAFF is a type II transmembrane protein of the tumor necrosis factor (TNF) ligand super family (TNFSF). It is highly homologous to APRIL (a proliferation inducing ligand), which is another member of the TNF ligand family. **(67)** Both proteins are very homologous within the TNF homology domain (THD). BAFF exists in two forms, a transmembrane and a soluble form. The soluble BAFF is generated by cleveage of the transmembrane form by a furin-like protease. There is also a delta version of BAFF which is shorter and is generated by alternative splicing. **(68)** Delta BAFF lacks the furin protease recognition motif, which is why it only exists as a membrane bound form. BAFF usually form trimers, which are mediated by THD. Soluble BAFF can exist as a homotrimer **(69, 70)**, but it can also form clusters of up to 60 monomers. **(71)** BAFF can also form hetreotrimers with delta BAFF or APRIL.

BAFF-delta BAFF heterotrimers are only present as transmembrane proteins. It is thought that they inhibit the release of soluble BAFF trimers and thus regulate the function of BAFF. **(72)** Soluble BAFF-April heterotrimers are biologically active and can be found in patients with autoimmune disease. **(73)** How BAFF-April heterotrimers are formed is still unclear since APRIL is only initially transcribed as a transmembrane protein, but is then processed in the Golgi apparatus by a furin convertase and secreted as heterotrimer. **(74)** One possible explanation of how BAFF-APRIL heterotrimers could be formed includes another fusion protein. This fusion protein is called TWE-PRIL: It consists of the cytoplasmic and transmembrane domain of TWEAK, which is a TNFSF member, and the extracellular domain of APRIL. Full-length BAFF and TWE-PRIL form transmembrane heterotrimers, which upon cleavage of a protease release soluble BAFF-APRIL heterotrimers. BAFF itself is produced by myeloid cells like monocytes, macrophages and DC´s. It was also found in activated T cells or in LPS or CpG

activated B cells. This results in a MyD88 dependent upregulation of BAFF. **(75)** This is true for mice. Humans however have similar expression of BAFF. hBAFF is produced by macrophages and DC´s and upon activation with IFNγ or G-CSF also by neutrophils. **(76)** If stimulated with IFNα, IFNγ, CD40L or LPS, human DC´s and monocytes upregulate hBAFF. **(77)** It has been shown that upon activation also other cell types are able to produce hBAFF also. These cell types would be airway gland epithelial cells **(78)**, salivary gland epithelial cells **(79)**, fibroblast-like synoviocytes **(80)**, astocytes **(81)**, by VCAM$^+$ stromal cells from hBM **(82)** and even osteoclasts. **(83)**

3.4.2. The BAFF receptors BAFF-R, TACI and BCMA

Soluble BAFF can bind to three receptors **(Figure 9)**. These proteins are BAFF-R, TACI (transmembrane activator and CAML (calcium modulator and cyclophilin ligand) interactor) and BCMA (B cell maturation antigen). BAFFR can only bind BAFF. TACI and BCMA can bind sBAFF as well as APRIL.

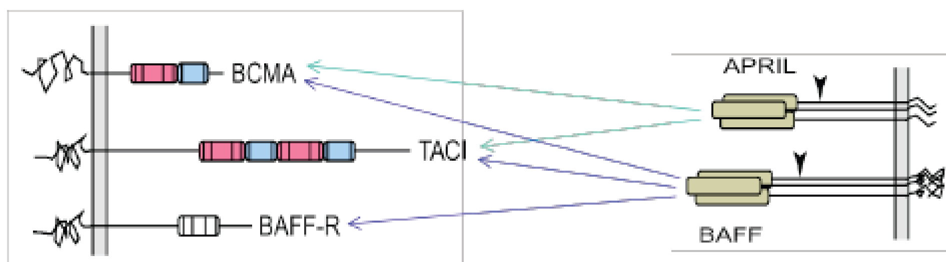


Figure 9: BAFF and APRIL and their receptors. *BAFF can bind all three receptors, APRIL only binds to TACI and BCMA. BCMA (B cell maturation antigen); TACI (transmembrane activator and CAML interactor); BAFF-R (BAFF receptor); APRIL (A Proliferation Inducing Ligand); BAFF (B cell activating factor belonging to the TNF family).*

TACI and BCMA can also bind TWE-PRIL but BAFF-APRIL heterotrimers only bind to TACI. **(73)** BAFF-R cannot only bind to the soluble form of BAFF but also the transmembrane bound form. The same is probably true for BCMA. TACI on the other hand can only be activated by the membrane bound or

oligomeric form of BAFF. Also April can bind to TACI, but not the soluble trimeric form. **(68)** The three receptors belong to the TNF receptor superfamily (TNFRSF), which have by definition a cysteine-rich extracellular domain. They are type III transmembrane proteins that are expressed on B cells. **(84)** In mice the BAFF-R is expressed on splenic B cells and a subset of $CD4^+$ T cells. TACI is highly expressed on splenic T2 and MZB cells and also by Fol B cells, but not on T1 B cells nor on resting or activated $CD4^+$ cells. **(85)** BCMA can be found on plasmablasts and plasma cells. **(86)** All BAFF receptors signal via interactions with TNF receptor associated factors (TRAFs). There are six TRAF molecules known. They all have a conserved C terminal TRAF domain, which enables the interaction with various receptors and intracellular signaling molecules. **(87)** BAFF-R only interacts with TRAF3 **(88)** and activates the alternative NF-κB pathway. **(89)** If BAFF is absent and BAFF-R signaling does not take place, TRAF3 interacts with the NF-κB inducing kinase (NIK). Therefore, NIK is degraded in the proteasome and due to that the NF-κB2 pathway is initiated. If now BAFF-R binds a ligand, TRAF3 is recruited to the receptor and then degraded by TRAF2 dependent proteolysis. **(90)** Therefore NIK is intact and activates the IκB kinase α (IKKα), which results in the processing of the NF-κB2 p100 precursor protein to p52 and the translocation of the p52/RelB dimer to the nucleus. **(91)** BAFF-R signaling also inhibits the nuclear translocation of the pro-apoptotic protein kinase Cδ(PKCδ) **(92)**, which also seems to be mediated by TRAF3 since B cell specific deletion of TRAF3 results in decreased levels of nuclear PK Cδ. **(93)** It has been claimed that PK Cδ interacts with a still unknown factor inhibiting its nuclear translocation, while the interaction of TRAF3 with the same factor releases PK Cδ from the complex. This would allow PK Cδ to enter the nucleus. **(87)** BAFF-R also activates the kinase AKT and the MAPK pathway with c-RAF and ERK, resulting in downregulation of the pro-apoptotic protein BIM. **(94, 95)** It was described that BAFF-R can also be found in the nucleus interacting with H3 IKKB and NF-κB/c-Rel. Thus, it functions as a transcriptional regulator. **(98)** In vitro TACI interacts with TRAF2, TRAF5 and TRAF6. **(140)** BCMA can bind TRAF1,2,3 and activates the MAPK patway with JNK and p38 as well as with the transcription factors NF-κB and ELK-1. **(141)**

However this is all true for the in vitro situation, but how the signaling pathway activated by TACI or BCMA function under in vivo conditions still has to be verified.

3.4.3. Biological activity of BAFF and its receptors

BAFF is a survival and maturation factor for B cells beyond the stage of immature B cells. To be more precise, BAFF is active from the transitional stage (T1) onwards. BAFF tg mice show an accumulation of B cells from the T1 stage onwards. These mice have enlarged spleens, LNs and PPs resulting from the massively expanded B cell population. The B cell progenitor numbers in the BM are not affected. The biggest increase can be seen in T2, MZB and splenic B220^{+} CD5^{+} B cells. The FolB cell numbers are increased to a lesser extent.

Another effect in BAFFtg mice is the increased levels of serum immunoglobulin of all isotypes and IgG subclasses. Another very important effect of BAFFtg mice is the development of autoimmune diseases. This disease shows similar symptoms like the human systemic lupus erythematosis (SLE), anti-nuclear and anti-dsDNA autoantibodies, circulating immune complexes and Ig deposition in the kidney resulting in glomerulonephritis, kidney destruction and proteinuria. **(65, 99, 100)**

BAFF-/- mice on the other hand show a reduced mature B cell compartment. Splenic Fol and MZB cells as well as T2 cells are almost completely absent. T1 B cells are only slightly reduced. B-1a and B-1b cells from the peritoneal cavity are present in normal numbers. The serum Ig levels are extremely low in these mice. The few mature B cells still present in these mice are not able to mount a normal TD or TI immune response. **(101)** Several in vitro studies showed that BAFF can prolong the survival of immature and mature B cells by preventing apoptosis. It could also be shown that BAFF acts synergistically with IL-6 to maintain BM plasma cell survival in vitro. **(86)** BAFF shows significant effects either in the transgene or in deficient mice. Mice deficient in APRIL, on the other hand, show

normal B cell numbers. However, an impaired class switch to IgA and enlarged GCs can be observed in these mice. **(102)** This shows that April is not important for mature Fol or MZB cells, but for the antigen dependent developmental process. Only the BAFFR$^{-/-}$ mice show a similar phenotype like BAFF$^{-/-}$ mice. Thus BAFF-R was considered the main receptor responsible for the earlier described B cell survival and maturation effects of BAFF. Similar to the BAFF deficient mice, the BAFFR$^{-/-}$ mice have reduced numbers of splenic T2 and mature FolB and MZB cells. However, peritoneal B-1 BM progenitors and splenic T1 B cells show no difference regarding cell number or function. The serum levels in these mice are reduced, but in contrast to BAFF$^{-/-}$ mice, immune responses to TI-II antigens and IgM responses against some TD antigens are nearly identical to WT mice. **(103)** BCMA-/- mice have normal mature B cell compartment, with the normal splenic architecture. However they have an impaired survival of long lived BM plasma cells. Also BM progenitor B cells and peritoneal B-1 cells are in normal numbers. The serum Ig levels are unchanged and primary and secondary immune response against TD and TI antigens proceed normally. **(101, 104)** TACI-/- mice have a phenotype similar to the BAFFtg mice. They show enlarged lymphoid organs and increased numbers of mature B cells. The serum Ig levels are normal; only IgA levels are decreased. Td immune responses are normal, but IgM and IgG responses to TI-II atigen are reduced. These mice develop an SLE-like autoimmune disease. **(105, 106)** Thus TACI signaling has an inhibitory function for peripheral B cell survival. Both APRIL and BAFF mediate the isotype switch to IgA, IgE and IgG1 in part in synergism with IL-4, by engagement of BAFFR and TACI respectively **(107)**, and signaling via APRIL-TACI pathway is important for the regulation of IgA production. **(108)** Due to TLR activation, BAFF-R and TACI expression is upregulated in Fol B, MZB and B1a cells. This leads to increased proliferation and Ig secretion **(109, 110)** which connects the innate and the adaptive immune system.

Aim of Thesis

In the first project the role of BAFF in autoimmune and immune deficient patients should be determined. It is known that B cells are a very important component of the adaptive immune system and the development of mature and functional B cells is crucial for the humoral immunity. Numerous facts are known about the process of differentiation of HSCs into mature B cells, including intermediate stages and their regulation by transcription factors, cytokines and interleukins.

However some aspects of the differentiation are still unsolved. A better understanding of the mechanisms might help to develop new therapies for the treatment of human disorders known to be associated with a dysregulated B cell development, including autoimmune diseases and malignant disorders such as lymphomas and leukemias.

The finding that increased expression of BAFF in mice results in the development of autoimmune disorders like SLE **(142)** or Sjögren's syndrome led to the idea that increased BAFF levels in humans could be the cause of these autoimmune diseases **(158, 159)**. To further elucidate the role of BAFF in human autoimmune diseases and other disorders associated with perturbation of B cell homeostasis, monoclonal antibodies against hBAFF were produced and a very sensitive ELISA for hBAFF was developed. With this ELISA, the reported observations could be confirmed and serum hBAFF levels in further disorders could be analyzed in order to determine an involvement of BAFF in selected human autoimmune, infectious and cancerous diseases.

In the second project the potential of TRegs in preventing GvHD was analyzed. It is known, that fms-like tyrosine kinase ligand (FLT3L) treatment, increases DC numbers and through that indirectly leads to expansion of peripheral NTreg. The increased number of NTreg is due to proliferation of pre-existing NTreg, and there favoured interactions with the increased number of DC. We investigated the therapeutic potential of FLT3L and IL-2/αIL-2 complexes on induced acute and chronic GvHD. These data hopefully reinforce the relevance of FLT3L and/or IL-2/αIL-2 treatment in transplantation

or autoimmune settings by its ability to increase both the number of immature tolerating DC and NTreg.

In the third project the effect of FLT3L or IL-2/αIL-2 on skin transplant rejection was evaluated. A major goal in transplantation medicine is to achieve allograft tolerance without the need for aggressive immunosuppressive therapy. One of the most sought ways is the artificial increase of Tregs in order to maintain the state of graft local immunotolerance. To test whether Flt3L treatment was effective in prolonging allograft survival we used the transplant model where the tail skin from a B6 $H\text{-}2^{bm12}$ mouse was transferred to the trunk of B6 mice. An aIL2/IL2 complex treatment previously shown to be highly effective in a pancreatic islet allograft transplant model was used as a control. These data hopefully reinforce the relevance of FLT3L and/or IL-2/αIL-2 treatment in transplantation models

2 Results

2.1 Soluble BAFF levels inversly correlate with peripheral B cell number and the expression of BAFF receptor.

Soluble BAFF Levels Inversely Correlate with Peripheral B Cell Numbers and the Expression of BAFF Receptors

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Matthias Kreuzaler, Melanie Rauch, Ulrich Salzer, Jennifer Birmelin, Marta Rizzi, Bodo Grimbacher, Alessandro Plebani, Vassilios Lougaris, Isabella Quinti, Vojtech Thon, Jiri Litzman, Michael Schlesier, Klaus Warnatz, Jens Thiel, Antonius G. Rolink and Hermann Eibel

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Soluble BAFF Levels Inversely Correlate with Peripheral B Cell Numbers and the Expression of BAFF Receptors

Matthias Kreuzaler,* Melanie Rauch,* Ulrich Salzer,† Jennifer Birmelin,‡ Marta Rizzi,† Bodo Grimbacher,‡ Alessandro Plebani,§ Vassilios Lougaris,§ Isabella Quinti,¶ Vojtech Thon,‖ Jiri Litzman,‖ Michael Schlesier,† Klaus Warnatz,† Jens Thiel,† Antonius G. Rolink,*,[1] and Hermann Eibel†,[1]

The TNF family member protein BAFF/BLyS is essential for B cell survival and plays an important role in regulating class switch recombination as well as in the selection of autoreactive B cells. In humans, increased concentrations of soluble BAFF are found in different pathological conditions, which may be as diverse as autoimmune diseases, B cell malignancies, and primary Ab deficiencies (PAD). Because the mechanisms that regulate BAFF levels are not well understood, we newly developed a set of mAbs against human BAFF to study the parameters that determine the concentrations of soluble BAFF in circulation. Patients with PAD, including severe functional B cell defects such as BTK, BAFF-R, or TACI deficiency, were found to have higher BAFF levels than asplenic individuals, patients after anti-CD20 B cell depletion, chronic lymphocytic leukemia patients, or healthy donors. In a comparable manner, mice constitutively expressing human BAFF were found to have higher concentrations of BAFF in the absence than in the presence of B cells. Therefore, our data strongly suggest that BAFF steady-state concentrations mainly depend on the number of B cells as well as on the expression of BAFF-binding receptors. Because most patients with PAD have high levels of circulating BAFF, the increase in BAFF concentrations cannot compensate defects in B cell development and function. *The Journal of Immunology*, 2012, 188: 497–503.

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The B cell-activating factor of the TNF family (BAFF, BLyS) binds with different affinities to three different TNFR-like proteins expressed by B cells termed BAFF-R, TACI, and BCMA (1). Binding to BAFF-R activates the classical and the noncanonical NF-κB signaling pathways, resulting in the expression of a series of downstream genes that are essential for B cell survival (2–4). In humans, homozygous deletion of the BAFF-R gene results in severe B lymphopenia caused by the arrest of B cell development at the stage of transitional B cells (5). As a consequence, more mature B cell subsets, including follicular, marginal zone, and memory B cells, are present only in very small numbers and T-independent immune responses are severely impaired, proving that BAFF-R is one of the central survival receptors for human B cells (5). In mice, homozygous deletion of the BAFF-encoding Tnfsf13b gene interrupts B cell development at the stage of transitional B cells (6), whereas overexpression of BAFF results in a phenotype sharing some of the autoimmune manifestations found in human systemic lupus erythematosus (SLE) (7). Elevated BAFF levels have also been reported in patients suffering from different autoimmune diseases, including Sjögren's syndrome, rheumatoid arthritis, and SLE (8–10). Disruption of BAFF/BAFF-R interactions by BAFF-blocking Abs depletes naive $IgM^{+}IgD^{+}CD27^{-}$ B cells in humans as well as in mice (11–13), and in a subgroup of SLE patients, the BAFF-blocking mAb belimumab has been found to be effective in reducing disease activity (12). Therefore, anti-BAFF therapy has recently been approved for SLE treatment.

Mutations in the BAFF-encoding TNFSF13B gene have not been found so far in patients with primary antibody deficiencies (PAD). Analysis of BAFF serum concentrations in patients suffering from common variable immunodeficiency (CVID)—the most frequent PAD syndrome characterized by low levels of serum IgG and IgA and/or IgM concentrations, impaired in vivo B cell function, and lack of plasma cells (14, 15)—showed markedly increased BAFF levels but no relationship to clinical parameters, B cell numbers, and subsets (16). In a different study (17), an age-related increase in BAFF serum concentrations was reported in healthy donors and CVID patients, but the regulatory circuits of these apparently paradox findings (high BAFF levels and impaired or absent B cell function) remained unclear.

*Division of Developmental and Molecular Immunology, Department of Biomedicine, University of Basel, 4058 Basel, Switzerland; †Center of Chronic Immunodeficiency, University Medical Center Freiburg, 79106 Freiburg, Germany; ‡Royal Free Hospital, University College London, London NW3 2PF, United Kingdom; §Pediatric Clinic and A. Nocivelli Institute for Molecular Medicine, Spedali Civili, University of Brescia, 25123 Brescia, Italy; ¶Department of Molecular Medicine, University of Rome La Sapienza, 00185 Rome, Italy; and ‖Department of Clinical Immunology and Allergology, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, 656 91 Brno, Czech Republic

[1]A.G.R. and H.E. contributed equally to this study.

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Address correspondence and reprint requests to Prof. Hermann Eibel or Prof. Antonius G. Rolink, Center of Chronic Immunodeficiency, University Medical Center Freiburg, Breisacher Strasse 66, 79106 Freiburg, Germany (H.E.) or Department of Biomedicine, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland (A.G.R.). E-mail addresses: hermann.eibel@uniklinik-freiburg.de (H.E.) and antonius.rolink@unibas.ch (A.G.R.)

Abbreviations used in this article: CLL, chronic lymphocytic leukemia; CRP, C-reactive protein; CVID, common variable immunodeficiency; PAD, primary antibody deficiency; SLE, systemic lupus erythematosus.

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To gain further insight into the relationships between steady-state BAFF serum levels and maintenance of B cell homeostasis in humans, we systematically studied BAFF serum concentrations, B cell subsets, and numbers in relationship to clinical parameters in 309 CVID patients. To address whether the steady-state BAFF concentrations are mainly regulated by BAFF binding to receptor-bearing B cells or by other parameters such as inflammation or monocyte activation, we included other primary immunodeficiencies affecting B cell function such as BAFF-R, TACI, BTK, AID, CD40L, and ICOS deficiency. Samples from patients receiving anti-CD20 treatment and after splenectomy were included as a comparison for severe changes affecting B cell numbers or critical organs for B cell maturation, whereas samples from healthy donors as well as from cord blood and from the corresponding mothers served as controls. In this study we report that long-term steady-state BAFF serum concentrations in patients with PAD depend primarily on B cell numbers and on the presence of BAFF-R but not on inflammatory parameters, splenomegaly, or autoimmunity.

Materials and Methods

Animals

Female Lewis rats were purchased from RCC (Fuelinsdorf, Switzerland). Rats were used at 8 wk age. BALB/c, BALB/c Rag2-deficient, BALB/c human BAFF transgenic, and BALB/c Rag2-deficient human BAFF transgenic mice were maintained and bred in our animal facility under specific pathogen-free conditions. Human BAFF transgenic mice were generated in our laboratory using the β-actin promoter to drive the expression of the transgene. All animal experiments were carried out in accordance with institutional guidelines (permission nos. 1887 and 1888).

Patients

In total, 421 serum and plasma samples from patients between 1 and 79 y of age from centers in the Czech Republic, Germany, Italy, and in the United Kingdom were analyzed. The cohort included 2 AID (HIGM2)-deficient patients, 7 patients with CD40L deficiency (HIGM1), 2 with BAFF-R deficiency, 14 with homo- or heterozygous BAFF-R mutations, 3 TACI-deficient patients, 5 patients with ICOS deficiency, 1 patient with Wiskott-Aldrich syndrome, 20 patients with X-linked agammaglobulinemia (BTK deficiency), 3 patients with myotonic dystrophy type 2 caused by expansion of CCTG repeats in intron 1 of the Znf9 gene, 5 patients with Good's syndrome, 8 with IgA deficiency, 7 patients with selective IgG deficiency, 1 patient with IgM deficiency, and 347 patients with CVID who were between 2 and 79 y old with a median age of 42 y. The female/male ratio of CVID patients was 1:1. Sera from 23 immunocompetent but splenectomized patients, from 11 anti-CD20 (rituximab)–treated SLE patients, and from 21 chronic lymphocytic leukemia (CLL) patients were used for comparison, and sera from 50 adult healthy individuals and of 12 newborns and of their mothers were used as controls. The study was approved by the Ethics Committee of the Albert-Ludwigs-University Freiburg (no. 78/2001), and all patients, parents, and control persons agreed by written informed consent.

Generation of anti-BAFF mAbs

Anti-BAFF mAbs were generated by immunizing Lewis rats with recombinant Fc-tagged human BAFF generated in our laboratory (18). Upon fusion with the myeloma cell line Sp2/0, hybridomas were tested for the production of anti-BAFF mAbs by ELISA using plates coated with Fc-BAFF or FLT3L-Fc. Binding was revealed by mouse anti-rat IgG alkaline phosphatase (Jackson ImmunoResearch Laboratories). Those hybridomas that produced IgG binding to Fc-BAFF but not to FLT3L-Fc were subsequently tested for the binding to commercial human BAFF (PeproTech) by ELISA. This analysis revealed 49 hybridomas producing anti-human BAFF mAbs that were either of the rat IgG2A or IgG2B isotype. Of these, 15 hybridomas produced mAbs that were able to block the binding of BAFF to its receptor. Different combinations of blocking and non-blocking anti-BAFF mAbs were used to establish the ELISA showing the highest sensitivity to soluble human BAFF.

BAFF ELISA

BAFF concentrations were measured by a sandwich ELISA using two different rat mAbs recognizing different BAFF epitopes. Serum or plasma was diluted in PBS containing 4% BSA and 0.2% Tween 20 (ELISA buffer) and applied to ELISA plates coated with 10 μg/ml mAb 2.81 in PBS. Plates were incubated for 2 h at room temperature and washed five times with H_2O containing 0.2% Tween 20. After adding biotinylated mAb 4.62 at 2 μg/ml in ELISA buffer, plates were incubated for 2 h at room temperature, washed five times, incubated with streptavidin-conjugated alkaline phosphatase (Amersham Biosciences, Buckinghamshire, U.K.) in ELISA buffer for 1 h at room temperature, washed five times, and developed with DNP-phosphate (1 mg/ml) in diethanolamine substrate buffer at pH 9.8. After 30 min the reaction was stopped by adding 1 M NaOH and the OD at 405 nm was determined. The standard curve using recombinant BAFF (PeproTech) is shown in Fig. 1. The detection limit was ~0.1 ng/ml BAFF, and differences between soluble BAFF concentrations in serum or plasma samples collected from the same individuals were not found.

Statistical analysis

Correlations between BAFF concentrations and other parameters were calculated with GraphPad Prism using the two-sided *t* test within a 95% confidence interval or the Spearman correlation.

Results

BAFF levels in serum of adult healthy controls and newborns

To determine reference values for soluble BAFF, we first analyzed BAFF concentrations in sera of 52 adult healthy controls. As shown in Fig. 2*A*, the median BAFF level found in 52 adults was 0.6 ng/ml, ranging from 0.3 to 2.25 ng/ml. Sera from cord blood (Fig. 2*B*) contained more BAFF (2.1 ng/ml; range, 0.6–4.5 ng/ml) than the sera from the blood of the corresponding mothers (0.6 ng/ml; range, 0.3–2.25 ng/ml), suggesting that BAFF concentrations are higher in individuals when the immune system and the B cell repertoire are not yet fully developed.

BAFF concentrations in patients with PAD

Soluble BAFF was detected in all patient samples except for three CVID patients who had BAFF concentrations at background levels (Fig. 2*C*). Because very low BAFF concentrations might result from mutations in the TNFSF13B gene, the coding regions of the alleles were sequenced but found to be normal (data not shown). Most of the samples from non-CVID patients contained elevated BAFF levels except for the AID-deficient individuals and for one sample from a patient with IgAD, two patients with persistent polyclonal B lymphocytosis, and two patients with mytonic dystrophy due to mutations in ZNF9. From the patients of the CVID cohort, 48 of 347 analyzed samples (13.8%) had normal BAFF concentrations (mean ± SD: 0.6 ± 0.2 ng/ml; HD, 0.6 ± 0.3 ng/ml), whereas all others had increased BAFF concentrations with peak levels at 90 ng/ml (mean, 9.8 ± 15 ng/ml), and 44 (12.6%) samples contained BAFF at concentrations >20 ng/ml. In CLL patients, BAFF serum concentrations were very similar to healthy controls (*t* test, $p = 0.8445$).

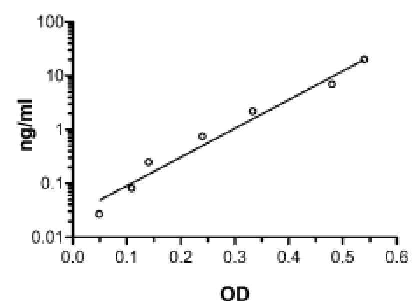

FIGURE 1. Standard curve and detection limits. The ELISA was standardized using recombinant human BAFF. The detection limit is at 0.1 ng/ml soluble human BAFF.

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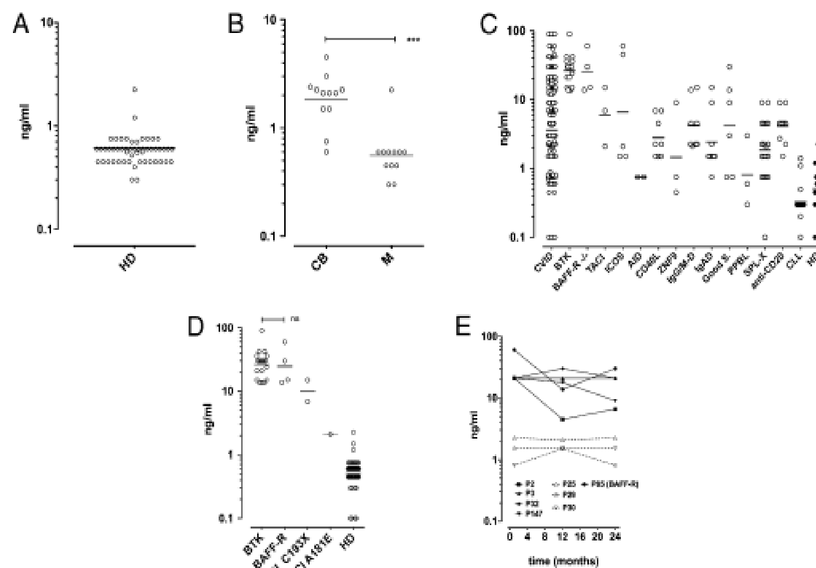

FIGURE 2. Soluble BAFF concentrations in healthy donors and patients with PAD. In healthy donors (*A*), BAFF concentrations are generally very low with a mean of 0.61 ± 0.27 ng/ml (SD; $n = 52$). Soluble BAFF concentrations are significantly higher ($p = 0.0003$) in the cord blood of newborns ($n = 12$) than in the blood of the corresponding mothers (*B*). Patients with PAD show elevated BAFF concentrations (*C*). The plot shows BAFF serum concentrations in 347 CVID samples, in 20 samples from patients with BTK, 4 with BAFF-R, 8 with CD40L, 2 with AID, 5 with ICOS, 8 with IgA (IgAD), 8 with selective IgM/G, 3 with TACI, 3 with ZNF9 deficiency, 3 with persistent polyclonal B lymphocytosis (PPBL), 23 samples from patients after splenectomy (SPL-X), 21 patients with CLL, and 11 patients under anti-CD20 (rituximab) treatment. Sixty samples from healthy donors served as controls. BAFF levels in BTK-, BAFF-R–, and TACI-deficient patients are shown in *D*; changes in BAFF concentration for eight CVID- and one BAFF-R–deficient patient within a period of 2 y are shown in *E*. Statistic differences were determined by the two-sided *t* test for unpaired samples in a 95% confidence interval. Geometric means are shown as horizontal lines.

Very high BAFF concentrations (≥10 ng/ml) were detected in samples of BTK patients, in patients with a homozygous BAFF-R deletion, in patients carrying heterozygous C193X TACI mutations, and in two ICOS patients with very low (≤2%) percentage of B cells (Fig. 2*C*, 2*D*). Intermediate levels (2–10 ng/ml) were found in patients with CD40L deficiency, myotonic dystrophy type 2, Good's syndrome, selective IgG, IgA, or IgM deficiency, in splenectomized patients, and in anti-CD20 treated patients. Healthy controls and CLL patients had significantly lower BAFF concentrations (0.1–2 ng/ml) (Fig. 2*A*, 2*C*). As reported before (19–21), short-term depletion of B cells by anti-CD20 treatment correlated with an increase of BAFF levels in patients under treatment, which, however, did not reach the concentrations found in BTK and CVID patients. Long-term aspleny after splenectomy also led to an increase of BAFF, which was comparable to anti-CD20 treatment (Fig. 2*C*). These data suggest that cells residing in the human spleen are not the major source of soluble BAFF found in circulation.

Because soluble BAFF concentrations may change during aging (17), in response to infection (22), or with monocyte activation (23, 24) we compared BAFF concentrations of seven CVID patients during a 2-y period (Fig. 2*E*). Although up to 2-fold changes in BAFF levels were observed for four CVID patients, those who started with BAFF levels >10 ng/ml retained high levels and those who had intermediate BAFF concentrations ranging from 0.8 to 2.3 ng/ml remained at lower levels, indicating that in different individuals BAFF concentrations remain remarkably stable over long periods of time.

It was reported before that elevated BAFF serum concentrations associate with autoimmunity as well as with primary and acquired immunodeficiencies (5, 16, 17, 25). It was also proposed that in CVID, elevated BAFF levels result from increased constitutive production and/or underlying immunoregulatory or inflammatory conditions (16).

We therefore analyzed whether BAFF levels change with clinical complications frequently associated with PAD such as autoimmunity, splenomegaly, granuloma, and lymphoproliferation, but we failed to detect differences between patients with or without these complications (Fig. 3*A*). The comparison between BAFF concentrations and two parameters changing during infection or inflammatory, namely the percentage of neutrophils and the concentration of C-reactive protein (CRP) (Fig. 3*B*), only revealed a weak association with CRP ($p = 0.0243$, $r = 0.198$). Analyzing the data of 270 CVID patients, a weak inverse correlation was found between BAFF levels and age (Fig. 3*B*; $p = 0.036$, $r = -0.128$), although there were no differences in the distribution of BAFF concentrations comparing very young patients at ages <15 y ($n = 22$) and patients at ages >64 y ($n = 23$, data not shown).

Because we noted that BAFF concentrations were high in all BTK-deficient patients, who have extremely few B cells, but were very low in CLL patients, who have highly increased numbers of circulating and BAFF-R– and TACI-expressing cells (Fig. 2*C*, 2*D*), we analyzed whether BAFF levels in CVID patients with B cell pools of different size would correlate to the percentage and numbers of B cells (Fig. 4*A*, 4*B*). As suspected, an inverse, nonlinear correlation was found between soluble BAFF concentrations and the percentage ($p < 0.0001$, $r = -0.5228$) and number ($p < 0.0001$, $r = -0.224$) of circulating B cells. In particular, from the group of 50 CVID patients with ≤50 B cells/µl (normal range, 80–450 B cells/µl), only 13 (26%) had BAFF concentrations <10 ng/ml, whereas 28 (56%) patients had soluble BAFF concentrations >20 ng/ml. For patients carrying mutations affecting either directly or indirectly B cell development or dif-

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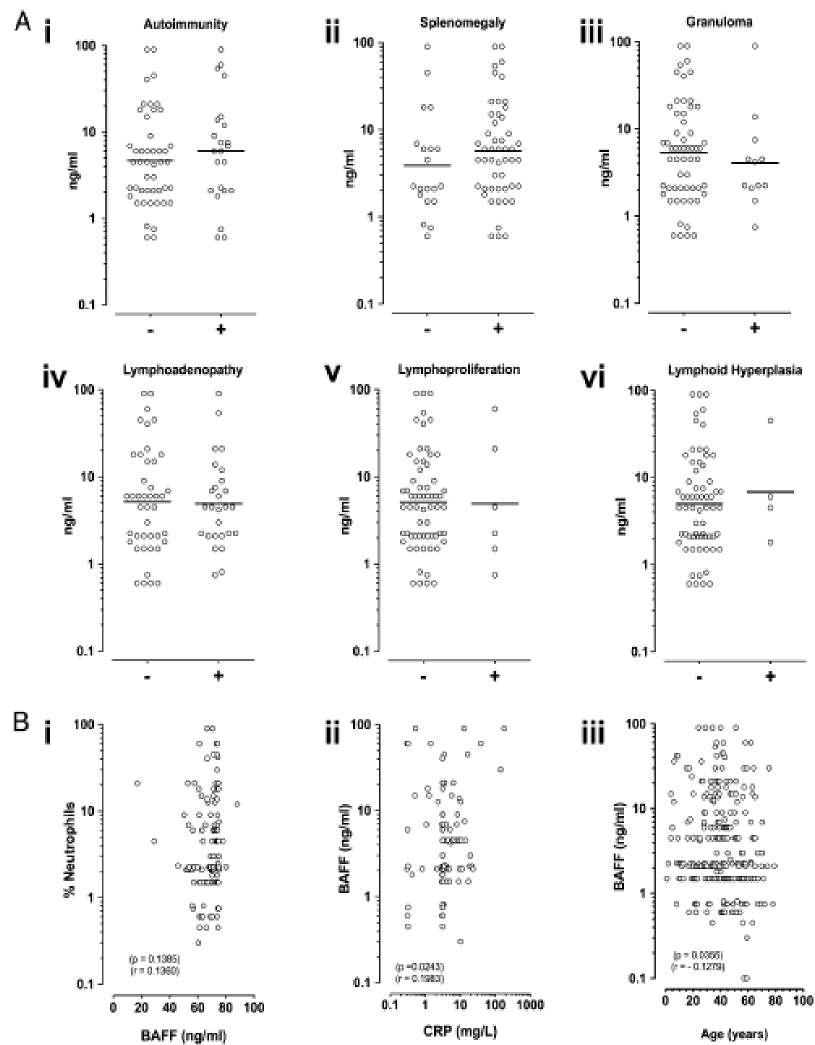

FIGURE 3. Differences in BAFF concentrations between patients with or without clinical complications. *A*, Differences in BAFF concentrations (ng/ml) in a cohort of 70 CVID patients with a complete clinical and immunological record between individuals with or without autoimmunity (*i*), splenomegaly (*ii*), granuloma (*iii*), lymphoadenopathy (*iv*), lymphoproliferation (*v*), and lymphoid hyperplasia (*vi*). Significant statistical differences between groups of patients without or with these complications were not found by the two-tailed *t* test within a 95% confidence interval for unpaired samples with different variances. *B*, Correlations between BAFF concentration, the percentage of circulating neutrophils (*i*), the concentration of CRP (*ii*), or age (*iii*) determined by the Spearman correlation within a 95% confidence interval ($n = 70$). A weak but significant correlation was found between BAFF serum levels and CRP (*ii*; $p = 0.0243$, $r = 0.1983$) or age (*iii*; $p = 0.0356$, $r = 20.1279$). Horizontal lines represent geometric means.

ferentiation (BTK, CD40L, ICOS) and BAFF binding (BAFF-R, TACI), the correlation between BAFF concentrations and the percentage (Fig. 4*C*; $p < 0.0001$, $r = -0.8$) or numbers of B cells (Fig. 4*D*; $p = 0.0001$, $r = -0.7$) was even stronger, and in BTK deficiency, similar BAFF levels were found in young (5 y) as in old (47 y) patients. These data suggest that the number of B cells, and thus the number of available BAFF-binding sites, is one of the most critical parameters in regulating the steady-state concentrations of soluble BAFF.

BAFF concentration in the serum of wild-type and Rag2-deficient human BAFF transgenic mice

The results described above strongly indicated that soluble BAFF concentrations in humans are to a large extent determined by the number of B cells, in that individuals with low B cell numbers have higher circulating BAFF levels than do those with normal B cell numbers. The most simple explanation for this finding would be that "consumption" of BAFF by B cells regulates the concentrations of soluble BAFF. To test this hypothesis in a system where BAFF production is constant but the number of B cells is varying, we determined BAFF levels in the sera of wild-type (BALB/c) and Rag2/Il2rg double-knockout mice on a BALB/c background expressing a human BAFF transgene under the control of the β-actin promoter. Clearly, human BAFF was undetectable in wild-type and Rag2/Il2rg-deficient mice lacking the human BAFF transgene (Fig. 5), whereas human BAFF transgenic wild-type mice had soluble BAFF concentrations at ~10 ng/ml. In marked contrast, ~5-fold higher BAFF levels (55 ng/ml) were measured in

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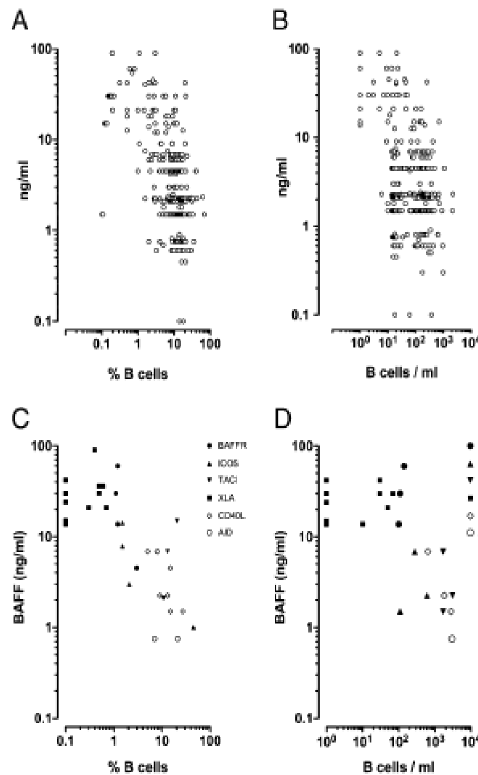

FIGURE 4. Correlation between BAFF levels and the percentage and numbers of B cells in patients with PAD. The relationships between BAFF levels (ng/ml) and the percentage of circulating B cells (*A*), the number of B cells/ml (*B*) in CVID patients, and the percentage (*C*) and numbers (*D*) of B cells in patients with genetically caused PAD were calculated by the Spearman correlation. An inverse correlation between BAFF concentrations was found for the percentage ($p < 0.0001$, $r = -0.5228$) and the number of B cells ($p < 0.0001$, $r = -0.2224$) in CVID patients and in patients with genetically defined PAD ($p < 0.0001$, $r = -0.7966$ for the percentage and $p = 0.0001$, $r = -0.6768$ for B cell numbers).

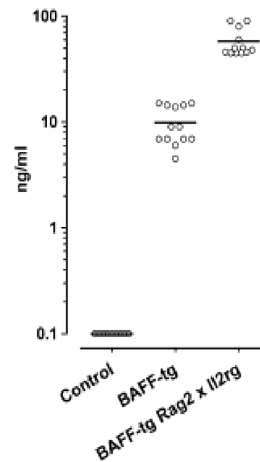

FIGURE 5. Soluble BAFF levels in human BAFF transgenic mice in the presence and absence of B cells. Human BAFF transgenic mice have ~10 ng/ml BAFF on a BALB/c background with normal B cell numbers and 55 ng/ml on the Rag2/Il2rg background, which completely lacks B cells.

transgenic human BAFF Rag2/Il2rg-deficient mice. Because human BAFF binds to murine BAFF-R and TACI expressed by the B cells of wild-type mice, and because the Rag2/Il2rg-deficient mice completely lack B lymphocytes, this result strongly supports our hypothesis that steady-state BAFF levels are to a large extent regulated by its consumption by B cells.

Although the concentrations of human BAFF in wild-type human BAFF transgenic mice were ~4- to 30-fold higher than in healthy controls (0.3–2.25 ng /ml), we did not observe any signs of autoimmunity. However, increased BAFF concentrations led to the 2- to 3-fold expansion of the B cell pool (data not shown), which is consistent with the survival promoting function of BAFF (26).

Discussion

BAFF-induced signals are essential for the development of a functional B cell compartment (1), and the treatment of patients with autoimmune diseases such as SLE with the BAFF-neutralizing Ab belimumab showed that transitional and naive $IgD^{+}CD27^{-}$ B cells are much more dependent on BAFF-induced survival signals than are $IgD^{-}CD27^{+}$ switched memory or IgD^{+} $CD27^{+}$ marginal zone-like B cells (11).

BAFF exists as membrane-bound protein and in a soluble form (27), which has been shown to aggregate into 3-mers or 60-mers depending on the primary structure of the protein (27–33). The cytokine is produced by hematopoietic (23) as well as by nonhematopoietic cells (34), and its expression has been found to be upregulated by proinflammatory responses (23, 27, 34, 35), during viral infections (25, 36, 37), and in various autoimmune conditions (8, 10, 38–40).

From studies of small cohorts of patients suffering either from common variable immunodeficiency, selective IgA deficiency, or X-linked agammaglobulinemia, increased levels of soluble BAFF have been reported in adolescent as well as in adult patients (16, 17). However, it remained unclear whether the increase in BAFF levels correlated to the size of the B cell pool, the developmental potential of B cells, or to immunodeficiency-related inflammatory conditions (16). BAFF-supported survival of transformed B cells and increased BAFF concentrations have also been reported for B lymphomas and leukemia (41–47), but the direct comparison of these results remains difficult since different BAFF-specific Abs and standards were used.

In this study we report the generation of a new set of mAbs against human BAFF, which we used to study factors regulating soluble BAFF concentrations in humans. To reveal whether the developmental stage of the immune system, the size of the B cell pool, and the availability of BAFF-binding receptors regulate soluble BAFF levels we tested samples from healthy newborns and adults, from patients with severe functional B cell defects such as BTK, BAFF-R, TACI, and AID deficiency, from patients with reduced B cell numbers after splenectomy or during anti-CD20–mediated B cell depletion, as well as in CLL patients who have a highly expanded, clonal B cell population.

By analyzing cord blood, we show that BAFF is present from birth on to support the maturation and survival of human B cells. The vast majority of B cells in newborns, who have in general ~2-fold higher B cell numbers per blood volume unit than do adults, are transitional B cells, which express less BAFF-R than do mature B cells and are more susceptible to apoptosis. Thus, higher BAFF levels in newborns and small children (17) may be a physiological response to ensure the survival of transitional B cells and to support the expansion of a growing B cell compartment.

In healthy adults, soluble BAFF levels are very low (0.6 ± 0.3 ng/ml) or even below the lowest sensitivity limit of our assay,

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suggesting that only as much BAFF is produced as is needed to provide sufficient survival signals to maintain and limit the size of the steady-state pool of B lymphocytes. This finding implies that feedback mechanisms exist that adjust BAFF production to the requirements of BAFF-dependent B cell subsets. One of the critical feedback loops seems to be the presence of BAFF-binding receptors. This interpretation is strongly supported by the >5-fold decrease in BAFF levels in human BAFF transgenic mice with a normal B cell compartment compared with their $Rag2/Il2rg^{-/-}$ counterparts. In BTK-deficient patients, who lack mature BAFF-R^{+} B cells from birth on, BAFF concentrations are up to 150-fold higher than in healthy controls. In BAFF-R–deficient patients, who have up to 10-fold less circulating B cells lacking the receptor with the highest affinity for BAFF, BAFF levels are increased up to 100-fold, whereas in patients with heterozygous TACI C193X mutations, who show normal BAFF-R but reduced TACI expression and ligand binding (48), soluble BAFF levels are increased only up to 25-fold. In contrast, BAFF concentrations are close to normal in the patient carrying a heterozygous TACI A181E mutation, which allows almost normal TACI expression and ligand binding.

The regulation of soluble BAFF levels by steady-state B cell numbers is further supported by the results obtained from analyzing other genetically caused PAD. In this study, BAFF levels were inversely correlated with the percentage of circulating B cells but were independent of the mutations in AID, CD40L, or ICOS affecting different cells and functions required for the generation of switched memory B and plasma cells. Similar observations were made for other, genetically not yet defined primary immunodeficiencies such as Good's syndrome and selective IgM, IgG, and IgA deficiencies. Again, BAFF levels inversely correlated to the numbers and the percentage of circulating B cells.

For CVID patients, a similar relationship between low numbers and percentage of circulating B cells and high BAFF levels was found, although it was not as pronounced as for the genetically defined primary immunodeficiencies since some patients showed both increased BAFF concentrations and normal or even increased B cell numbers. Elevated BAFF concentrations in splenectomized individuals and the lack of correlation between BAFF levels and splenomegaly, which is a sign of splenic dysfunction, indicate that spleen function is not required to maintain BAFF serum concentrations.

In none of the analyzed cohorts of PAD did BAFF concentrations correlate to autoimmunity, splenomegaly, lymphadenopathy, or acute inflammation, as reflected by CRP levels or increased percentage of neutrophils. Therefore, the size of the B cell pool and the availability of BAFF receptors seem to be the primary factors regulating steady-state concentrations of soluble BAFF, although a long-term increase in BAFF levels in response to chronic infections and inflammation cannot be excluded. The differences between our human BAFF transgenic mice and other BAFF transgenic mouse lines in developing autoimmunity may be due to different genetic (7) backgrounds (BALB/c versus C57BL/6) and to differences in BAFF concentrations, which were 10- to 75-fold higher (49) than in our transgenic mice.

In conclusion, our results suggest that the number of B cells and the presence of BAFF-binding receptors determine the concentrations of soluble BAFF. Although multiple parameters that are not well understood act on BAFF-producing cells, the number of B cells and thus of available BAFF receptors seems to be one of the most important regulatory factors adjusting BAFF levels and the size of the B cell pool in adults. Many patients with PAD have low numbers of circulating B cells that are blocked in differentiating into switched memory B cells or plasma cells. Our study shows that in these patients defects in B cell development and function cannot be compensated by increased BAFF concentrations.

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Disclosures

The authors have no financial conflicts of interest.

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2.2 The effect of FLT3L and IL-2/αIL-2 complex treatment on cells of the immune system in normal mice

Introduction

T regulatory cells (Tregs) can down-regulate effector T cells and therefore may help control autoimmune responses. For obvious reasons it is of major interest to understand and manipulate these particular cells in vivo, in order to gain a therapeutic tool for autoimmune diseases.

The in vivo Treg population may be expanded by several different methods including cytokines.

Thus, it was shown, that FLT3L treatment resulted in significant increases of several different haematopoietic cell populations **(181)**, among others, Tregs.

FMS-like tyrosine kinase or FLT3L is a type III receptor tyrosine kinase. This family is characterized by an extracellular domain, consisting of 5 Ig-like domains and a cytoplasmic domain that is a spilt tyrosine kinase motif.

FLT3L injection into normal mice leads to an expansion of hematopoietic progenitor cells (HPCs) and a stimulation of hematopoiesis. This results in splenomegaly, BM hyperplasia and enlarged LN.

Another important cytokine that has an effect on Tregs and is even necessary for their survival, is IL-2. The sources of IL-2 are activated naïve T cells, Th1 and some $CD8^+$ cells. If a T cell first encounters their specific Ag in the presence of a co-stimulatory molecule, the cell will go into cell cycle. This will also induce the synthesis of IL-2 in some cells as well as the α chain (CD25) of the IL-2R in most activated T cells.

It was described that when IL-2 is injected as a complex of IL-2/αIL-2 mAB, several lymphoid cell populations increase significantly. Depending on which antibody was used for the complex formation, Tregs increased as well.

In this study, we have compared the cellularity of lymphoid organs from different mouse strains treated either with FLT3L or IL2/αIL-2 mAB complexes.

Material and Methods

Buffers and solutions

ACK (red blood cell lysis buffer)

0.15 M NH_4Cl

1.0 mM $KHCO_3$

0.1 mM EDTA

sterile filter through a 0.22 µm filter

Coomassie blue staining solution

0.25 g Coomassie brilliant blue R-250

45 ml H_2O

45 ml methanol

10 ml glacial acetic acid

Coomassie destaining solution

45 ml H_2O

45 ml methanol

10 ml acetic acid

DNA loading buffer (6x)

0.25% bromphenol blue

0.25% xylene cyanol

1 mM EDTA

30% glycerol

PBS (10x)

1.37 M NaCl

27 mM KCl

15 mM KH_2PO_4

80 mM Na_2HPO_4

pH 7.2

TAE

1 mM EDTA

40 mM Tris-acetate, pH 8.0

TE

1 mM EDTA

10 mM Tris/HCl, pH 8.0

IMDM medium, SF

176.6 g IMDM powder (Gibco BRL)

30.24 g $NaHCO_3$ (Fluka)

100 ml Penicillin/Streptomycin solution (100x) (Gibco BRL)

100 ml MEM Non Essential Amino Acids (100x) (Gibco BRL)

10 ml Insulin (5 mg/ml) (Sigma)

10 ml β-Mercapthoethanol (50 mM) (Fluka)

30 ml Primatone RL (10%) (Quest International)

adjust to pH 7.0 with NaOH (Fluka)

fill up to 10 l with fresh H_2O, double-distiled

sterile filter through 0.22 µm filter

Freezing medium

10% DMSO in FCS

FCS from Amimed

Ciproxin®

2 mg/ml solution (Bayer AG)

IL6-supernatant

IL6 producing X63 cells (X63-IL6, Karasuyama et al) were grown in 2% FCS/SF-IMDM medium for 7-10 days, then the suspension was centrifuged to remove cells and thereafter sterile filtered through a 0.45μm Millipore filter. The resulting cleared supernatant was kept at 4°C and used at a final concentration of 2% for growth supporting activity.

Trypsin-EDTA solution (10x) from Gibco BRL

Mice

C57BL/6, DBA/2, (C57BL/6xDBA/2)F_1(BDF1), (BM1xBM12)F_1 and (B6xBM12)F_1 mice were bred under SPF conditions in our animal unit. All mice were used between 7 to 16 weeks of age and were maintained in specific pathogen–free conditions. All animal experiments were carried out within institutional guidelines. Mice were killed by CO2 inhalation and organs removed by standard procedures.

Antibodies and flow cytometric analysis

Commercial antibodies were diluted in FACS buffer and used at a final dilution of 1:200.

anti-mouse CD4	BD Pharmingen
anti-mouse CD8α	BD Pharmingen
anti-mouse CD11b	BD Pharmingen
anti-mouse CD11c	BD Biosciences

anti-mouse CD25 α-chain	BD Pharmingen
anti-mouse CD122 (β-chain)	BD Pharmingen
anti-mouse CD132 (common gamma)	BD Pharmingen
anti-mouse CD44	BD Biosciences
anti-mouse NK1.1	BD Biosciences
Streptavidin-PE used at 1:1000	BD Biosciences
anti-mouse FoxP3	ebioscience

BD Biosciences (Allschwil, Switzerland).

Cell preparation

Single-cell suspensions from spleen or lymph nodes were prepared by pressing the organs through a 100-μm nylon mesh into SF-IMDM containing 2-5% FCS. For flow cytometry, cells were washed and resuspended in PBS containing 2% FCS and 0.1% sodium azide (FACS buffer). Viable cells were stained with trypan blue (0.4% trypan blue in 0.81% Nacl and 0.06% KH_2PO_4) and counted in an improved Neubauer hemocytometer. The total number various lymphocytes subpopulations were calculated from the frequency estimated by FACS analysis and the total number of living cells recovered per organ.

Lysis of red blood cells

The pelleted cells were resuspended in 1-2 ml of ACK buffer and incubated at RT for 1 min. To stop the lysis reaction, 10 ml of medium were added. After centrifugation at 1200 rpm, 4°C for 10 min the cells were resuspended in the appropriate medium.

Flow cytometry

Intracellular staining for FoxP3 was performed according to the manufacturer's instructions (eBioscience).

For staining of cell surface antigens, 50-100 µl cell suspension (1-2 x 10^7 cells/ml) are mixed together with the appropriate volume of antibody dilution in a 96-well round bottom plate and incubated at 4°C for 30 min in the dark to protect the the fluorescent dyes. To wash the cells, 100 µl of FACS buffer are added to each well and the plate is centrifuged at 1200rpm (250xg) and 4°C for 3min. The supernatant was then "flicked" out quickly and the plate was dried carefully on a paper towel. The cell pellets were resuspended in the remaining supernatant by vortexing the plate. If necessary, a further staining step with a Streptavidin-coupled fluoreszent dye was performed. In this second step the incubation was done for 30 min at 4°C, followed by another washing step. Finally, cells were resuspended in an appropriate volume of FACS buffer. Unless PE-Cy7 was used as fluorescent dye, 10 µg/ml propidium iodide (PI) were added to the FACS buffer, in order to be able to exclude dead cells. Negative and single stained compensation controls were always included. FACS analysis was carried out on a FACSCalibur (Becton Dickinson) and data was analyzed using ™FlowJo (Tree Star, Inc.). Cells were gated on the lymphoid FSC/SSC gate and on PI-negative living cells, if PI was included.The appropriate dilutions of the antibodies that were produced in the lab were determined by titration beforehand. Commercial antibodies were mostly used at a final concentration of 1:200. Strep-PE was used at a final concentration of 1:1000, Strep-APC at 1:200 and Strep- PE-Cy7 at 1:400.

Freezing and thawing of cells

For freezing, the pelleted cells were resuspended in cold freezing medium at a cell density that was dependent on the cell line, and 1 ml aliquots were transfered to 2 ml cryotubes (Nunc), cooled down in freezing chambers and incubated at -80°C for at least 12 h. Subsequently, the tubes were transfered to their final storage place in a liquid nitrogen tank.

For thawing, cells were unfrozen in a 37°C water bath and transferred immediately to 10 ml of the appropriate medium. After centrifugation at 1200 rpm and 4°C for 10 min, the cell pellet was gently resuspended in the appropriate medium and transferred to a culture dish.

FLT3L mass production and purification

For FLT3L mass production, transfected CHO cells (CHO hFLT3L-Fc 2nd sort) were thawed and cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. After 24h the selection marker G418 was added to the culture. After additional 24 hours the media was aspirated and the cell washed with 8ml sterile PBS. The PBS was aspirated and then 2ml trypsinEDTA solution added. The flasks went back into the 37°C incubator for 5min. When the cells started to detach from the plastic, 5ml of media was added and the cells were rinsed and collected in a 15ml falcon tube. The falcon tube was filled to a total volume of 15ml with media. The cells were then centrifuged at 1200rpm, 4°C for 10min. Media was removed and the cell pellet was washed in 10ml media and then centrifuged again at 1200rpm, 4°C for 10min. The media was aspirated and the pellet re-suspended in media. The cells were transferred into a medium sized flaks. After 24-48 hours the cells were split as described before and expanded to a large flak. Cells were expanded in big flasks till a large quantity of flaks was present. Each big flask was transferred into a roller bottle with 500ml medium containing 2-4% FCS (low IgG) and 0,5% cyproxin. The roller bottles were transferred to a roller in a 37°C room. The cells stayed in the roller bottles for 2 weeks constantly turning.

After this time, the cell suspension was centrifuged for 10min at 3500rpm at RT. The supernatant was sterile filtered through diatomaceous earth (Highflow Super Cell Medium, Fluka).

The sterile suspension was stored at 4°C till further use.

The FLT3L suspension was run through a Protein G-Sepharose® column (GE Health Care). Prot G is a bacterial cell membrane protein that binds the Fc part of IgG. The recombinant FLT3L had an IgGFc-tag, which was bound by the prot G and the media ran through.

The column was washed with sterile PBS and then eluated with 0,1 M citric acid, ph 2,2. The fractions were collected in 2ml eppendorf tubes containing 200µl Tris pH 8,9 to neutralize the solution. The concentration af the fractions were evaluated by Thermo Scientific NanoDrop 1000™. The positive fractions were collected in a dialysis tubing and dialysed against sterile PBS. After 24h the PBS was exchanged by a fresh bottle of sterile PBS. Now the concentrations of the fractions were measured again and aliquoted. The aliquots were stored at -20°C till further use.

Monoclonal anti-IL-2 IgG antibody; mass production and purification

The monoclonal IgG anti-mouse IL-2anti JES6-1A12 or S4B6 were cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. The expansion and the production of the anti-IL-2 mAB hybridomas is the same like for the FLT3L transfectants. Also the purification via a Protein G-Sepharose® column is the same as described before.

The different mAbs were stored at- 20°C till further use.

rIL-2 mass production and purification

For IL-2 mass production, the hybridomas (X63 IL-2, Karasuyama et al) were thawed and cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. The expansion and the production of the IL-2 hybridomas is the same like for the FLT3L transfectants or anti-IL-2 mAb

hybridomas. The purification of the IL-2 was done on an S4B6 column, which is an anti-IL-2 mAB coupled to cyanogenbromide activated Sepharose beads (GE Health Care) according to the description of the manufacturer. The IL-2 binds to the anti-IL-2. By 0,1M citric acid the fractions were eluated as described before. The IL-2 was stored at-80°C till further use.

FLT3L treatment

For FLT3L treatment, mice generally received 10-20 µg rFLT3L (in 0.2 mL PBS) by intraperitoneal injection daily for 10 days.

IL-2/αIL-2 complex treatment

To form antigen-antibody complexes, 2,5µg of the IL-2 and 7,5µg of the αIL-2 were mixed together in a 1,5ml eppendorf tube. The tube was then incubated in a 37°C incubator for 30min. After the complexes had formed PBS was added to reach the final volume.

Each mouse received 10µg (2,5µg IL-2 + 7,5µg αIL-2) of the complex in 200µl PBS/mouse as the final volume.

Results

2.2.1 Effects of FLT3L treatment in BDF1 mice

BDF1 mice were injected daily with 10µg/mouse of recombinant human FLT3L intra peritonealy for 10 days. Human FLT3L can act sufficiently on mouse FLT3 receptor and induce the appropriate response **(181)**. After 10 days, which was shown to be the optimal time for expansion of the different cell populations, the mice were sacrificed and analyzed by FACS. **Figure 10** shows the percentage and absolute numbers of the respective population.

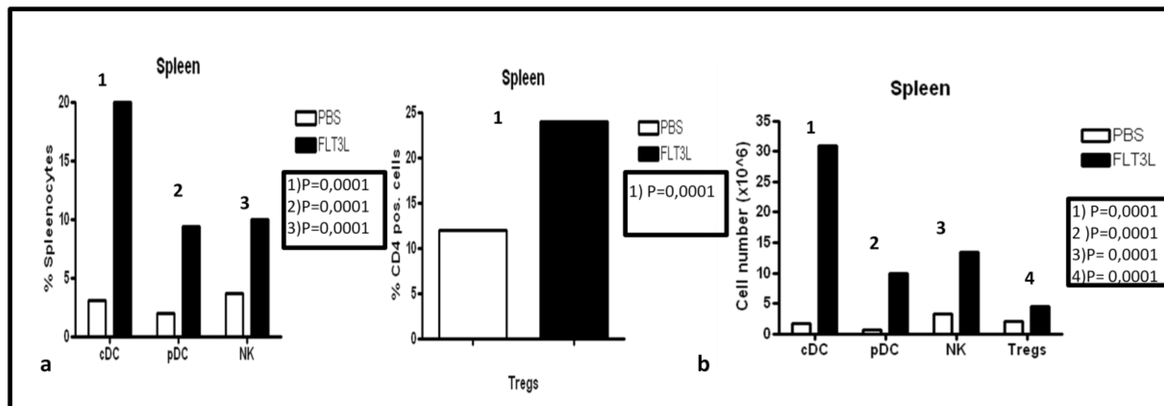


Figure 10, BDF1 mice FLT3L treated: *The amount of cells in per cent (a) and in absolute numbers (b) is depicted in these graphs. The white bars indicate the PBS injected control mice (UT), the black bars represents the FLT3L (10µg/mouse for 10 days) treated mice. The cDCs (UT=3,1/treated=20,0), pDCs (UT=2,0/treated=9,4), NK (UT=3,7/treated=10) and Tregs (UT=12,4/treated=24,0) increased in per cent, as well as in absolute numbers (x10^6) due to FLT3L treatment cDCs (UT=1,7/treated=31,4, pDCs (UT=0,7 /treated=10), NK (UT=3,3/treated=13,4), Tregs(UT=2,0/treated=4,5).*

The most striking effects of FLT3L treatment could be observed in the spleen where the conventional DCs (UT=1,7x10^6 cells, treated=31x10^6 cells) as well as the plasmocytoide DCs (UT=0,7x10^6 cells, treated=10x10^6) showed dramatic increases. Also, the number of NK cells increased dramatically (UT=3,3x10^6, treated 13,4x10^6).

The DC compartment was increased in the spleen **(Fig. 10)**, the BM, the thymus and the LNs (data not shown) of the treated mice. There was no significant difference in the $CD4^+$ and the $CD8^+$ T cell population. However the Treg population in the spleen and LNs were increased by 2-3 fold (UT=12%, treated=24%) (UT=2x10^6, treated 4,5x10^6). This can be explained by the increased number of DCs in the system. It is well established that DCs can support nTreg proliferation in an IL-2 dependent manner, but require additional cell contact dependent signals supplied by DCs **(181)**.

It could be excluded, that the increased numbers of Tregs are of thymic origin, but instead develop extrathymically in the periphery. **(181)**

2.2.2. Effect of FLT3L treatment on (BM1xBM12)F1 or (B6xBM12)F1 mice

Since it is always a problem when experiments are carried out with mice of two different genetical backgrounds, we choose two mouse strains with B6 background but, which carry different mutations in their MHC.

The one strain was the (BM1xBM12)F1 mouse. This mouse has a mutation in MHC class I and MHC class II, namely in $H2K^b$ and $H2I\text{-}A^b$. Due to these mutations, when injected with B6 wt lymphocytes, these mice develop acute GvHD symptoms.

The second strain is the (B6xBM12)F1 mouse, which only has a mutation in the MHC class II; in the $H2I\text{-}A^b$ gene. When injected with B6 wt lymphocytes, these mice develop lupus like symptoms, which are symptoms of a chronic GvHD.

In order to test the effect of FLT3L on these different mouse strains, FLT3L was injected daily for 10 days with 10µg/mouse. After 10 days, the mice were sacrificed and spleens analyzed by FACS for changes in lymphoid cell populations.

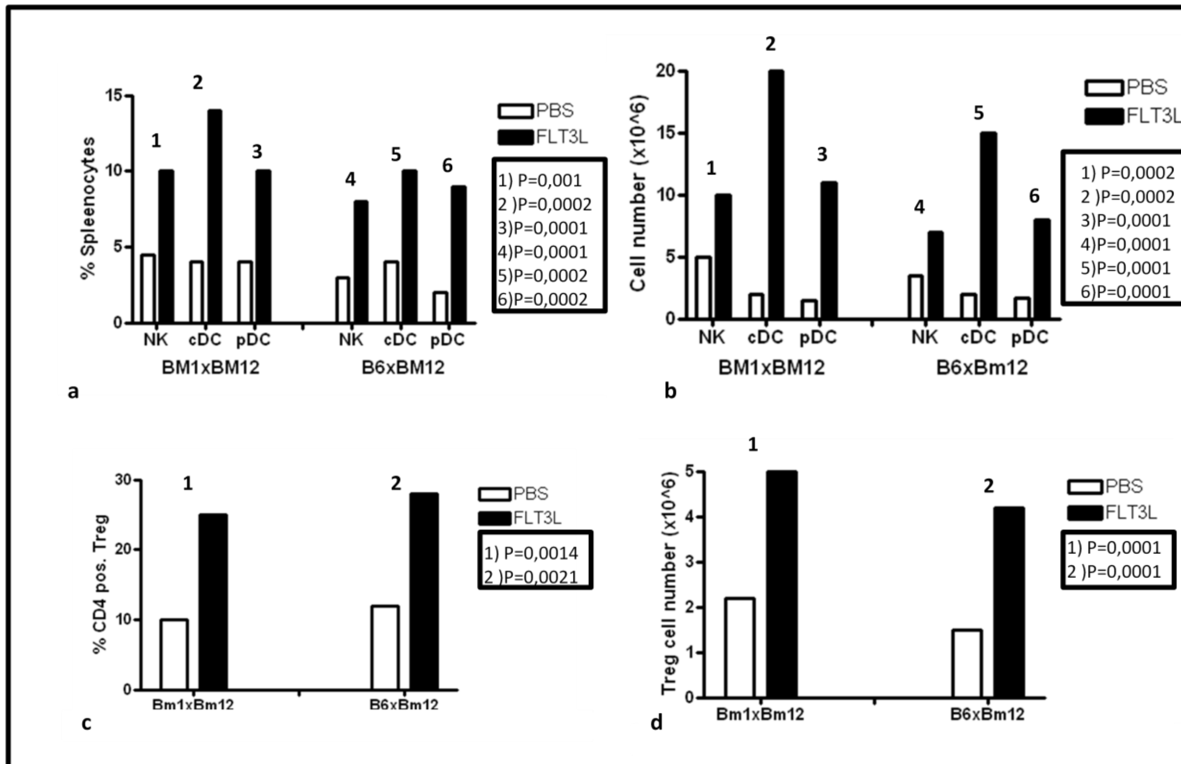


Figure 11, FACS analyses of spleen from FLT3L treated (BM1xBM12)F1 and (B6xBM12)F1 mice: *shows NK cells, pDCs, cDCs in relative (a) and absolute (b) numbers as well as the relative (c) and absolute numbers (d) of Tregs. In both mice, BM1xBM12 and in B6xBM12 the increase in the different cell populations was significant. In every case white bars are PBS control mice and black bars are FLT3L treated mice.*

Figure 11 shows the results of this analysis in relative and absolute cell numbers. Several cell populations were increased in these mice. Similar to the BDF1 mice, FLT3L treatment increased the NK cell population (UT=5%, treated=12%) as well as conventional (UT=4%, treated=14%) and plasmocytoid dendritic cells (UT=4%, treated=10%) in the BM1xBM12 mice. In absolute numbers the increase was also significant. NK cells (UT=5x10^6 cells, treated=10x10^6), cDC´s (UT=2,5x10^6, treated=20x10^6 cells) and pDC´s (UT=2x10^6 cells, treated 11x10^6 cells) all increased significantly due to the FLT3L treatment.

Similar results were obtained in the B6xBM12 mice. Again NK (UT=3%, treated=8%), cDC´s (UT=4%, treated=10%) and pDC´s (UT=2%, treated=9%) increased in relative numbers. Also the absolute cell

numbers increased. NK cells (UT=3,5x10^6, treated=8x10^6), cDC´s (UT=2x10^6, treated=15x10^6) and pDC´s (UT=1,8x10^6, treated=8x10^6 cells) all increased due to FLT3L treatment.

Another cell population that increased were the regulatory T cells. In the BM1xBM12 mice they increased from 10% UT to 25% in the treated mice. In total number they increased from 2,2x10^6 cells to 5x10^6 cells.

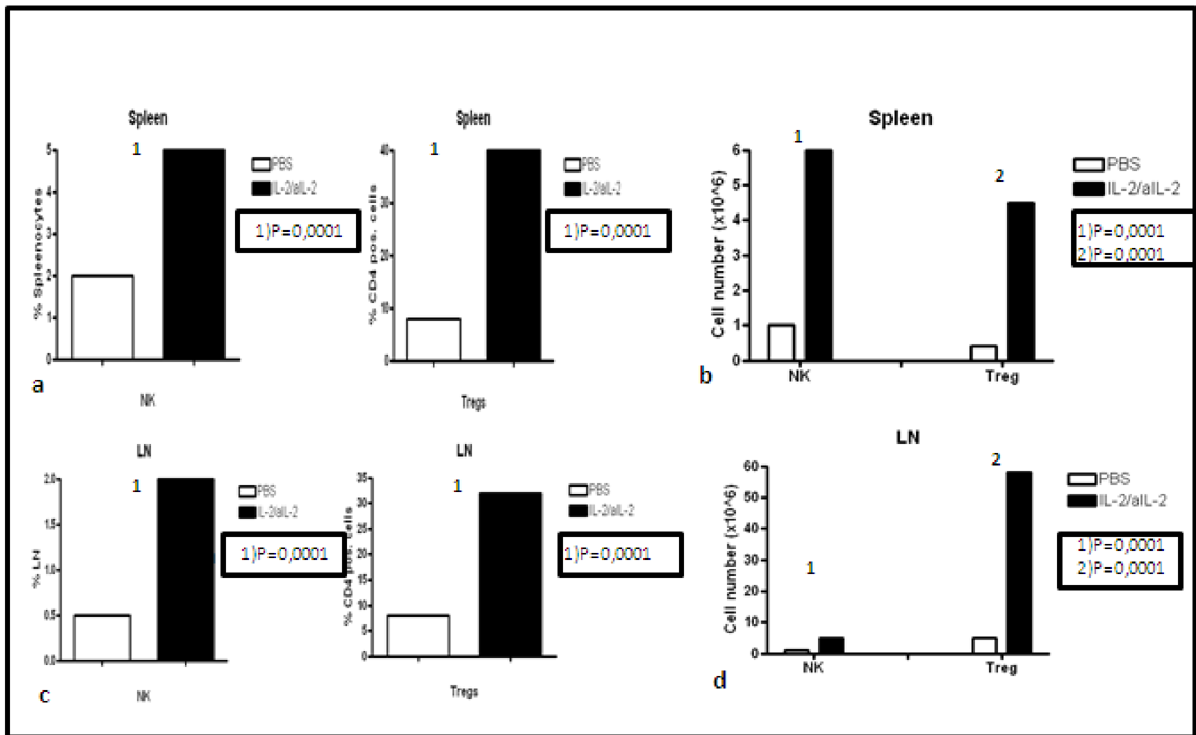
In the B6xBM12 mice the effect was similar in this population. The Tregs increased from 12% in the untreated mice to 28% in the treated mice. The increase in absolute numbers was from 1,5x10^6 in untreated to 4,2x10^6 cells in the FLT3L treated mice. All in all, the effect of FLT3L on the different cell populations in the different mice was as expected and quite similar.

2.2.3. Effects of IL2/αIL-2 mAB complex treatment on the different mouse strains.

Recently it was shown by Sprent et al, that the regulatory T cell compartment can be expanded in vivo using an IL-2/αIL-2 mAB complex. The complex treated mice also showed an increase in NK cells, however, unlike with FLT3L, the different DCs were not affected. The study shows, that upon treatment of mice with IL-2/αIL-2 mAB complexes, long term acceptance of islet allografts can be achieved. Two mABs were particularly interesting to us because of their specific increase of the regulatory T cell compartment. The S4B6 and especially the JES6-1A12 mAB. **(182)**

These two mAbs were produced and purified using standard procedures. The mAbs were complexed with recombinant mouse IL-2 was, produced in our laboratory. All mice were treated with

10µg/mouse (7,5µg αIL-2/2,5µg IL-2) of the complex. Mice were injected for 3 consecutive days. For comparison, control mice were injected with PBS. After the treatment, the mice were sacrificed and cellsuspensions from spleen and lymph nodes (LN) were analyzed by FACS.



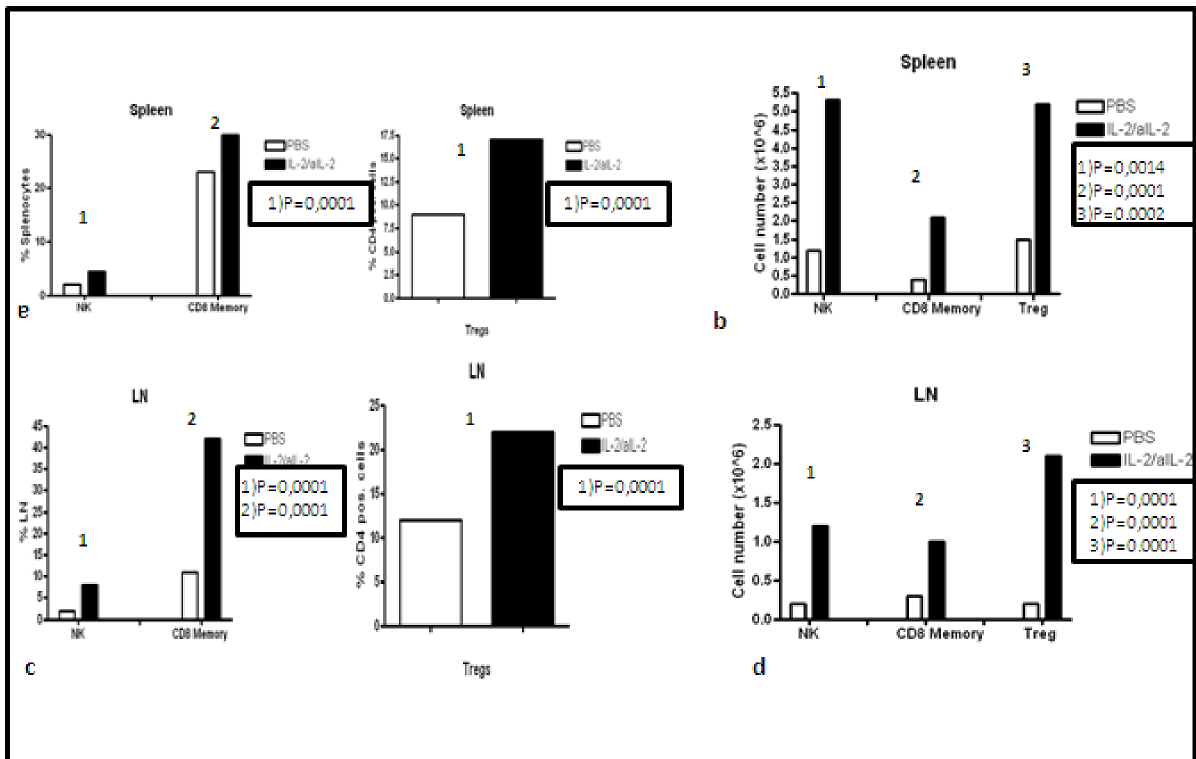


***Figure 12: FACS analyses of normal mice treated with IL-2/αIL-2 complexes. The figure** shows NK cell and Tregs in the JES6-1A12 treated mice (upper part) in the spleen in relative (a) and absolute (b) numbers.It also shows the effect of the same mAb on these cells in the LN (c=relative numbers) (d=absolute numbers). Also NK cells, CD8 memory and Tregs numbers are shown with the S4B6 mAb (lower part) in the spleen (e=relativ numbers e)(f=absolute numbers) and in the LN (g=relative numbers)(h=absolute numbers). White bars show PBS control mice and black bars are IL-2 complex treated mice.*

Figure 12a shows the relative amount of NK cells in the spleen of mice treated with IL-2/αIL-2 (JES6-1A12) complexes and with PBS as control. It also shows the relative numbers of Tregs in the spleen with and without IL-2 complex treatment. It is obvious, that the relative number of NK and Tregs increase significantly. NKs rise from 2% to 5%, Tregs from 8% to 40%. Even more important, the absolute numbers **Fig. 12b** of NKs and Tregs increase significantly in the spleen as well. NKs show 1x10^6 cells in the spleen and PBS control group and 6x10^6 cells in the complex treated mice. In the Treg compartment it is even more impressive. From 0,4x10^6 cells in the PBS mice to 4,5x10^6 cells in the IL-2 complex treated mice. This means a 10 fold increase in the Treg compartment. Just like in the spleen, these cell numbers also increased in the LNs of these mice. NK cells increased from 0,5% up to 2%, Treg´s from 8% to 32%. **Fig. 12c**

In absolute numbers (**Fig 12d)** NK´s went from 1x10^6 cells to 5x10^6 cells. Treg cells increased from 0,6x10^6 to 3,2x10^6 cells. So, there was a significant increase in spleen and LNs in the NK and Treg compartment. This was true for the JES6-1A12 mAB. In the next step we had to analyze the effects of the second mAB, namely S4B6.

Fig. 12e shows the relative numbers of NK cells, CD8 memory and Tregs in the spleen of PBS control and S4B6 complex injected mice. There is an increase in the NK population from 2% UT to 4,5% complex treated. CD8 memory cells increased from 23% UT to 30% treated. The Tregs also increased from 9% UT to 17% in the treated mice. In absolute numbers, **(Fig. 12f)** NK cells increased from 1,2x10^6 cells to 5,3x10^6 cells. CD8 memory cells increased from 0,4x10^6 cells to 2,1x10^6 cells. Tregs also increased from 1,5x10^6 cells to 5,2x10^6 cells.

The picture In the LNs was similar. NKs increased from 2% to 8%, CD8 memory T cells from 11% to 42% and Tregs from 12% to 22%. **(Fig. 12g)**

Figure 12h shows the increase of NK cells from 0,2x10^6 cells to 2,1x10^6 cells , CD8 memory T cells increased from 0,3x10^6 cells to 1x10^6 cells in the LN. Also Tregs increased with this complex from 0,2x10^6 cells to 2,1x10^6 cells.

These results show that the IL-2/αIL-2 complex treatment with either mAb JES6-1A12 or with mAb S4B6 increased the Treg compartment significantly, with the JES6-1A12 mAb being more efficient.

This was true for all the mouse strains tested, including BDF1 mice, as well as the B6 strains (BM1xBM12)F1 and (B6xBM12)F1.

Discussion

The regulatory T cell compartment of the FLT3L treated mice was significantly increased in the BDF1 mice as well as in the two B6 mice (BM1xBM12)F1, (B6xBM12)F1. The increased number of Tregs is not due to increased thymic production, but due to conversion of pre-existing $CD4^+$ $FoxP3^-$ cells **(181)**.

The FLT3L-induced Treg expansion could be either due to a direct signaling of the cytokine on the Treg population or to an indirect effect mediated by other cells.

Neither CD4 helper nor nTregs express detectable FLT3 at the protein level. It is also known that sorted nTregs do not proliferate in vitro upon anti-CD3 stimulation in presence of FLT3L. Therefore, the effect of FLT3L on nTreg expansion is likely to be indirect.

FLT3L treatment also increased both NK and DC numbers in the spleen. But FLT3L-induced expansion of nTregs took place even when mice were depleted of NK cells by prior injection of the mice with the anti-NK1.1 mAb PK 136 (data not shown), suggesting that NK cells were not involved in the expansion of nTregs.

Thus, it is likely, that Tregs expand due to the FLT3L induced expansion of DCs.

The IL-2/αIL-2 complex treatment is a different story. The exact mechanism on how the complex induces proliferation is not fully understood. However, it is known that nTregs depend on IL-2 for their survival. It is also known that they carry an IL-2R just like effector T cells. It is assumed that due to the binding of the mAb to the IL-2, the half-life time of IL-2 in the circulation is prolonged and thus, the bio-availability for IL-2 is increased. Also the duration of the IL-2 interaction with the IL-2R could be longer and of higher affinity due to stabilizing effects of the crosslinked complex. So the IL-2 can give an increased and longer signal, meaning the signal in the cell is stronger and leads to increased proliferation.

So the effect of the IL-2 complex is a direct one, which is increased by the enhanced binding capacities of the complex to the receptor.

NK cells also increase significantly after the IL-2/αIL-2 complex treatment. This is no surprise because NK cells also express an IL-2 receptor.

This means that Tregs, NKs and effector T cells only need a low amount of IL-2 to be activated. With the increased and prolonged availability of IL-2 due to its complex with the anti-IL-2 mAb, these cells can proliferate to a maximum extent.

2.3 The influence of FLT3L and IL-2/αIL-2 complex treatment on acute GvHD

Introduction

Graft versus host disease (GvHD) is a major complication after allogenic bone marrow transplantation. About 50% of the allo-BM transplantations lead to GvHD. However, 90% of these cases are very mild, with skin rashes as the most severe symptom. The remaining 10% however lead to significant morbidity and mortality in humans undergoing such transplantations.

A GvHD occurs when donor derived alloreactive T cells recognize and react to histo-incompatible Ags on recipient cells and secrete cytokines and chemokines. Usually an acute GvHD occurs within 100 days post transplantation. Mononuclear phagocytes and other leukocytes are thought to be responsible for the initiation of GVHD and for the subsequent injury to host tissue. Regulatory T cells may down regulate such alloreactive effector T cells. We assume, that increasing the number of Treg's might downregulate the activity of alloreactive T cells in the GvHD. In the previous chapter, it was shown that treatment of mice with FLT3L or IL-2/αIL-2 complex injections caused an increase of the Treg compartment. However, not just Tregs are increased in numbers, but also other lymphoid cell populations like NKs and DCs are also increased. These cells cannot be neglected in the analysis of the acute GvHD models. In this study we have investigated the potential prophylactic effects of cytokine induced Tregs, DCs and NK cells on the outcome of acute GvHD.

Material and Methods

FLT3L mass production and purification

For FLT3L mass production, transfected CHO cells (CHO hFLT3L-Fc 2nd sort) were thawed and cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. After 24h the selection marker G418 was added to the culture. After additional 24 hours the media was aspirated and the cell washed with 8ml sterile PBS. The PBS was aspirated and then 2ml trypsinEDTA solution added. The flasks went back into the 37°C incubator for 5min. When the cells started to detach from the plastic, 5ml of media was added and the cells were rinsed and collected in a 15ml falcon tube. The falcon tube was filled to a total volume of 15ml with media. The cells were then centrifuged at 1200rpm, 4°C for 10min. Media was removed and the cell pellet was washed in 10ml media and then centrifuged again at 1200rpm, 4°C for 10min. The media was aspirated and the pellet re-suspended in media. The cells were transferred into a medium sized flaks. After 24-48 hours the cells were split as described before and expanded to a large flak. Cells were expanded in big flasks till a large quantity of flaks was present. Each big flask was transferred into a roller bottle with 500ml medium containing 2-4% FCS (low IgG) and 0,5% cyproxin. The roller bottles were transferred to a roller in a 37°C room. The cells stayed in the roller bottles for 2 weeks constantly turning.

After this time, the cell suspension was centrifuged for 10min at 3500rpm at RT. The supernatant was sterile filtered through diatomaceous earth (Highflow Super Cell Medium, Fluka).

The sterile suspension was stored at 4°C till further use.

The FLT3L suspension was run through a Protein G-Sepharose® column (GE Health Care). Prot G is a bacterial cell membrane protein that binds the Fc part of IgG. The recombinant FLT3L had an IgGFc-tag, which was bound by the prot G and the media ran through.

The column was washed with sterile PBS and then eluated with 0,1 M citric acid, ph 2,2. The fractions were collected in 2ml eppendorf tubes containing 200µl Tris pH 8,9 to neutralize the solution. The

concentration af the fractions were evaluated by Thermo Scientific NanoDrop 1000™. The positive fractions were collected in a dialysis tubing and dialysed against sterile PBS. After 24h the PBS was exchanged by a fresh bottle of sterile PBS. Now the concentrations of the fractions were measured again and aliquoted. The aliquots were stored at -20°C till further use.

rIL-2 mass production and purification

For IL-2 mass production, the hybridomas (X63 IL-2, Karasuyama et al) were thawed and cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. The expansion and the production of the IL-2 hybridomas is the same like for the FLT3L transfectants or anti-IL-2 mAb hybridomas. The purification of the IL-2 was done on an S4B6 column, which is an anti-IL-2 mAB coupled to cyanogenbromide activated Sepharose beads (GE Health Care) according to the description of the manufacturer. The IL-2 binds to the anti-IL-2. By 0,1M citric acid the fractions were eluated as described before. The IL-2 was stored at-80°C till further use.

rIL-2 mass production and purification

For IL-2 mass production, the hybridomas (X63 IL-2, Karasuyama et al) were thawed and cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. The expansion and the production of the IL-2 hybridomas is the same like for the FLT3L transfectants or anti-IL-2 mAb hybridomas. The purification of the IL-2 was done on an S4B6 column, which is an anti-IL-2 mAB coupled to cyanogenbromide activated Sepharose beads (GE Health Care) according to the

description of the manufacturer. The IL-2 binds to the anti-IL-2. By 0,1M citric acid the fractions were eluated as described before. The IL-2 was stored at-80°C till further use.

NK1.1 mAb mass production and purification

The anti- NK1.1 hybridoma (PK136) was expanded as described before. Also the purification via an Pretein G-Sepharose® column was identical to earlier described procedures. The aliquots were stored at 4°C till further use.

FLT3L treatment

For FLT3L treatment, mice generally received 10-20 µg rFLT3L (in 0.2 mL PBS) by intraperitoneal injection daily for 10 days.

IL-2/αIL-2 treatment

To form antigen-antibody complexes, 2,5µg of the IL-2 and 7,5µg of the αIL-2 were mixed together in a 1,5ml eppendorf tube. The tube was then incubated in a 37°C incubator for 30min. After the complexes had formed PBS was added to reach the final volume.

Each mouse received 10µg (2,5µg IL-2 + 7,5µg αIL-2) of the complex in 200µl PBS/mouse as the final volume.

NK1.1 treatment

The NK1.1 mAb (PK136) that was produced in our lab was injected before FLT3L or IL-2 complex treatment into the mice. 200µg/mouse of PK136 were injected i.v. into the tail vain at day -4 and -2. At day 1 the treatment with the respective other cytokines started. The mice were analyzed every week and scored appropriately.

Acute GvHD induction

GvHD was induced by intravenous injection of pooled cell suspension of lymph node and spleen cells from C57Bl/6 into BDF1 (C57Bl/6 x DBA/2) F1 mice or (BM1xBM12)F1. Animals were followed on a daily basis and euthanized when necessary.

Results

2.3.1 Effects of FLT3L and IL-2/αIL-2 treatment acute on GvHD in BDF1 mice

An acute GvHD was induced in (C57/BL6xDBA/2)F1 mice by i.v. injection of a pooled cell suspension of LNs and spleen from C57/Bl6 mice. After injection, the mice were monitored daily and were examined regarding weight and survival.

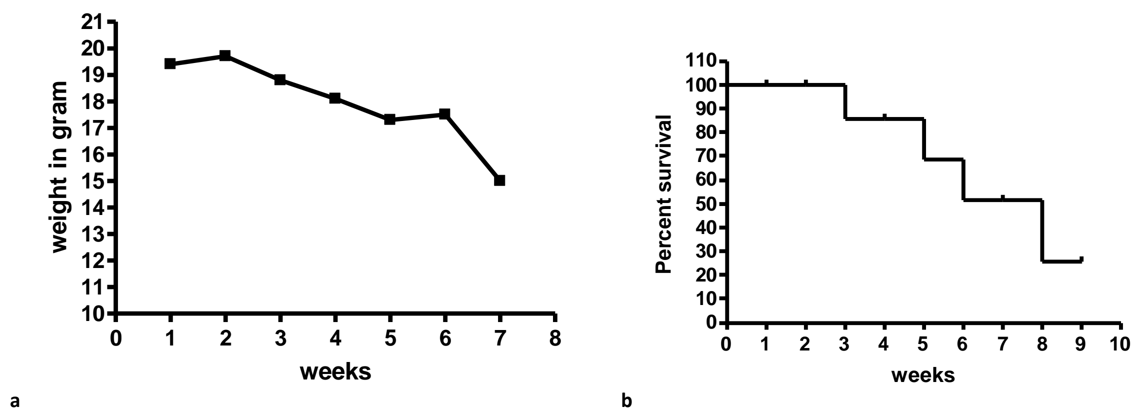


Figure 13, weight and survival of BD1 mice: *shows the weight of acute GvHD BDF1 mice over a timeperiod of eight weeks (n=7). The weight is dipicted in grams. The mice lost 4,4 grams within 7 weeks post induction (a). Also the survival curve of a chronic GvHD BDF1 population is shown during a time period of 9 weeks (n=7). After 9 weeks only 25% of the original population was still alive (b).*

In Figure 13a and b the weight and the respective survival curve of the BDF1 mice can be observed. 7 mice were analyzed in this experiment. After 20 days the mice started to lose weight. The weighing was carried on until the mice had lost 20% of their primary weight. At that point the mice had to be sacrificed. The weight loss in the mice was severe. After 5 weeks nearly all the mice had lost 20% of their primary weight and had to be sacrificed. Hence, the survival curve drops at day 30. At day 50 all mice were dead due to severe effects of the GvHD.

After establishing that we could reliably induce acute GvHD in our mice, FLT3L treatment was combined with the acute GvHD induction. BDF1 mice were injected with 10 daily injections i.p. at

10µg/mouse. At day 11 the mice were chalanged with pooled LN and Spleen cells from C57/Bl6 mice for induction of GvHD. The recipient mice were then scored for haematokrit, weight loss and survival.

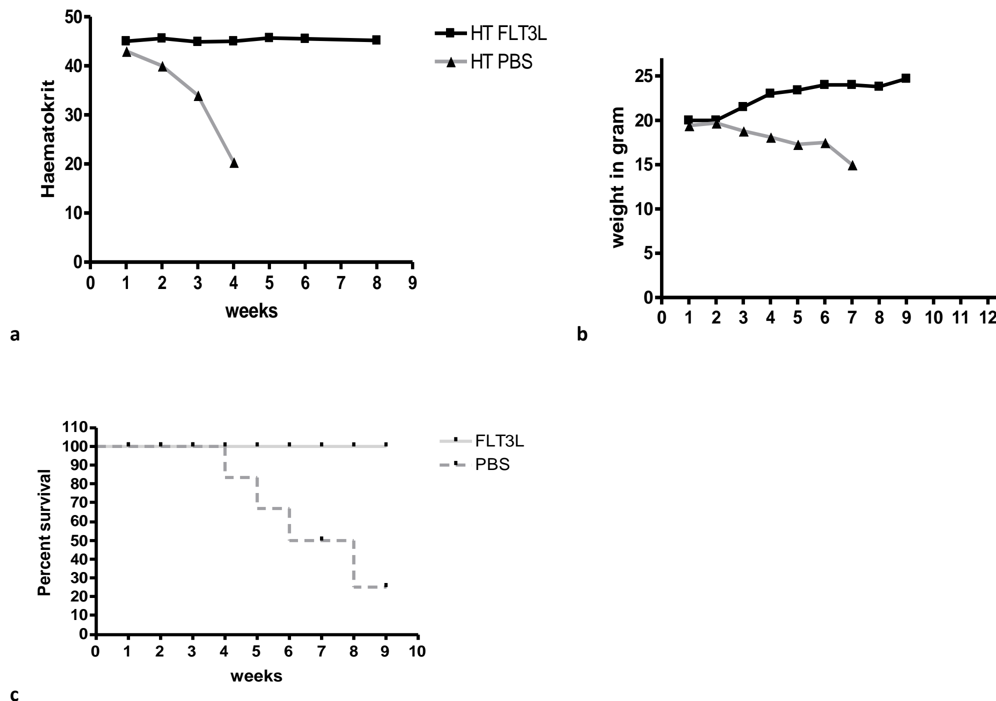


***Figure 14, FLT3L treatment in acute GvHD mice:** shows the Haematokrit in acute GvHD mice treated with FLT3L (n=8) and PBS (n=7). The Haematokrit was distinguished every week for 8 weeks. The haematokrit is dipicted in per cent. PBS treated mice decreased within a time periode of 4 weeks post induction. The FLT3L treated mice kept a stable haematokrit value over 9 weeks. **(a)** In **(b)** the weight of these mice is shown over the timeperiode of eight weeks. The weight is dipicted in grams. PBS mice lost 5 grams within 7 weeks, FT3L treated mice gained 4 grams in 9 weeks. This is all summed up in the survival assay **(c)** which shows, that PBS mice started to die (had to be sacrificed) after 4 weeks and after 8 weeks 75% of the mice were dead. In the FLT3L treated group 100% of the mice survived until week 9.*

Figure 14a shows the Haematokrit of FLT3L and PBS treated mice in weeks post GvHD induction. The HT of FLT3L BDF1 mice (Black line) did not change significantly within 8 weeks post GVHD induction. The HT stayed within the “normal” HT for mice between 40-50%. The control mice had “normal” HT of 43% to begin with. However after 4 weeks the HT had dropped to 20%. This was a very fast and drastic drop. The mice had to be sacrificed at this point. The FLT3L treated group had 8 mice and the PBS control group had 7 mice.

Figure 14b shows the weight of FLT3L treated mice (black) and the PBS control group (grey). Flt3L treated mice gained 4,7 grams within a time period of 9 weeks post induction. On the other hand PBS control mice lost 4,4 grams within 7 weeks. At this point the mice had to be sacrificed. It is important to know that the control mice had developed severe diarrhea at this point.

Figure 14c shows the survival rate of FLT3L and PBS treated mice in acute GvHD mice. Here it was encountered whether mice had to be sacrificed due to weight loss or a severe reduction of the Haematokrit (HT). The FLT3L mice did not show any signs that made it necessary to sacrifice them. The PBS treated control group showed 4 weeks post induction the first severe signs and part of the cohort had to be sacrificed. At week 9 only 25% of the original population was still alive.

These experiments show that FLT3L treatment can protect BDF1 mice from the onset of GvHD.

We obtained similar data for the IL-2/αIL-2 complexes in BDF1 mice. Mice were injected for three consecutive days with 10µg/mouse (7,5µg αIL-2/2,5µg IL-2) of the JES6-1A12 complex or PBS as a control. The timeline of three days was chosen due to the toxic effects of IL2 (vascular leakage) during longer treatment.

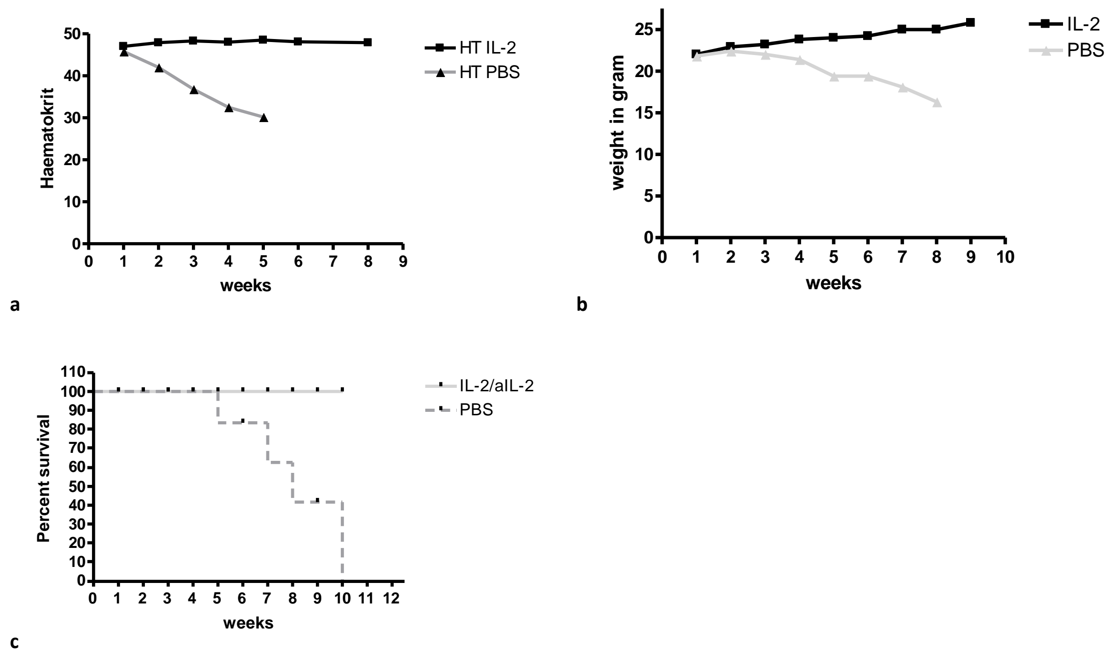


Figure 15, IL-2/αIL-2 treatment in acute GvHD mice: *shows the haematokrit in acute GvHD mice treated with IL-2/αIL-2 (n=7) and PBS (n=7). The haematokrit (% erythrocytes of the total blood volume) was determined every week for 8 weeks. PBS treated mice decreased within a time periode of 4 weeks post induction to a haematokrit of 30%. The IL-2/αIL-2 treated mice kept a stable haematokrit valu of 47% over a timeperiod of 9 weeks* ***(a)****. In* ***(b)*** *the weight (grams=g) of these mice is shown over the timeperiod of 9 weeks. PBS treated mice lost 5,9 g within 8 weeks, IL-2/αIL-2 treated mice gained 3 g in 9 weeks. These data are combined in the survival assay* ***(c)*** *which shows, that PBS treated mice started to die (had to be sacrificed) after 5 weeks and after 10 weeks 100% of the mice were dead. In the IL-2/αIL-2 treated group 100% of the mice survived more than 10 weeks.*

Figure 15a shows the haematokrit (HT) of actute GvHD mice treated with and without the IL-2/αIL-2 complex. The HT was stable in the IL-2/αIL-2 treated mice. The HT stayed within the normal range of 47% for 9 weeks. The control mice showed a severe reduction of the HT values after 3 weeks post GvHD induction. They dropped from 45,8% at week one to 30,2% after week 5.

Figure 15b shows that the IL-2/αIL-2 treated mice gained weight over an 8 week period. They started at an average weight of 22,0 grams and ended at an average weight of 25,8 grams. The PBS control mice, however, lost weight significantly. They started at an average weight of 21,8 grams and ended at an average weight of 16,3 grams. These mice had to be sacrificed.

Figure 15c shows the survival rate of IL-2 complex (JES6-1A12) treated mice compared to the PBS control group. The IL-2/αIL-2 treated mice survived over the period of 10 weeks post GvHD induction. The PBS control mice, however, needed to be sacrificed after 5 weeks because of the GvHD effects. After 10 weeks post induction, 100% of the mice were dead. 7 mice were used in each group.

These data suggest that FLT3L treatment as well as IL-2/αIL-2 complex treatment can prevent the onset of the acute GvHD in BDF1 mice.

2.3.2 Effect of FLT3L and IL-2/αIL-2 on acute GvHD in (BM1xBM12)F1 mice

In order to prevent that genetic background differences of the mice (donor and recipient) interfere with the outcome of the experiment we used the (BM1xBM12) F1 mouse.

These mice are B6 mice with two different mutations in the MHC alleles. To induce GvHD in these mice, pooled LN and spleen B6 wt cells were injected i.v. into the (BM1xBM12)F1 recipient mice. Prior to GvHD induction, these mice were treated i.p. with FLT3L, IL-2/αIL-2 or PBS according to the standard protocol.

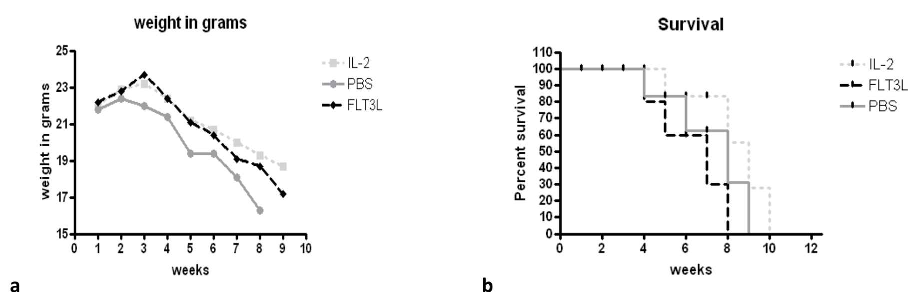


***Figure 16, FLT3L and IL2/αIl-2 treatment in (BM1xBM12)F1 mice:** shows the weight (g) of acute GvHD mice treated with FLT3L (n=7), IL2/αIl-2 (n=7) and PBS (n=7) over the timeperiode of 9 weeks. PBS mice(grey line) lost 5,6 g within 8 weeks, FLT3L treated mice (broken grey line) lost 5 g within 9 weeks and IL-2/αIL-2 treated mice (black line) lost 4,3 g in 9 weeks.**(a)** In **(c)** the survival assay of these mice is shown. PBS mice (black) started to die (had to be sacrificed) after 4 weeks and after 9 weeks 100% of the mice were dead.FLT3L treated mice started to die at week 4 and by week 8 100% of the mice were dead. The IL-2/αIL-2 treated group mice started to die after 5 weeks and after 10 weeks 100% of the mice had died.*

The results in the (BM1xBM12)F1 were different to the ones observed in BDF1 mice.

Figure 16a shows the weight of acute GvHD induced mice pre-injected with PBS (grey line), FLT3L (broken grey line), and IL-2 complex (black line). All mice, regardless of what treatment they obtained, lost weight. PBS mice started with 21,8 g and after 8 weeks had lost 5,5 g to 16,3 grams. FLT3L mice had 22,2 g and lost 5 g to 17,2 grams within 9 weeks. The IL-2/JES6-1A12 complex injected mice started at 22 g and lost 3,3 g to 18,7 g after 9 weeks.

Figure 16b shows the survival curve of (BM1xBM12)F1 mice treated with IL-2/αIL-2 (JES6-1A12), FLT3L or PBS. Unlike in the BDF1 mice, the (BM1xBM12)F1 mice were not protected against the onset of GvHD. After 4 weeks, the PBS (black line) control mice showed effects of the GvHD induction that made it necessary to sacrifice them. The FLT3L mice (broken grey line) also showed the same effect at week 4. At week 5, the IL-2 complex treated mice (grey line) dropped as well. 100% of FLT3L treated mice were dead by week 8 and PBS treated mice by week 9. After 10 weeks, 100% of the IL-2 complex treated mice had to be sacrificed due to the severe GvHD effects. Each group contained 7 mice.

This was unexpected since the BDF1 mice had shown full protection from the onset of acute GvHD with FLT3L or IL-2 complex treatment, Because this was not the case in the (BM1xBM12)F1 mice, we continued with a different experiment.

We injected in parallel BDF1 and (BM1xBM12)F1 mice with PBS, IL-2/αIL-2 (JES6-1A12), IL-2/αIL-2 (S4B6) and FLT3L. The haematokrit, which is the most sensitive method to detect acute GvHD, was determined every week for all mice.

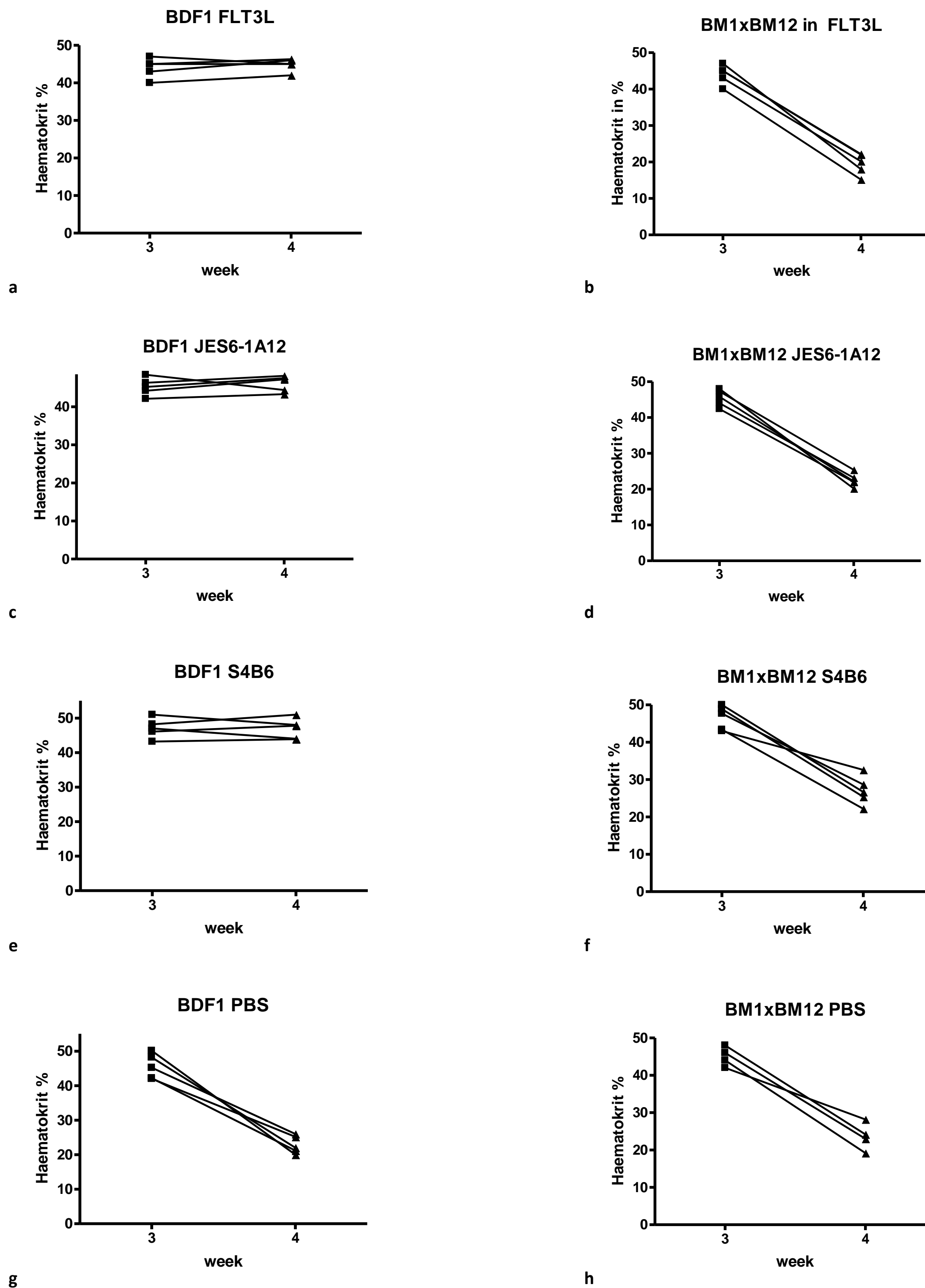


Figure 17, Haematocrit of chronic GvHD BDF1 and (BM1xBM12)F1 mice: *shows the haematokrit between week 3 and 4 post acute GvHD induction in BDF1 and (BM1xBM12)F1 mice treated with PBS, FLT3L and IL2/αIL-2 complexes (S4B6 and JES6-1A12). In* ***(a)*** *FLT3L treatment in BDF1 mice is shown, the HT values stay within the "normal" range. In* ***(b)*** *FLT3L treatment in (BM1xBM12)F1 mice is shown, the HT drops from normal levels (40-50%) to (20-30%). In* ***(c)*** *JES6-1A12 treatment in BDF1 mice is shown, the HT values stay within the "normal" range. In* ***(d)*** *JES6-1A12 treatment in (BM1xBM12)F1 mice is shown, the HT drops from normal levels (40-50%) to (20-30%). In* ***(e)*** *S4B6 treatment in BDF1 mice is shown, the HT values stay within the "normal" range. In* ***(f)*** *S4B6 treatment in (BM1xBM12)F1 mice is shown, the HT drops from normal levels (40-50%) to (20-35%). In* ***(g)*** *and* ***(h)*** *PBS treatment in BDF1 and (BM1xBM12)F1 mice is shown, the HT values drop from the normal levels (40-50%) to (20-28%) and from (40-50%) to (20-30%) respectively*

Figure 17 a-h shows the haematocrit (HT) values in per cent erythrocytes of total blood volume of the different treated groups. The BDF1 mice show full protection against the onset of acute GvHD when injected with IL-2 complexes (JES6-1A12 and S4B6) as well as with FLT3L. For the PBS treated mice the HT dropped from 40-50% to 20-30% between weeks 3 and 4 in this group.

In the (BM1xBM12)F1 mice the treatment with FLT3L as well as with the different IL-2 complexes could not protect the mice from a fatal drop in the HT. FLT3L treated mice showed the strongest reduction of HT in all groups. At week 3 the HT value was between 40-50%. At week 4 two of the mice had a HT below 20%, which was the lowest HT measured in these experimental sets.

The results show that treatment with FLT3L or IL-2 complexes can rescue BDF1 mice from the onset of acute GvHD. However, these same treatments fail to protect (BM1xBM12)F1 mice.

2.3.3 NK depletion in FLT3L treated mice prior to acute GvHD induction

Since the FLT3L and IL-2/αIL-2 treatment did not show the expected effect in the (BM1xBM12)F1 as they did in the BDF1 mice, we had to evaluate our experiments.

Both treatments increased the regulatory T cell compartemnt significantly. Tregs down-regulate the immune or autoimmune responses. Thus, Tregs may not play a significant role on acute GvHD in these mice, but instead another cell population might be responsible.

As seen in **Figure 11,** due to FLT3L or IL-2 complex treatment not only Tregs, but also NK cells increased to a great extent. To investigate if the NK cells were the cells to protect mice from acute GvHD, we depleted NK cells prior to treatment and induction of GvHD.

The αNK1.1 mAb PK136 was used for NK cell depletion. This mAb was produced in our facility using the standard protocols. BDF1 mice were injected twice on day -6 and -3 with 200µg/mouse of PK136

mAb prior to FLT3L treatment. At day 0 the mice were injected with FLT3L for 10 consecutive days with 10µg/mouse. At day 11 the mice were challenged with polled B6 wt LN and spleen cells. After that the mice were scored with the appropriate methods.

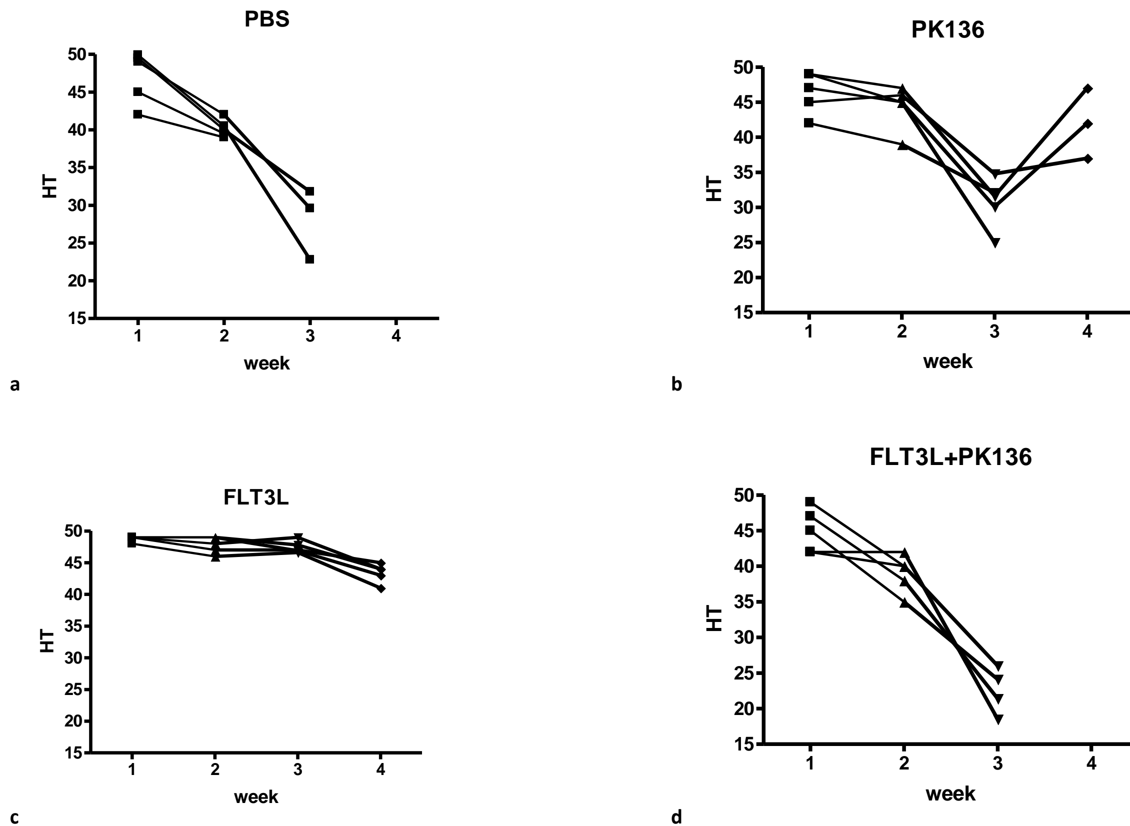


***Figure 18, haematocrit of BDF1 mice NK depleted prior to FLT3L treatment:** shows the HT values of PBS treated, NK depleting mAB (PK136) treated, FLT3L treated and FLT3L+PK136 treated mice. In **(a)** PBS treated mice show a drop in HT from normal levels (40-50%) to levels between 22-32%. In **(b)** the HT of PK136 treated mice is shown. The levels drop from 40-50% to 25-35% at week 3. At week 4 the levels increased to between 35-45%. In **(c)** FLT3L treat ed mice are shown. The HT values stay stable between 40-50%. In **(d)** FLT3L +PK136 treated mice are shown. The values drop from normal levels to levels between 17-27%.*

Figure 18 shows the Haematocrit of the NK depleted BDF1 mice. The HT of the PBS treated mice decreased significantly within three weeks. They dropped from "normal" levels of 40-50% to levels between 20-30%. Only the FLT3L treated mice showed full protection against the onset of chronic GvHD. The HT levels in these mice stayed in the "normal" range between 40-50%.

Mice injected with only the NK1.1 depleting mAb showed a significant drop of the HT values. They went from the "normal" levels to 30-40% after three weeks. However, the values increased at week four again to about 40%. Why the HT values were increasing again in this group is still unclear.

The mice that were injected with the NK1.1 depleting mAb and FLT3L showed the most severe effect where the HT decreased to 20-30% after three weeks. The only difference between the FLT3L treated and the NK1.1 mAb+FLT3L treatment was the lack of NK cells in the second group.

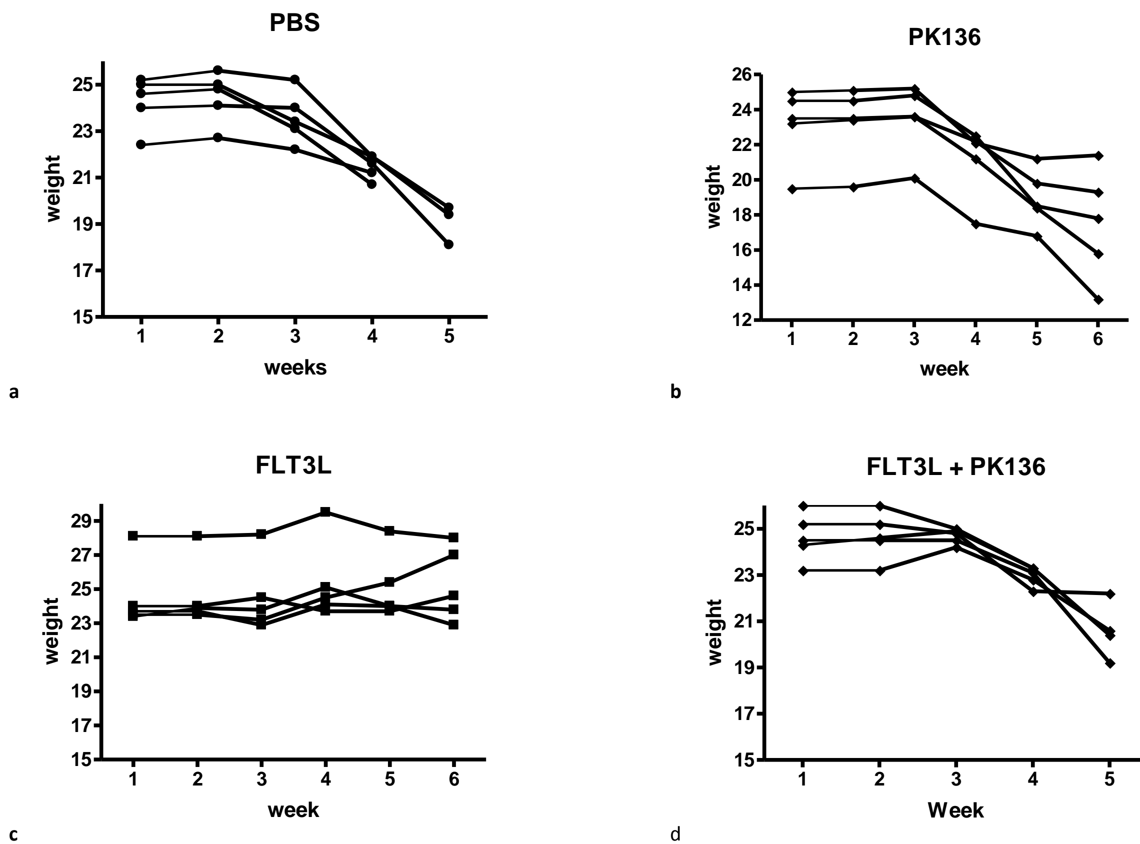


Figure 19, weight of BDF1 mice NK depleted prior to FLT3L treatment: *shows the weight in gram of PBS treated, NK depleting mAB (PK136) treated, FLT3L treated and FLT3L+PK136 treated mice. In* ***(a)*** *PBS treated mice show a drop in their weight. In* ***(b)*** *the weight of PK136 treated mice is shown. The weight of these mice dropes aswell. In* ***(c)*** *the weight of FLT3L treated mice is shown. In these mice the weight stays stable, increases even. In* ***(d)*** *FLT3L +PK136 treated mice are shown. The weight of these mice drops within 5 weeks significantly.*

Figure 19 shows the weight of the NK depleted BDF1 mice. These data sets confirm the HT results. The PBS tretaed mice lost 5 grams within 5 weeks indicating acute GvHD. The mice treated only with FLT3L gained 3-4 grams within the same amount of time. PK136 (NK1.1mAb) treated mice lost 4-5

grams within 5 weeks. The NK1.1mAb +FLT3L treated mice lost 5 grams within 5 weeks. Thus, BDF1 mice are protected from the onset of GvHD when treated with FLT3L. However, when NK cells are depleted, the same mice are not protected.

Discussion

BDF1 mice were protected against acute GvHD due to treatments that increased regulatory T cells. Therefore, it was believed that these cells were responsible for the protection. However, when (BM1xBM12)F1 mice showed no protection, regardless of Treg increase, the outstanding role of Tregs in this system had to be questioned. (BM1xBM12)F1 mice were chosen because of genetic proximity to the B6 wt mice that were used to induce acute GvHD in both strains. Using the (BM1xBM12)F1 mouse ensured that the observed effects were not due to genetic differences.

These experiments suggest that Tregs are most likely not responsible for the protection of acute GvHD. Their role, if any, in this system has to be re-evaluated.

However, we found that NK cells play an important role in the protection of these mice. BDF1 mice have one B6 MHC allele (H-2^b) and one DBA2 MHC allele (H-2^d). When B6 lymphocytes, homozygous for H-2^b, are injected into BDF1 mice, some NK cells of the recipient will always recognize the injected B6 cells as foreign ("missing self",namely the DBA/2 H-2^d allele) and kill these cells. Since the NK cells increased in numbers by the FLT3L or IL-2/anti-IL-2 treatments, there are more cells to kill off the injected B6 wt cells, so that no effector cells develop for induction of the GvHD. Also it is known, that BDF1 mice have very active NK cells, which are increased due to the treatment. **(188)**

In the (BM1xBM12)F1 mice both alleles are b. The B6 wt cells injected are also of the b type. Since the mutations in class I and II are point mutations in MHC, which do not affect NK-cell recognition, the NK cells of the host do not recognize the B6 wt cells as foreign and do not kill these cells. The

mutations are only recognized by T cells and sufficient to induce an alloreaction, leading to GvHD. **(189)**

In conclusion, Tregs that increased due to the FLT3L or IL-2 complex treatment were not responsible for the protection. The protection of BDF1 mice from GvHD could be due to the genetic disposition of host and donor and the presence of accumulated host NK cells, recognizing injected alloreactive B6 T cvells as having "missing self" and killing them, resulting in the absence of GvHD.

2.4. The effect of FLT3L and IL-2/αIL-2 treatment on chronic GvHD

Introduction

GvHD is a common side effect of allogenic BM transplantation. There are two forms of GvHD, acute (chapter 2.3) and chronic. Chronic GvHD occurs in humans approximately 100 days post BM transplantation. Usually the chronic disease display milder symptoms and is not as severe as the acute form. However, in rare cases it may also lead to the failure of inner organs and death. In general, chronic GvHD has severe effects on the quality of life of the patients.

Inflammation of joints (arthritis) or muscles (myositis) or inner organs, such as the heart (endocarditis or pericarditis), kidneys (glomerulonephritis) or visceral pleura (pleuritis) can be effected. However, psychological symptoms like fatigue or loss of appetite can also be observed in chronic GvHD patients.

It is impossible to predict which symptoms will occur, since they come in no significant order or do not show at all.

The cause of chronic GvHD is not fully understood. However, there are several theories on how the disease arises. There is more than one factor which triggers the disease. It is likely that the "bad genes, bad day" hypothesis has a role in the onset of chronic GvHD. This theory suggests that there

must be both a genetic disposition as well as several environmental factors present in order to acquire the disease.

One of the major problems of this disease is the subsequent tissue injuries. The binding of autoantibodies leads to a constant activation of the immune system and through that to chronic tissue degeneration.

Autoantibodies (anti nuclear antibodies, ANA) are a very important factor for the diagnosis of this disease. This ANA analysis is the standard method to confirm a chronic GvHD.

Material and Methods

ELISA substrate buffer for alkaline phosphatase

0.1 g $MgCl_2 \times 6 H_2O$

10 mM NaN_3

10% diethanolamine

adjust to pH 9.8 with HCl

fill up to 1l with ddH_2O

ELISA substrate stock solution (100x)

100 mg/ml Dinitrophenylphosphate

in H_2O

stored at -20°C

FACS buffer 2% FCS

10 mM NaN_3

in PBS

ELISA buffer

4% BSA

0.2% Tween 20

10 mM NaN_3

In sterile PBS

FLT3L mass production and purification

For FLT3L mass production, transfected CHO cells (CHO hFLT3L-Fc 2nd sort) were thawed and cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. After 24h the selection marker G418 was added to the culture. After additional 24 hours the media was aspirated and the cell washed with 8ml sterile PBS. The PBS was aspirated and then 2ml trypsin-EDTA solution added. The flasks went back into the 37°C incubator for 5min. When the cells started to detach from the plastic, 5ml of media was added and the cells were rinsed and collected in a 15ml falcon tube. The falcon tube was filled to a total volume of 15ml with media. The cells were then centrifuged at 1200rpm, 4°C for 10min. Media was removed and the cell pellet was washed in 10ml media and then centrifuged again at 1200rpm, 4°C for 10min. The media was aspirated and the pellet re-suspended in media. The cells were transferred into a medium sized flask. After 24-48 hours the cells were split as described before and expanded to a large flask. Cells were expanded in big flasks till a large quantity of flaks was present. Each big flask was transferred into a roller bottle with 500ml medium containing 2-4% FCS (low IgG) and 0,5% cyproxin. The roller bottles were transferred to a roller in a 37°C room. The cells stayed in the roller bottles for 2 weeks constantly turning.

After this time, the cell suspension was centrifuged for 10min at 3500rpm at RT. The supernatant was sterile filtered through diatomaceous earth (Highflow Super Cell Medium, Fluka).

The sterile suspension was stored at 4°C till further use.

The FLT3L suspension was run through a Protein G-Sepharose® column (GE Health Care). Prot G is a bacterial cell membrane protein that binds the Fc part of IgG. The recombinant FLT3L had an IgGFc-tag, which was bound by the prot G and the media ran through.

The column was washed with sterile PBS and then eluated with 0,1 M citric acid, ph 2,2. The fractions were collected in 2ml eppendorf tubes containing 200µl Tris pH 8,9 to neutralize the solution. The concentration af the fractions were evaluated by Thermo Scientific NanoDrop 1000™. The positive fractions were collected in a dialysis tubing and dialysed against sterile PBS. After 24h the PBS was exchanged by a fresh bottle of sterile PBS. Now the concentrations of the fractions were measured again and aliquoted. The aliquots were stored at -20°C till further use.

rIL-2 mass production and purification

For IL-2 mass production, the hybridomas (X63 IL-2, Karasuyama et al) were thawed and cultured in 7ml SF-IMDM media with 2-4% FCS and 0,5% cyproxin in a small cell culture flask. The expansion and the production of the IL-2 hybridomas is the same like for the FLT3L transfectants or anti-IL-2 mAb hybridomas. The purification of the IL-2 was done on an S4B6 column, which is an anti-IL-2 mAB coupled to cyanogenbromide activated Sepharose beads (GE Health Care) according to the description of the manufacturer. The IL-2 binds to the anti-IL-2. By 0,1M citric acid the fractions were eluated as described before. The IL-2 was stored at-80°C till further use.

FLT3L treatment

For FLT3L treatment, mice generally received 10-20 µg rFLT3L (in 0.2 mL PBS) by intraperitoneal injection daily for 10 days.

IL-2/αIL-2 treatment

To form antigen-antibody complexes, 2,5µg of the IL-2 and 7,5µg of the αIL-2 were mixed together in a 1,5ml eppendorf tube. The tube was then incubated in a 37°C incubator for 30min. After the complexes had formed PBS was added to reach the final volume.

Each mouse received 10µg (2,5µg IL-2 + 7,5µg αIL-2) of the complex in 200µl PBS/mouse as the final volume.

Chronic GvHD induction

GvHD was induced by intravenous injection of pooled cells suspension of lymph node and spleen cells from DBA2 mice into BDF1 (C57Bl/6 x DBA/2) F1 mice or by injection of pooled cells suspension from lymph node and spleen cells from C57Bl/6 mice into (B6xBM12)F1. Animals were followed on a daily basis and euthanized when necessary.

Bleeding of mice

Mice were brought into a bleeding chamber and fixed. The tail was exposed and could be handled safely. With a fresh razor blade the *Vena caudalis mediana* was cut. The blade was removed and the dripping blood was collected in serum tubes. After the collection of the blood, the tail was compressed with a tissue to stop the bleeding. The mice were released in a fresh cage. The serum tubes were incubated at RT for 15min. Then the tubes were centrifuged at 8000rpm for 10min at RT. The serum had separated from the cells was could be easily accessed. Until further use, the serum was stored at 4°C (if used within the next couple of days) or at -20°C.

Kidney sections

One half of a kidney of a RAG2$^{-/-}$ mouse was imbedded in tissue teck and solidified on a dried ice block. The kidneys were stored at -80°C till further use. 30min before use the kidneys were brought to the cryostate and stored there at -20°C. The kidneys were sectioned in 5µm thick slices and transferred onto a microscope glass slide. After this the slides with the kidney sections were incubated in acetone for 10min at RT to get rid of the water in the sections. Then the sections were dried for 5min and until further use frozen at -20°C.

ANA analysis

The serum of the mice was diluted in FACS buffer 1 in 2 starting at 1:20 until 1:1280. The dilutions were incubated on a kidney section of a RAG2$^{-/-}$ mouse for 30min at RT in the dark. Then the sections were washed twice for 10min in PBS. Then the sections were incubated with anti-FC mAB wich was labeled with FITC for 30min at RT in the dark. The sections were washed twice for 10min in PBS. The sections were dried and one drop of 10% Glycine in PBS was added on each section. A cover slip was put on the sections and fixed with nail polish on the side of the slip. The samples where then analyzed under a fluorescent microscope for “positive” green nuclei.

Proteinurie test

For the Proteinurie test “Albustix” were used. These Albustix were designed for humans and are usually used in the clinic. The mice were fixed in one hand and turned on their backs. When the mice started to pee, the Albustix was brought into the fresh pee. If positive the Albustix changed color to dark blue.

dsDNA ELISA

Coating: Poly-L-Lysine 0,1% w/v = 0,1mg/ml

For one plate 1ml Poly-L-Lysine + 4ml buffer

buffer 20ml Tris (40mM, ph 7,5) + 5ml EDTA (5mM) + 475ml demin H_20 = 500ml buffer

Add 50µl/well

Incubate over night at 4°C or for 2h at 37°C

Flick and dry by tapping on a tissue

Add 50µl/well of Calf Thymus DNA (per plate: 0,05ml DNA (stock is 2mg/ml) in 4,95 ml Tris-EDTA buffer.

Store at 4°C till further use.

Measure:

Add 50µl/well of Sample (in various dilutions) on to the plate.

Incubate for 1-2h

Add second mAb and incubate for 1-2h.

Add substrate and measure at 405nm.

Results

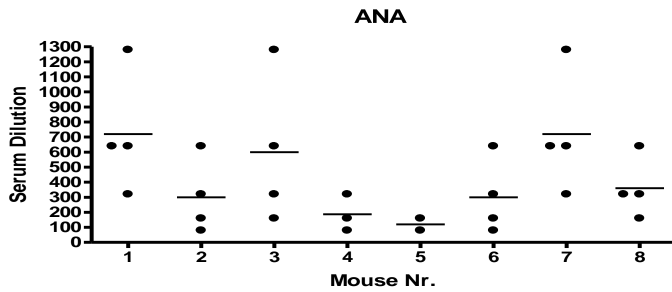
2.4.1 Induction of chronic GvHD in BDF1 and (B6xBM12)F1 mice

Two different mouse strains were used to test the potential therapeutic role of FLT3L or IL-2 complex on chronic GvHD. The methods of induction will be revealed here.

To induce chronic GvHD in BDF1 mice, pooled cells from DBA/2 spleen and LN were injected i.v. .

The other mouse strain used was the earlier described (B6xBM12)F1 mouse.

The mice were monitored and analyzed with the appropriate methods. Chronic GvHD mice were tested for delopment of IgG anti-nuclear antibody titers as well as the presence of protein in their urine. The presence of proteins (mainly albumin) in the urine is a sign of glomerulonephritis, which is a specific indicator for chronic GvHD. Also, the amount of serum IgG antibodies to dsDNA was determined by a specific ELISA.

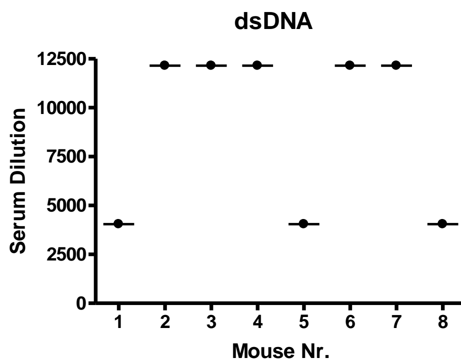


a

Table 1 shows the number of mice that were positive, respectively negative in the Proteinurie assay. (Immunglobulins in the urine)

weeks post GvHD induction	9 weeks	12 weeks	15 weeks	18 weeks
Amount positive mice	2	5	6	6
Amount negative mice	5	2	1	1

b



c

Figure 20, chronic GvHD in untreated BDF1 mice: *shows the results of the analysis of chronic GvHD induced mice. In **(a)** the titers of anti nuclear antibodies (ANA) in the serum of the mice is shown. What can be seen is the dilution in which the serum of the mice was still positive in the ANA analysis. 3 mice were positive at the highest dilution of 1.1280, another 3 mice were positive at a dilution of 1:640. One mouse was positive at a dilution of 1:320 and one at 1:160. In **(b)** the proteinurie test is shown starting from week 9 until week 18. At week 9 two mice were positive, at week 12 five, at week 15 and 18 six mice were positive. In **(c)** the titers of dsDNA are shown. Six of the eight mice are positive at the highest titers (dilution 1:12500). Two mice developed intermediate titers at a dilution of 1:4050.*

The results of the ANA analysis 9 weeks post GvHD induction in untreated BDF1 mice is shown in **Figure 20a**. In this experiment 8 mice were analyzed. After 9 weeks, three mice had the highest titers of ANA. These samples gave a positive signal at a dilution of 1:1280. Three mice showed positive signals at a dilution of 1:640. One mouse was positive at 1:320, and the last mouse was positive at 1:160. All mice had significantly increased levels of anti nuclear antibodies 9 weeks post induction.

The proteinurie test **(Table 1 Figure 20b)** measures proteins accumulation in the urine due to kidney failure in chronic GvHD mice. At week 9, two of the 8 mice showed positive results for immunoglobulins in the urine. At week 12 post induction, 5 mice showed positive results, and at week 15, six mice showed positive results. Several weeks thereafter, these 6 mice continued to be positive for proteins in their urine.

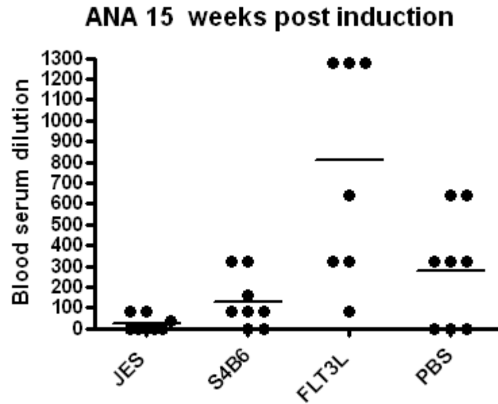
In **Figure 20c** the results of an ELISA for IgG antibodies directed against dsDNA in the serum of chronic GvHD mice at week 10 post induction is shown. Five of the eight mice are positive at the highest titers (dilution 1:12500). Three mice developed intermediate titers at a dilution of 1:4050.

Thus, it could be shown, that injected mice developed chronic GvHD after 9-12 weeks post GvHD induction. Similar results can be seen for both mouse strains.

2.4.2 The effect of FLT3L and IL-2/α IL-2 complex treatment on chronic GvHD

Prior to inducing chronic GvHD in (B6xBM12)F1 mice, they were injected with FLT3L (10µg for 10 days), IL-2/αIL-2 (2,5µg/7,5µg = 10µg for 4 days) complex and PBS as a control. Due to this treatment, the Treg compartment increases and potentially helps to down regulate the alloreactive effector T cells.

To analyze the status of the disease anti-nuclear antibody titers in the serum, proteins in the urine and IgG anti-dsDNA titers in the serum were measured. All these methods are also used for human diagnosis of chronic GvHD. The mice were bled and tested every three weeks.

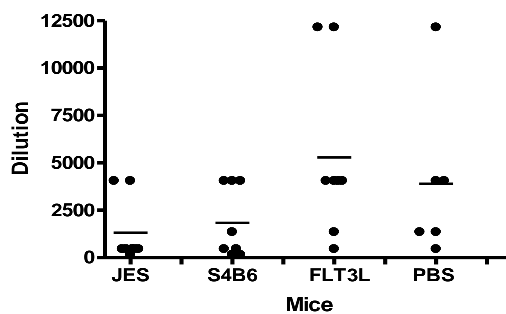


a

Table 2 shows the number of mice that were positive, respectively negative in the Proteinurie assay within the different treated groups.

weeks post GvHD induction	12 weeks			
Treatment	JES6-1A12	S4B6	FLT3L	PBS
Amount positive mice	0	0	2	0
Amount negative mice	8	8	6	8
weeks post GvHD induction	15 weeks			
Treatment	JES6-1A12	S4B6	FLT3L	PBS
Amount positive mice	3	6	6	5
Amount negative mice	5	2	2	3
weeks post GvHD induction	18 weeks			
Treatment	JES6-1A12	S4B6	FLT3L	PBS
Amount positive mice	0	0	3	8
Amount negative mice	8	8	5	0

b



c

Figure 21, chronic GvHD symptoms in treated mice: *shows the results of the analysis of chronic GvHD induced mice in the different treated mice. In* ***(a)*** *the titers of IgG anti-nuclear antibodies (ANA) in the serum of the mice is shown. What can be seen is that in the FLT3L treated groups the highest titers can be seen. The titers in the PBS treated group are increased as well. In the two IL-2 complex treated mice the titers are lower than in the two other groups. In* ***(b)*** *the proteinurie test is shown starting from week 12 until week 18 is shown. At week 12 only in the FLT3L treated mice positive results could be seen. At week 15 in all groups mice showed positive results for proteinurie. At week 18 only in the FLT3L and the PBS group mice showed positive results for proteinurie. In* ***(c)*** *the titers of IgG anti-dsDNA in the serum of the mice is shown. Prophylactic treatment with JES6-1A12-complexed IL-2 and S4B6-complexed IL-2 had intermediate levels of dsDNA in the serum. FLT3L and PBS mice had increased levels of dsDNA in the serum.*

Figure 21a shows the titers of anti-nuclear antibodies in all mouse groups 15 weeks post treatment. Each group contained 8 mice.

Three mice had significantly incraesed titers when treated with the IL-2 (JES6-1A12) complex. Two of these mice were positive at a 1:80 dilution. One mouse had a positive signal at a 1:40 dilution.

For the second IL-2 (S4B6) complex, six mice were positive. Three of these mice were positive at a 1:80 dilution. One was positive at the 1:160 dilution and two at a 1:320 dilution. The PBS treated group showed positive results in five of the mice. Three were positive at a 1:320 dilution and two at a dilution of 1:640.

The FLT3L injected mice showed the highest titers of all treated mice. All eight mice of this group were positive for the ANA analysis. One mouse had a positive signal at a 1:80 dilution, two at a 1:320 dilution and one at a 1:640 dilution. Three of these mice had the highest measurable titers at 1:1280.

Figure 21b (Table 2) shows the proteinurie results of the different treated groups at week 12, 15 and 18 post induction. At week 12, two mice of the FLT3L group show positive results.

At week 15 some mice of every group were positive. For JES6-1A12 treated mice, three were positive, for S4B6 treated mice, six were positive, for FLT3L treated mice, six were positive, and for the PBS control group, five mice were positive. At week 18 only the FLT3L group with three mice and the PBS control group with eight mice were positive.

Figure 21c shows the titers of IgG anti-dsDNA in the sera of these mice. A continuous dilution series of the blood serum was carried out. The largest dilution giving a positive signal is plotted in this graph. The average dilution to give a positive signal in the JES6-1A12 treated mice was 1:1250. For the S4B6 treated mice the average dilution was 1:2400. The PBS control group had an average

dilution of 1:4000. FLT3L treated mice had an average dilution of 1:5500. The FLT3L treated mice had the highest titers of dsDNA in the serum of all groups.

These results suggest that the IL-2/mAb complex treatment (JES6-1A12 and S4B6) protects mice from chronic GvHD to some extent. However, FLT3L pre-treatment may make the onset of the GvHDdisease even more severe.

Discussion

What seems obvious throughout the experiments is that there is some protection against chronic GvHD due to the IL-2/mAb complex treatment. The titers of anti-nuclear antibodies in the JES6-1A12 treated group are lower than the titers in the PBS control group. Hence, the IL-2 complex treatment may provide some protection against the onset of chronic GvHD. However, the mice still show significant titers, which indicates that there is no “full” protection.

This trend is confirmed by the ELISA for anti- dsDNA as well as for the proteinurie assay. In both cases the JES6-1A12 treated group was more protected then the PBS control group. Hence there was a lower titer of anti-dsDNA antibodies in the serum and a lower amount of positive mice in the proteinurie assay.

The same is true for the S4B6 anti-IL-2/IL-2 complex treated group. The mice show lower titers of ANAs and anti-dsDNA antibodies as well as a lower incidence ofproteinurie positive mice. However, the titers are higher than the ones with JES6-1A12 complex treated mice. This means that the IL-2/JES6-1A12 complex provides a better protection than the IL-2/S4B6 complex treated mice.

Thus, the JES6-1A12 mAB is better than the S4B6 mAB when it comes to providing protection of chronic GvHD. This is not a surprise considering the superior effect of the JES6-1A12 mAb on augmentation of Tregs. The JES6-1A12 mAb complex increases the Treg compartment approximately 10 fold, whereas, the S4B6 mAb increases the Treg compartment only 4-5 fold. These numbers suggest the superior role of the JES6-1A12 mAb in preventing the onset of chronic GvHD. However, it is not clear why there is no full protection.

The number of Tregs is higher than the number of Teff cells. The amount of Tregs should be sufficient to prevent the Teff cells from causing harm to the host, but this is not the case. Thus, it seems that the Tregs might not be sufficient to "stop" the onset of the disease themselves.

The results of FLT3L treatment show a different story. Despite the increase of the Treg compartment in these mice, they were not protected at all. It seems that the FLT3L treatment actually aggravates the chronic GvHD symptoms. These mice had the highest titers of ANAs as well as high titers of IgG anti-dsDNA antibodies. The proteinurie assay also revealed that the FLT3L mice showed more severe damage to the kidneys than any other group.

FLT3L treatment may not protect against chronic GvHD because of the other cell populations, which this treatment increases, such as NK cells, B cells, cDCs and pDCs. Especially the DC compartment increases to a great extent. It was shown that the FLT3L treatment produces pDCs which then produces IFNα, which is a pro-inflammatory cytokine that might activate immune cells. In humans, IFNα therapy for SLE (Systemic Lupus Erythematosis) patients leads to an aggravation of the symptoms. Due to IFNα and the subsequent increase of mature DCs and the decrease of immature DCs might tilt the fate of auto-reactive T cells from deletion to activation. In a "normal" system, immature DCs capture apoptotic cells. The DCs then present apoptotic cell derived antigens to auto-reactive T cells in a manner that results in their silencing/deletion. Mature DCs, which are induced by IFNα, mightl activate and expand these auto-reactive T cells. **(183)**

This effect described in humans might be the reason why our FLT3L treatment of mice leads to enhanced symptoms of the induced GvHD disease.

In conclusion, treatment with the IL-2/Mab complexes helps protect mice from the onset of chronic GvHD. These mice show reduced symptoms compared to the PBS group. However, FLT3L treatment which also increases the Treg compartment could not provide protection against the onset of GvHD, instead it enhanced the symptoms.

2.5. The effect of FLT3L and IL2/αIL-2 on skin transplant rejection

Introduction

Transplantation is a very important method in the surgical field to replace a defective organ with a healthy variant of the same kind. Not only whole organs can be transplanted, but also cells or tissues.

There are different forms of transplantation. During an autologous transplantation, donor and recipient is the same person. This method is used often in plastic surgery. In a syngenic transplantation, the donor and the recipient are identical twins, which means no immunosuppressant is necessary, since there is no difference in MHC. In an allogenic transplantation, donor and acceptor of the graft are of the same species but with different MHC. In a xenogenic transplantation, donor and acceptor are from different species.

The allogenic transplantation system is important for this work. A successful transplantation is hindered by the allograft rejection, where alloreactive T cells mediate the rejection. There are two possible ways on how a transplant can be recognized as foreign and thus be rejected.

The first way is called direct allorecognition in which T cells of the host recognize unprocessed allogeneic MHC molecules on the grafted cells directly. The second way is the alloantigen presentation, where the APC of the host takes up and process allogeneic MHC molecules and presents them to an alloreactive T cell.

The strength of the immune response is dependent on the number of mismatched alleles between donor and host.

Nowadays a pre- and post-transplant therapy consisting of a whole battery of immunosuppressive drugs is necessary in order to prevent rejection of the allograft. Cyclosporin, Corticosteroids, αCD3 or αCD20mAb are examples for immunosuppressive drugs that are used. More advanced is Cyclosporin (Everolimus). However, the danger of immunosupression is that there can be subsequent opportunistic infections and sometimes also tumors developing in the transplanted patients.

These risks are the reason why an alternative therapy would be beneficial. A possible treatment could be one which increases regulatory T cells. Thus, a model in which the Tregs outnumber the effector cells that are responsible for the rejection of the graft has to be developed.

It is known that FLT3L is a crucial regulator of DC development. Mice deficient for either FLT3L or FLT3 have a severely reduced DC compartment while monocyte development is not affected. Increasing the availability of FLT3L by injection results in the expansion of the pDC and the DC compartments as well as NK and Treg cell compartments. However, the increase of NKs and Tregs is due to an indirect activation through DCs. This emphasizes the role of DCs to establish and maintain sufficient numbers of Tregs for obtaining peripheral tolerance.

It was shown by Sprent et al that due to IL-2/αL-2 complexes induced Treg expansion the rejection of allogeneic pancreatic islets is prevented. **(165)**

C57Bl/6 ($H2^b$) mice were first treated with streptozotocin to destroy the pancreatic insulin-secreting β cells. When the mice started to become diabetic, they were treated with IL-2 complexes for three

days. After that, the mice were grafted under the kidney capsule with fully MHC mismatched BALB/c ($H2^d$) islets.

Our aim is to test whether IL-2 complex treatment could protect mice from rejecting a skin transplant from an allogeneic donor.

Material and Methods

Mice

Male and female C57BL/6 were either bred in our animal facility or purchased from
Janvier (Le Genest Saint Isle, France). C57/BL/6 mice transgenic for human Flt3L were produced as previously described and kept in our animal facility. All mice were used between 7 and 16 weeks of age and were maintained in specific pathogen-free conditions. All animal experiments were carried out within institutional guidelines, with the approval of the Cantonal Veterinary Office of Basel, Switzerland. Mice were humanely killed by CO_2 inhalation, and organs were removed by standard procedures.

Flt3L and aIL2/IL2 immune complex production and treatments

Recombinant Flt3L was produced as a fusion protein with the Fc fragment of the human IgG1 antibody as previously described (BAFF paper). Primers used to amplify cDNA coding for human Flt3L were as follows: 3'-CACCTGACTGTTACTTCAGCC-5' and 3'-CCTGGGCCGAGGCTCTG-5'. In all

experiments, if not otherwise specified, mice received 20ug of rFLT3L (0.2 mL) by intraperitoneal injection daily for 10 days prior to transplant and 4 injections after the transplantation every second day.

Hybridoma cell line, JES6.1, producing antibody specific for mouse IL-2 was a kind gift from Jonathan Sprent (The Garvan Institute of Medical Research, Sydney, Australia). Mouse recombinant IL-2 was purified in the laboratory on a CnBr sepharose column coupled to the S4B6 antibody as previously described. Recombinant mouse IL2 and aIL-2 JES6.1 antibody were mixed at the molar ratio of 3:1, respectively, and incubated on 37°C to form the immune complex (IC). Mice received seven daily i.p. injections of maximum 10ug of the IC (0.2 mL) before the transplantation and two injections after every second day.

Cell preparation

Spleen single-cell suspensions were prepared by pressing the organ through a 100-µm nylon mesh into 2% fetal calf serum (FCS) Iscove modified Dulbecco medium (IMDM). Cells were centrifuged and resuspended in PBS containing 2% FCS and 0.1% sodiumazide (FACSwash). Total cell numbers were calculated using the frequency estimated by fluorescence-activated cell sorter (FACS) analysis, and the total number of living cells that was counted using the hemocytometer and trypan blue dye.

FACS

The following mAbs were used: anti-CD4 (GK1.5) and anti-Helios (22F6) were purchased from BioLegend (San Diego, CA); ICOS (DX29) was purchased from BD (San Jose, CA); CD103 (2E7), anti-FoxP3 (mAb FJK-16S), CD44 (IM7) were purchased from eBioscience (San Diego, CA); CD62L (MEL-14) was purified from the hybridoma supernatant and labeled by standard methods. Cell surface staining was performed as previously described, and analyses were performed on a FACSCanto and analysed

using FlowJo (TreeStar, Ashland, OR) software. Dead cells were excluded from analysis by a combination of light scatter and/or absence of propidium iodide staining. Intracellular staining for FoxP3 and Helios was performed according to the manufacturer's instructions (eBioscience).

Real-time quantitative PCR

Skin from accepted and rejected skin grafts as well as normal tail skin was harvested from euthanised mice. Skin samples were homogenised with Polytron (Kinematica, AG, Switzerland) and RNA was obtained through Trizol purification according to the manufacturer's instructions. RNA concentration and purity was determined on a Nanodrop spectrophotometer. cDNA was synthesized using Superscript III (Invitrogen) and random hexamers according to manufacturer's instructions. Primer pairs were as follows: IL-10 3'-CCCTTTGCTATGGTGTCCTT-5', Foxp3 3'-AGGAGCCGCAAGCTAAAAGC-5', TGF-β 3'-ACCATGCCAACTTCTGTCTG-5' were obtained from Sigma-Aldrich. qPCR was performed on the ABI StepOneplus PCR machine (ABIsystems). Values were normalized to HPRT expression in each sample, and fold expression was normalised to the normal tail skin samples calculated by a comparative CT method.

Transplantation of skin graft

The mice used for this experiment were B6 H-2^{BM12} mice as donor of the skin graft and C57Bl/6 as recepeints. Recepeints mice were treated post transplantation. FLT3L treatement startet 10 days pre transplantation with 20µg/mouse i.p. and IL-2/αIL-2 treatment startet 7 days pre transplantation. On the day of the transplantation B6 H-2^{BM12} were sacrifised and skin grafts from the tail were prepared. The C57Bl/6 mice were anestezised and the back of the mice was shaved and desinfected. A 0,5cm by 0,5cm square of skin was reemoved with sharp scissors from the back of the mouse. The graft was brought into the wound and spread into place. Then the graft was glued at the edges of the

incision. A plaster was placed on the operation site. Then the mice were given pain medication and were woken from the anesthesia and transfered into normal cages. FLT3L mice received 4 shots every two day with 20µg/mouse post transplantation. IL-2/αIL-2 complex treated mice received 2 shots post transplantation. After 7 days the plaster was removed and the mice were scored every day for rejection and ecceptance.

Results

C57Bl/6 wt mice were treated with FLT3L (10 days with 10µg/mouse) or with IL-2/αIL-2 (JES6-1A12) complex (7 days with 10µg/mouse) i.p.. At day 10 (FLT3L) or day 7 (JES6-1A12) respectively the mice received a skin graft transplant on their trunk from the tail of a BM12 donor mouse. To protect the tarnsplantation site, the mice received a plaster. The FLT3L treated mice received 4 additional shots of FLT3L on day 2, 4, 6 and 8 post-transplantation. The IL-2 complex treated mice received two additional shots of the complex on day 4 and 8 post-transplantation.

On day 8 the plaster was removed and the mice were scored for rejection or acceptance of the graft.

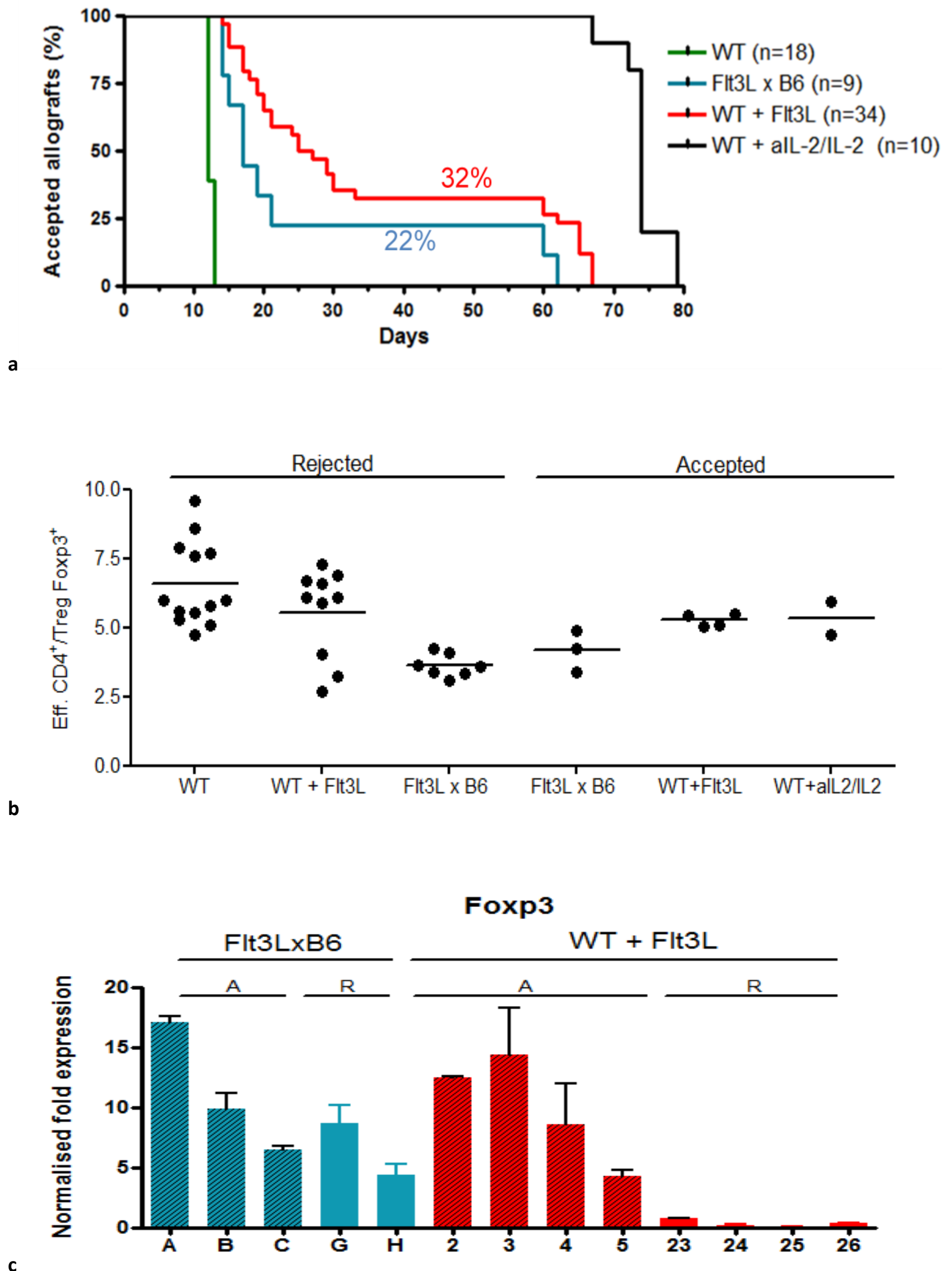


***Figure 22, FLT3L and IL2/αIL-2 influence on skin transplant rejection: (a)** shows the amount of accepted allografts in the different groups in percent is shown. In **(b)** the ratio in cell counts of $CD4^+$ effector T cells(Teff) and Treg $FoxP3^+$ cells in rejected and accepted grafts is shown. In**(c)** the qPCR analysis of skin grafts of FLT3L tg and FLT3L treated mice in accepted and rejected grafts with regards to the Treg marker FoxP3 is shown.*

In **Figure 22a** the amount of accepted allografts in percent is shown.

All PBS treated B6wt mice rejected the graft within 12 days (N=18). On the other hand, all IL-2/αIL-2 treated mice kept their allografts for a maximum of 70 days +/- 5 days (N=10).

In the cohort of mice treated with 14 injections of FLT3L, 11.7% (4 out of 34) rejected the graft with similar kinetics as the PBS treated mice. However, 56% (19 out of 34) showed a rejection that was delayed by 4-14 days. 32% (11 out of 34) had a longer graft survival similar to IL-2/αIL-2 treated mice (60 to 70 days).

FLT3L tg mice showed similar acceptance of the graft as FLT3L injected mice with 22% graft survival up to 60 days (Fig. 21c).

When the number of Treg cells in FLT3L treated mice was reduced by aCD25mAb injection, the graft survival was similar to the one observed in PBS treated mice (data not shown). This suggests that the FLT3L induced prolonged graft survival was due to increased numbers of Tregs.

Figure 22b shows the ratio in cell counts of $CD4^{+}$ effector T cells(Teff) and Treg $FoxP3^{+}$ cells in rejected and accepted grafts. In the rejected grafts, the ratio of Teff and Treg cells in the FLT3L treated B6 mice is about 5%. Wheras the ratio in WT mice is about 7%. The ratio of Teff cells vs Tregs in FLT3L tg mice is at 3%. In the rejected WT mice the number of effector cells is higher compared to FLT3L treated or in the FLT3L tg mice.

In the accepted grafts, the ratio of Teffs to Tregs in the FLT3L tg mice is about 4%. The FLT3L treated mice had an average ratio of 5%, and the IL-2/αIL-2 complex had an average ratio of 5%. This indicates that the Tregs are increased compared to the Teff cells. Hence, the Treg to Teff cell ratio is important for the acceptance of the graft by the host.

Figure 22c shows the qPCR analysis of the skin grafts of FLT3L tg and FLT3L treated mice with regards to the Treg marker FoxP3. It is distinguished between accepted and rejected graft. Each column represents one mouse. The FLT3LxB6 tg mice showed increased levels of FoxP3 expression in the

accepted skin graft. Mouse A had a 17 fold increase in FoxP3 expression compared to a “normal” mouse. Mouse B had a 10 fold increase and mouse C an 8 fold increase.

There was no significant difference in the FoxP3 levels in the rejected skin of FLT3L tg mice compared to the accepted ones. Mouse G had a 9 fold increase and mouse H had a 5 fold increase in FoxP3.

In the FLT3Ltreated mice, however, the results were different. The accepted grafts had increased FoxP3 levels. Mouse 2 had a 12.5 fold increase, mouse 3 a 15 fold increase, mouse 4 a 9 fold increase and mouse 5 a 5 fold increase in FoxP3 expression levels. The mice that had rejected their grafts showed lower amounts of FoxP3 expression and therefore lower Treg numbers. Mice 23-26 had a maximum of a 2 fold increase in FoxP3 expression far lower than in the skin of mice that had accepted the graft.

Thus, in the FLT3L tg mice there was no significant difference in the FoxP3 expression levels between the accepted and the rejected grafts. However, in the FLT3L treated mice there was a difference. Grafts that were accepted showed increased numbers of FoxP3 expression. Whereas in the rejected grafts the FoxP3 levels where “normal”. This indicates that due to increased levels of Tregs by FLT3L the skin grafts are accepted. It is unclear why in some of the grafts the Treg (FoxP3) numbers diminish.

It was shown that due to IL-2/αIL-2 (JES6-1A12) complex treatment, 100% of allogeneic skin grafts were accepted for up to 70-80 days.

In both FLT3L treated and FLT3L-tg mice, some mice rejected the graft. However, the rejection, in both cases, was later than in control mice. Also, some mice accepted the skin graft. This acceptance was similar to the IL-2 complex treated mice. In the FLT3L treated mice the Tregs are likely to be responsible for the acceptance of the graft, as indicated by the FoxP3 expression levels in them. The rejected grafts had “normal” levels of FoxP3 expression.

When Tregs were depleted with a CD25 mAb, the mice rejected the graft within the same time as the PBS control mice (+/- 2 days), indicating the important role of Tregs in the acceptance of the graft.

These findings indicate that FLT3L treatment could be a possible therapy for preventing solid organ rejection.

Discussion

For a successful allogenic transplantation, the patient has to be immunosupressed in order to prevent rejection of the graft. The problem with this treatment is the subsequent opportunistic infections and tumors that may develop.

Treatments that increase Tregs, which might down regulate the immune response of effector T cells, carries strong hopes in the field of transplantation. In this work two possible treatments to increase the Treg compartment were analyzed for their effects on allogeneic skin transplants: injection with IL-2/αIL-2 (JES6-1A12) complexes and treatment with FLT3L.

The IL-2 complex treatment worked successfully in the sense that 100% of the grafts were not rejected within 70-80 days post transplantation. However, after 70-80 days the graft was rejected because the treatment with the IL-2 complex was stopped and the effects of the treatment are transient. 10 days after stopping the treatment, the Treg numbers go back to normal and the superior effect of these cells over the effector cells wears off. These results confirmed the findings of Sprent et al who used allogeneic pancreatic islets that were transplanted under the kidney capsule. Mice treated with IL-2 complex did not reject the grafts. (**165**)

The advantage of this system is that 100% of the mice are protected. However the mice would have to be challanged with the IL-2 complex again. This results in vascular leakage in the mice, and the

dose has to be tightly controlled because of its toxic effects. More than 7 daily injections leads to 100% lethality, which makes this treatment difficult for therapy.

On the other hand, FLT3L treatment shows no toxicity in mice. Even after numerous injections there are no observed deaths. The problem with this system is that only 32% of the mice accept the graft for up to 70 days. The remaining 68% reject the graft 2-12 days later then the control group. In the rejected grafts, the amount of FoxP3 transcript and therefore the number of Tregs are significantly lower compared to the accepted grafts.

This shows that if the Tregs are present in increased numbers at the site of transplantation, they can prevent rejection of the graft. Waldmann et al had described this phenomenon that Tregs disappear from the site of transplantation after a certain time. **(184,185)** However, the question to why the Tregs leave the transplantation site but not in all cases is still unknown.

In conclusion, the IL-2/αIL-2 complex treatment prevented the mice from rejecting the skin graft for up to 70 days. However, there are risks involved due to the toxicity effects of the IL-2 treatment. The dosage of the IL-2 complex can lead to severe side effects of toxicity.

FLT3L successfully works in 32% of the transplants, but due to the loss of Tregs in the rest of the transplants the grafts are rejected.

When the problem of losing Tregs at the site of transplantation can be solved, then the Treg increase due to FLT3L treatment could be an alternative therapy for future transplantation models.

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