IL-2/anti-IL-2 complexes: The resurrection of IL-2 as potential treatment for SLE-like murine chronic Graft-versus-Host Disease?

Inauguraldissertation

zur

Erlangung der Würde eines Doktors der Philosophie

vorgelegt der

Philosophisch-Naturwissenschaftlichen Fakultät

der Universität Basel

von

Stefan Heiler

aus Grenzach-Wyhlen, Deutschland

Basel, 2018

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

Prof. Dr. Ed Palmer

Prof. Dr. Daniela Finke

Basel, den 22 Mai 2018

Prof. Dr. Martin Spiess

Dekan

I. Table of Contents

II. Abbreviations

aGvHD	Acute Graft-versus-Host Disease
AIRE	Autoimmune regulator
ANA	Anti-nuclear antibody
anti-RBC	Isologous IgG anti-erythrocyte antibody
APC	Antigen presenting cell
APS-1	Autoimmune polyendocrinopathy syndrome type 1
B6	C57BL/6
BAFF	B cell activating factor
Bcl-6	B cell lymphoma protein 6
BCR	B cell receptor
BDF1	F1 generation of C57BL/6 and DBA/2 mice
BTK	Bruton's tyrosine kinase
CD	Cluster of differentiation
cGvHD	Chronic Graft-versus-Host Disease
cTEC	Cortical thymic epithelial cell
CTL	Cytotoxic T lymphocytes

CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
Dbl-tg	Double-transgenic
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle Medium
DP	Double-positive for CD4 and CD8 expression
dsDNA	Double-stranded deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
FCGR2	Fc-γ receptor 2
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead-box-protein P3
FTOC	Fetal thymus organ culture
GITR	Glucocorticoid-induced TNFR-related protein
GvHD	Graft-versus-Host Disease
GWAS	Genome wide association study
H-2	Murine major histocompatibility complex
HEL	Hen egg lysozyme

HLA	Human major histocompatibility complex
i.p.	Intraperitoneal
i.v.	Intravenous
ICGN	Immune complex glomerulonephritis
ICOS	Inducible T cell co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL	Interleukin
IL-2R	Interleukin-2 receptor
IMDM	Iscove's modified Dulbecco's Medium
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance syndrome
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon response factor
iTreg	Induced regulatory T cell
JAK	Janus kinase

JES6/IL-2	IL-2 bound to JES6.1 mAb
LN	Lymph node
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mTEC	Medullary thymic epithelial cell
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NZB	New Zealand Black
NZW	New Zealand White
NZW/B F1	F1 generation of NZW and NZB
OT1-BDF1	F1 generation of C57BL/6-Tg(TcraTcrb)1100Mjb and DBA/2 mice

OT2-BDF1	F1 generation of C57BL/6-Tg(TcraTcrb)425Cbn and DBA/2 mice
OVAp	Ovalbumin peptide
PALS	Periarteriolar lymphoid sheath
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PMA	Phorbol-12-myristat-13-acetat
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RAG	Recombination-activating genes
RT	Room temperature
S4B6/IL-2	IL-2 bound to monoclonal antibody S4B6
SCID	Severe combined immunodeficiency
SLAM	Signaling lymphocyte activation molecule
SLE	Systemic Lupus Erythematous
SP	Single-positive for CD4 or CD8
ssDNA	Single-stranded deoxyribonucleic acid
STAT	Signal transducer and activator of transcription
TACI	Transmembrane activator and CAML interactor

Tcm	Central memory T cell
TCR	T cell receptor
Tfh	Follicular helper T cell
TGF	Transforming growth factor
TLR	Toll-like receptor
TNFR	Tumor necrosis factor receptor
TRA	Tissue-restricted antigen
Treg	Regulatory T cell

III. Aim of the Thesis

This thesis tries to answer the following questions surrounding cGvHD:

- Is there a prophylactic or therapeutic potential of IL-2 complexes in SLE-like murine cGvHD induced by DBA/2 lymphocytes in BDF1 mice?
- Do donor $CD8^+$ T cells contribute to the pathogenesis in this cGvHD induced by DBA/2 lymphocytes?
- What is the influence of host T cells on graft-versus-host reactions induced by DBA/2 lymphocytes?
- What is the effect of a restricted host TCR repertoire on graft-versus-host reactions induced by the transfer of DBA/2 lymphocytes into BDF1 mice?

IV. Summary

It has been shown previously that administration of Interleukin-2 (IL-2) bound to anti-IL-2 monoclonal antibodies (IL-2 complexes) leads to a selective stimulation of T cell subsets based on the antibody used for the formation of the complexes. Moreover, the increased selectivity of IL-2 complexes prevents adverse side effects observed from administration of plain IL-2 and at the same time leads to an enhanced biological activity of this cytokine.

In this study we investigated the prophylactic and therapeutic effects of two IL-2 complexes, JES6/IL-2 and S4B6/IL-2, on murine chronic Graft-versus-Host Disease (cGvHD). Therefore, we used the p→F1 model of cGvHD and induced the disease by transfer of DBA/2 lymphocytes into BDF1 mice. The IL-2 complexes were either administrated prophylactically, before disease induction, or therapeutically, 4 weeks after disease induction.

We found that the JES6/IL-2 and S4B6/IL-2 have opposing effects on SLE-like symptoms in murine cGvHD. Whereas the prophylactic treatment with JES6/IL-2 had an ameliorating effect on disease symptoms accompanied by generally suppressed donor lymphocytes, prophylactic S4B6/IL-2 treatment resulted in a more severe cGvHD accompanied by a more activated donor compartment. When IL-2 complexes were administrated therapeutically (following the initiation of the disease), only S4B6/IL-2 complexes exhibited an effect and efficiently reduced disease symptoms. Our findings further suggest an important suppressive role of donor $CD8^+$ T cells in this model of cGvHD because (i) the beneficial effect of therapeutic treatment with S4B6/IL-2

complexes depended on the presence of this population and (ii) a more aggressive cGvHD develops in the absence of these cells.

Host T cells, especially $CD4^+$ $FoxP3^+$ regulatory T cells (Tregs), are thought to be critically involved in the control and regulation of disease driving mechanisms resulting in GvHD. To investigate the role of host T cells in graft-versus host reactions in the p→F1 model, we transferred DBA/2 lymphocytes into BDF1 mice with a restricted TCR repertoire (OT1-BDF1 and OT2-BDF1). Our findings that the transfer of DBA/2 lymphocytes (containing $CD8^+$ T cells) leads to an acute GvHD (aGvHD) in TCR restricted (TCR oligoclonal) BDF1 mice but cGvHD in normal BDF1 mice provides further evidence for an important influence of host T cells on graft-versus-host reactions. Likely as a consequence of the greatly reduced Treg compartment in OT1-BDF1 mice, these mice develop stronger aGvHD compared to OT2-BDF1 mice. Moreover, these findings show that donor $CD8^+$ T cells are central for the induction of acute GvHD and suggest that an insufficient control of donor $CD8^+$ T cells by a TCR restricted host might result in aGvHD in OT1- and OT2-BDF1 mice. When DBA/2 lymphocytes depleted of $CD8^+$ T cells are used to induce GvHD in TCR transgenic BDF1 mice, cGvHD developed, showing again more severe symptoms in OT1-BDF1 mice and milder symptoms in OT2-BDF1 mice. These results establish an important role of a diverse host T cell repertoire in the regulation of graft-versus-host reactions and the resulting GvHD.

Introduction

1. Immunological tolerance

Tolerance can be described as induced state of non-responsiveness to a specific antigen. The immune system has to discriminate between self and foreign in order to mount an immune response only against potentially harmful non-self antigens. Self-tolerance is established during lymphocyte development and constantly maintained in the mature compartment through antigen dependent mechanisms. Whether an antigen induces an immune response or tolerance (tolerogen) depends on many factors (e.g. chemical form, route of entry into the body, dose, tissue localization). If self-tolerance is broken by any means, immune responses against self-antigens may lead to autoimmune disease.

a) B cell tolerance

Tolerance in the B cell compartment is established by several mechanisms depending on the interaction of the B cell receptor (BCR) with the antigen, the developmental stage of the B cell, the anatomical location where the antigen is encountered and the presence of co-stimulatory signals [1].

i) Clonal deletion

The concept of clonal deletion has been introduced by Frank Macfarlane Burnet as part of his clonal selection theory on acquired immunity for which he was awarded the Nobel Prize in Medicine and Physiology in 1960 jointly with Peter Brian Medawar. Clonal deletion describes a process by which autoreactive B cells are physically eliminated upon engagement of their antigen receptor during development. At the IgM^+ immature B cell stage when developing B cells first express a functionally rearranged BCR, they are

tested for receptor fitness and self-reactivity. If the receptor binds self-antigens with too strong affinity, the B cell is induced to undergo apoptotic cell death.

Namazee and Bürki provided the experimental evidence for clonal deletion by using BALB/c (H-2K^{d}) mice expressing a transgenic BCR (3-83tg) that recognizes the major histocompatibility complex class-I (MHC-I) molecule H-2K^{k} [2]. The 3-83 antibody binds MHC-I from the H-2^{k} haplotype with high affinity and MHC-I from the H-2^{b} haplotype with much lower affinity. Crossing these 3-83tg mice to H-2^{k} expressing mice leads to deletion of developing B cells expressing the 3-83 BCR in the bone marrow (central deletion). Thus, no IgM^{+} B cells carrying the 3-83 idiotype are detected in the spleen and lymph node (LN) of H-2$^{d/k}$ 3-83tg mice; furthermore, 3-83 specific IgM is not detectable in the sera of such mice. In contrast, 3-83tg mice on the H-2$^{d/d}$ background show no such deletion. These experiments provide evidence, for deletion of immature B cells bearing a self-reactive BCR in the bone marrow upon encounter of membrane-bound, multivalent, highly BCR crosslinking self-antigens. Additionally, also low-affinity B cells can bind to multivalent self-antigens with high avidity. However, B cells of non-transgenic mice are capable of modifying their antigen receptor away from self-reactivity to escape the fate of clonal deletion.

ii) Receptor editing

The fate of B cells expressing a self-reactive antigen receptor does not ultimately result in clonal deletion. It was shown independently by several groups that tolerance mechanisms exist which rescue autoreactive B cells from clonal deletion [3, 4]. Developing B cells that receive signals through a self-reactive receptor upon antigen encounter are arrested in their developmental progression and reactivate the recombination-activating gene (RAG) protein machinery. Ongoing VJ recombination

within the Ig light chain (IgL) loci and successful pairing with the Ig heavy chain (IgH) can lead to a new receptor with non-self specificity; this allows the developing B cell to escape deletion and resume its' developmental progression. In the periphery, however, receptor editing does not occur upon auto-antigen encounter and self-reactive B cells are regulated by other mechanisms.

Receptor editing was demonstrated in mice expressing the transgene for the IgH derived from the 3H9 antibody specific for double-stranded (ds) DNA [5]. Most IgH and IgL combinations containing the V_H3H9 transgene are specific for ds or single-stranded (ss) DNA. However, only few hybridomas prepared from the spleen of those V_H3H9-only mice generated antibodies that bind ssDNA and not dsDNA. By analyzing the IgL repertoire of such hybridomas, it was found that some V_k genes were overrepresented and IgL containing J_k5 were frequent. Together this reflects the many combinations of V_H3H9 together with endogenous IgL chains to generate anti-DNA specific receptors and allows efficient editing or elimination of those anti-self specificities upon antigen encounter. The frequent use of J_k5 indicates several rounds of IgL editing in order to find an appropriate combination of IgH and IgL genes that do not generate an autoreactive specificity.

Another approach to demonstrate receptor editing of immature B cells compared centrally deleting 3-83tg x H-2^b mice to peripheral deleting 3-83tg x MT-K^b mice, expressing the K^b antigen only in liver, pancreatic islets and kidney. It was shown that immature anti-idiotype (54.1) specific B cells exhibited elevated RAG2 expression in contrast to non-self reactive B cells. Further, bone marrow cells of centrally deleting mice showed elevated IgL gene recombination, detected by excision products, and a higher frequency of lambda light chain rearrangements. Taken together, these

experiments support the idea that signaling through autoreactive BCRs in immature B cells induces further VJ recombination leading to secondary rearrangement of IgL genes and possibly a change in specificity away from self-reactivity, thereby rescuing cells from clonal deletion.

In hybridomas generated from 3H9 (IgH + IgL) transgenic B cells of adult mice, the B cell repertoire comprises receptors composed of transgenic IgH together with endogenous IgL [3]. Although mRNA of both, the transgenic IgL and the endogenous IgL are transcribed, endogenous IgL preferentially pair with the 3H9 IgH. The restricted usage of endogenous Igκ V genes reflects a selection of many IgL chains that pair with IgH from the 3H9 antibody. This form of receptor editing is the dominant tolerance mechanism, which is actively driven by antigen that acts on immature B cells in the bone marrow leading to a change in specificity at the genetic level.

iii) Anergy

Another mechanism for tolerance induction in B cells is functional inactivation, also known as clonal anergy. Tolerance by anergy was first demonstrated in mice transgenic for a soluble form of hen egg lysozyme (HEL), which were crossed to mice transgenic for a BCR specific for HEL. This HEL/anti-HEL double-transgenic (HEL-Dbl-tg) mouse model (MD4xML5) was developed by Goodnow [6]. In these mice, antigen specific B cells expressing a transgene-encoded BCR are not deleted in the bone marrow and found in normal numbers in the periphery. However, lysozyme specific antibodies are not detectable in the serum. Anergy induction in HEL-Dbl-tg B cells is accompanied by down-regulation of surface IgM levels whereas expression of IgD is maintained. Additionally, such B cells are unable to upregulate CD86 co-stimulatory molecules.

When anergic B cells are removed from their tolerogenic environment in HEL-Dbl-tg mice and "parked" in non-transgenic hosts (HEL-free), they progressively regain surface IgM levels comparable to those of transferred non-tolerant anti-HEL transgenic B cells. Moreover, functional recovery of anergic B cells was achieved by several days of LPS stimulation *in vitro* [7]. Thus, anergy is a reversible, intrinsically regulated state of induced unresponsiveness.

Induction of anergy requires a certain threshold concentration of antigen. This could be demonstrated in HEL-Dbl-tg mice by manipulation of the serum concentration of soluble lysozyme via an inducible metallothionein promoter in the HEL transgene [8]. It was calculated that concentrations at which only 4.5% of BCRs are occupied by lysozyme in HEL-Dbl-tg mice failed to induce tolerance whereas receptor occupancy of 45% resulted in anergic tolerance to HEL.

Anergic B cells have a reduced lifespan (only 2 to 3 days) and are excluded from splenic follicular and marginal zones through competition with non-self-reactive B cells [9]. Anergy represents an important mechanism by which autoreactive B cells are rendered tolerant and the reactivation of anergic B cell can lead to severe autoimmune responses.

A limitation of the original MD4xML5 model of anergy is the absence of competition with non-HEL-specific B cells. In MD4xML5 mice, HEL-specific B cells are found in the splenic follicles but not in marginal zones. In mixed BM chimera where Ig transgenic B cells represent a minor fraction, anergic B cells do not enter splenic follicles and have a reduced lifespan of less than 3 days [10]. In class-switch recombination competent HEL transgenic (SW_{HEL}-tg) mice, in contrast to HEL-tg mice, HEL-specific B cells are capable to switch antibody isotypes to IgG. Moreover, due to the targeted insertion at the

physiological site in the IgH locus and possibly due to the instability of rearranged V-regions in early B-cell development, a large population of "competing" non-lysozyme-binding B cells is present in these mice. Increased competition effects migratory properties and results in localization of HEL-specific B cells to extra-follicular locations (PALS). Additionally, HEL-specific SW_{HEL} x ML5 B cells show an immature phenotype (CD21/CD35^{lo} CD23^{lo}). This model more closely resembles a physiologic situation where self-reactive B cells represent only a small population and competition with non-self-reactive cells is high. HEL-specific B cells in SW_{HEL} x ML5 mice are anergic but can be stimulated to secrete class-switched IgG antibodies upon BCR-independent CD40 stimulation *in vitro*. Interestingly, in this model the accumulation of immature anergic SW_{HEL} x ML5 B cells can't be attributed simply to the higher level of receptor occupancy because MD4xAL3 mice, which express higher concentrations of soluble HEL compared to MD4xML5 mice, have HEL-specific B cells showing a more mature phenotype (CD21/CD35^{int} CD23^{hi}) despite almost complete receptor occupancy (89%) which localize in splenic follicles [9].

iv) Clonal deletion in the periphery

Mechanisms that ensure the establishment of self-tolerance in the primary lymphatic organs (central tolerance) are not perfect as evident from the presence of autoantibody producing B cells in the periphery [11]. Since tolerance induction is an antigen driven process, tolerogenic antigens need to be present during lymphocyte development at above-threshold concentrations at the sites of tolerance induction (in the bone marrow for B cells and in the thymus for T cells). For immature B cells, the tolerogenic antigen has to be either present at very high concentrations or in a multivalent form that causes extensive cross-linking of BCRs. Antigens that are expressed only in discrete tissues with

no access to primary lymphatic organs do not induce tolerance through central tolerance mechanisms. Furthermore, in contrast to T cells, B cells might experience a second wave of diversification by somatic hypermutation in germinal center reactions after they left the bone marrow. This implicates the need for additional tolerance mechanisms to control peripheral mature B cells that escaped central tolerance mechanisms or have altered their antigen receptors through hypermutation.

Non-tolerant mature HEL-specific B cells are rendered unresponsive when transferred to a HEL-tg host (ML5). Moreover, these HEL-specific B cells down-regulate surface IgM similar to B cells in HEL-Dbl-tg mice and do not respond to T cell-dependent immunization [8]. This indicates that in mature B cells, tolerance can be induced in the periphery depending on the concentration of the tolerogenic antigen.

The deletion of autoreactive B cells in response to antigens expressed exclusively at peripheral sites was demonstrated in 3-83tg mice with liver specific expression of K^b (MT-K^b/3-83tg mice) [12]. Such mice deleted K^b-specific B cells in the periphery but not in the bone marrow, thus only at sites where antigen was encountered. The complete absence of K^b-specific B cells in LNs reflects the efficient deletion of the recirculating B cell pool and suggests that those cells found in the spleen are newly formed B cells from bone marrow. In contrast, peripheral deletion of K^b-specific B cells is not observed when K^b is expressed under the keratin promoter in the periphery of 3-83tg mice. Similar findings (no tolerance induction to peripherally expressed antigens) were obtained from HEL-Dbl-tg mice expressing HEL under control of rat thyroglobulin promoter (rTg) [13]. The opposing outcome of tolerance induction in the MT-K^b and rTg-K^b model may be a consequence of different accessibility of B cells to the antigens as well as variable capacity of liver and thyroid cells to present those antigens.

Further evidence for a differential regulation of tolerance induction in respect to the autoantigen stems from autoimmune-prone, Fas-deficient, MRL/lpr H-2^d mice carrying the same MT-K^b and 3-83 transgenes [14]. Although Fas deficient, double-transgenic mice efficiently deleted 3-83 specific B cells, they produced elevated levels of IgG anti-chromatin antibodies. This indicates that induction of tolerance to membrane-associated autoantigens seems to be differentially regulated compared to nuclear antigens.

Under normal physiological conditions, most immature B cells arriving in the spleen are negatively selected and die by apoptosis during transition from immature T1 stage to the more mature T2 stage [15, 16]. B cell activating factor (BAFF), is a limiting factor and critically regulates this transition and maturation process [17, 18]. It was shown that B cells, from the immature stage in the bone marrow up to mature splenic B cells, respond to BAFF either by phenotypic maturation or increased Bcl-2 mediated survival [19]. Usually, anergic self-reactive B cells have a life span of 2 to 3 days and are excluded from splenic follicular and marginal zones due to extensive competition with non-self reactive B cells. In situations where BAFF is available in excessive amounts (in BAFF-tg mice), self-reactive B cells are rescued through BAFF generated survival signals. This leads to the development of self-reactive B cells with intermediate affinity beyond the T2 stage and their localization in splenic follicular and marginal zones [20]. Although high-affinity autoreactive B cells are efficiently deleted, B cells with intermediate affinity remain at sites where they can receive T cell help or mitogenic stimulation e.g. by LPS. Thus, excess of BAFF can rescue anergic self-reactive B cells with low-affinity receptors from deletion in the periphery and contributes significantly to the development of antibody-mediated autoimmune diseases.

b) T cell Tolerance

The thymus fulfills two important tasks for the establishment of self-tolerance. First, autoreactive specificities are removed from the developing T cell repertoire by the central mechanisms of receptor editing and negative selection (recessive tolerance). Second, the thymus produces regulatory T cells (Tregs) with suppressive capacity in order to control autoreactive T cells that escaped negative selection (dominant tolerance). For this, the thymus provides a specialized microenvironment of distinct cortical and medullar compartments through which thymocytes pass in an ordered sequence during their development. Two transcription factors play a central role for the induction of T cell tolerance: autoimmune regulator (AIRE) and forkhead-box-protein P3 (FoxP3). Mutations in the genes encoding these transcription factors result in the monogenetic disease autoimmune polyendocrinopathy syndrome type 1 (APS-1) and immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance syndrome (IPEX), respectively [21, 22].

Selection processes in the thymus reflect important differences between B and T cells in regard to development (release of fully mature T cells, CD4/CD8 lineage commitment), receptor formation (induction of allelic exclusion) and antigen recognition (MHC-restriction). ERK signaling is an important factor in the selection of T cell. Low-affinity T cell receptor (TCR) interactions generate sustained ERK activation and lead to positive selection of T cells, whereas high-affinity interactions induce transient ERK activation and promote negative selection resulting in the elimination of potentially dangerous T cells [23].

i) Positive and negative selection

During T cell development, self-tolerant and self-MHC restricted thymocytes are selected through interactions with (self-) antigen presenting cells (APC) of non-hematopoietic origin. This selection process takes place in specialized microenvironments of the thymus and is mediated by cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) as well as dendritic cells (DCs) [24]. In the cortex, double-positive (DP) thymocytes are positively selected if they express an antigen receptor that can recognize self-peptide:self-MHC (self-pMHC) complexes presented by cTECs [25, 26]. Thus, during positive selection, self-MHC restricted thymocytes are selected to survive and mature [27]. Both, the MHC molecule and the presented peptide strongly influence the selection outcome and therefor contribute to the formation of the TCR repertoire [28-30]. Peptide ligands with the capacity to promote positive selection of thymocytes were found to be of low affinity for the TCR, meaning they inhibit T cell responses or at best induce weak stimulation of mature T cells [31].

The affinity hypothesis has been put forward to explain the opposing outcome that can result from TCR signaling in developing thymocytes. This concept describes that TCR/co-receptor/pMHC interaction of low or intermediate affinity result in survival (positive selection) whereas interactions with apparent affinity above a certain threshold induce apoptosis (negative selection) of the thymocyte. It is thought that the thymocyte integrates TCR signals by kinetic proofreading (connecting the apparent affinity to the dissociation rate and the half-life of the TCR/co-receptor/pMHC interactions (dwell time)) and serial triggering. High affinity interactions are characterized by slow dissociation rates and long half-lives that result in full “zippering”

of TCR and co-receptors leading to complete phosphorylation of CD3 containing ITAMs whereas low affinity interactions result in only partial "zippering" and insufficient activation of the CD3 complex [32]. It was shown that MHC-I restricted thymocytes display an universal apparent affinity threshold for negative selection with a dissociation constant (Kd) ~ 6 μM and half-life ($t_{1/2}$) of 1 - 3 seconds [33]. The apparent affinity of MHC-I restricted TCR/pMHC interactions is particularly influenced by co-receptor binding (10 -15 fold increased by CD8) in contrast to MHC-II restricted interactions where CD4 contribution is weak (Kd of 10 – 60μM with or without CD4). Intracellular signaling events following high or low affinity interactions differ in the compartmentalization of Ras/MAPK signaling pathway components and the kinetics of Erk activation. Low affinity interactions with fast off-rates are characterized by a slow accumulation of activated Erk at the Golgi whereas high affinity interactions with slow off-rates induced rapid localization of activated Erk at the plasma membrane. In contrast, the activation kinetics and the localization of Jun, another important MAPK downstream of TCR signaling, is similar for high and low affinity signaling [23]. This compartmentalization of activated Erk and Jun forms the basis for the different selection outcome in response to high and low affinity TCR/pMHC interactions.

The cytosolic proteasomal degradation machinery of cTECs contains the ß5t subunit (processing of peptides presented on MHC-I) and together with lysosomal Cathepsin L (processing of peptides presented on MHC-II) cTECs have the ability process and present sets of peptides that are unique to this cell type [34, 35]. Commitment of DP thymocytes to either the $CD8^+$ or the $CD4^+$ T cell lineage depends on interaction with MHC-I or MHC-II molecules, respectively, and is controlled by the activity of the co-receptor associated tyrosine kinase Lck [36-38]. Furthermore, positive selection

terminates further α-chain rearrangement through down regulation of RAG protein expression, promotes survival by inducing Bcl-2 and leads to developmental progression to the single-positive (SP) stage [39, 40]. The majority of DP thymocytes will die by programmed cell death within 3 to 4 days because their receptors do not bind to self-pMHC, hence, no survival signal is transmitted (death by neglect) [41].

Subsequent to positive selection, thymocytes undergo phenotypic changes associated with maturation (e.g. TCR upregulation, CD69 expression) and migrate to medullary regions where development is completed. There, SP thymocytes encounter mTECs as well as DCs of intra- or extrathymic origin [42, 43]. mTECs have the unique feature to express tissue-restricted antigens (TRAs) from distinct tissues under the control of AIRE and can present the corresponding peptides on MHC molecules [44]. Expression of peripheral antigens is an essential part of central tolerance mechanisms to promote self-tolerance in the T cell compartment. Strong recognition of self-pMHC complexes by the TCR leads to thymocyte death by apoptosis (negative selection) or might induces Treg lineage deviation (agonist selection) [45, 46]. DCs in the thymic medulla are potent mediators of negative selection through induction of apoptosis via co-stimulatory molecules [47, 48]. In order to complete thymic selection, SP thymocytes reside in the medulla for another 4 to 5 days to scan multiple mTECs for potential high affinity self-antigens. Thereafter, thymocytes are exported as fully functional mature T cells to the periphery.

The requirements for positive selection and the influence of MHC molecules together with the presented peptides on the selection of the TCR repertoire have been demonstrated in several experimental systems.

The thymus selects a repertoire of TCRs biased to recognize peptides presented in combination with allelic variants of MHC molecules (haplotype) expressed by radioresistant cortical thymic epithelial cells (cTECs). Transfer of F1 bone marrow into lethally irradiated parental recipient mice provided experimental evidence that thymocytes are "educated" in the thymus and restricted to MHC molecules of the parental haplotype, expressed on radioresistant thymic stromal cells. F1 T cells from irradiation chimaera (F1 H-$2^{b/d}$ → BALB/c H-$2^{d/d}$) that were immunized with syngeneic minor antigens (B10 H-$2^{b/d}$) could only lyse target cells expressing the MHC haplotype of the recipient strain but not target cells expressing the MHC haplotype of the other parent [49]. Further support for self-MHC restriction was provided by studies investigating the selection of TCR transgenic thymocytes on different MHC haplotypes. The 2C TCR is specific for L^d antigen and is efficiently positively selected by H-$2K^b$ in the absence of its high affinity antigen, L^d. It was shown that the selection in an autoimmune environment (H-2^d, expressing L^d) or a non-selecting environment (H-2^s, lacking K^b) generates comparable fractions of $2C^+$ $CD8^+$ T cells in the periphery but those generated on the H-2^d background are functionally inactive. In the thymus of H-2^d mice, 2C thymocytes are found mainly in the double-negative population whereas in the H-2^s mice, DP thymocytes express the 2C TCR. There is no deletion of $2C^+$ thymocytes at the DP stage in H-2^s mice but positive selection is also not possible because of the lack of H-2^b [50].

Studies with irradiated bone marrow chimeras reconstituted with bone marrow from mice carrying the transgenic TCR specific for the male antigen (H-Y), show that antigen specific T cells are only positively selected if they mature in an environment that provides the appropriate MHC. Since H-Y specific TCRs are restricted to peptide

presentation by H-2D^b, positive selection of the H-Y specific receptor is only achieved in mice expressing H-2D^b molecule (e.g. B6, BDF1, B10.HTG). In the thymus of non-H-2D^b recipients (B10.BR (H-2D^k), B10.D2 (H-2D^d)), H-Y specific thymocytes are not selected because of the lack of H-2D^b expression. However, thymocytes that could edit their receptor to recognize H-2D^k or H-2D^d were selected. These thymocytes expressing an edited TCR still express the transgenic β-chain but in combination with an endogenous α-chain [51]. Efficient receptor editing was demonstrated in immature OT-I transgenic T cells in response to peptides of ovalbumin (OVAp) presented on the cortical thymic epithelium. Thymocytes in such mice are not deleted because they replace the transgenic α-chain by expression of an endogenous α-chain. On a RAG deficient background where rearrangement of endogenous α-chain genes is inhibited OT-I transgenic TCRs are efficiently deleted in response to OVAp [52].

In male H-Y TCR transgenic mice, deletion of developing TCR transgenic thymocytes occurs at the DP stage upon recognition of that male antigen. CD8$^+$ T cells found in the periphery of these mice, that escaped deletion, show reduced CD8 expression and do not differentiate into cytotoxic T lymphocytes (CTLs) in response to male (H-Y expressing) stimulator cells. Transgenic T cells in the periphery are mainly present in a population of T cells expressing neither CD4 nor CD8. The occurrence of such T cell populations in TCR transgenic H-2^d SCID mice indicates a selection process independent of H-2^b molecules [38] [53].

Fetal thymus organ cultures (FTOCs) prepared from non-transgenic $\beta_2m^{-/-}$ or OT-I TCR transgenic mice provide an elegant tool to investigate the peptide requirements for positive selection of a specific TCR. Using such FTOC approaches it was shown that OT-I specific CD8$^+$ T cells could only be positively selected if the required K^b molecule is

present. Selection on the variant K^{bm1} protein was not possible. Moreover, positive selection is very sensitive to MHC density and peptide concentration as well as peptide specificity. In FTOC it was also shown that variants of the antigenic peptides are able to induce positive selection and functional maturation of OVAp specific thymocytes. Interestingly, all these antigenic variants that induce positive selection are also antagonists that can inhibit CTL dependent lysis. One variant peptide (E1) was shown to mediate positive selection of the OT-I specific receptor at low ligand concentration ($\beta_2m^{-/-}$) and negative selection at high concentrations ($\beta_2m^{+/-}$)[31, 54].

ii) Promiscuous gene expression

Thymic medullary regions provide a special environment for the induction of central tolerance. There, various APCs of extra- and intra thymic origin can be found that act in concert to promote negative selection of self-reactive receptors. Thymocytes are selected in response to self-antigens derived from various sources. mTECs have a pivotal role in the induction of self-tolerance due to the ability to express antigens that are usually restricted to cells of specific tissues, so called tissue-restricted antigens (TRAs). This promiscuous gene expression of TRAs is mediated by AIRE proteins interacting with other transcription factors to promote DNA accessibility of normally closed chromatin regions. The molecular details, however, are still not fully understood. Although, each specific TRA is only expressed by a fraction of mTECs (1 - 3%), the repertoire of peripheral self-antigens is largely represented by this AIRE dependent expression. Indirect presentation of TRAs that have been transferred to DCs increase the chance that thymocytes interact with rare TRAs during selection in the medulla. Promiscuous gene expression might contribute to generation of Tregs specific for rare self-antigens. Central tolerance critically depends on AIRE and the regulation of

promiscuous gene expression. AIRE deficiency in mice leads to multi-organ autoimmune disease with lymphocyte infiltration resembling the rare human autoimmune disease APS-1 [55, 56].

iii) Treg development in the thymus

Tregs (referring to natural or thymus derived Tregs unless otherwise specified) represent an important subpopulation of suppressive $CD4^+$ T cells whose activity is dependent on their expression of the transcription factor FoxP3 [57-60]. Tregs derive from the same progenitor cells and also share the same maturation and differentiation process as conventional T cells in the thymus. The requirement for IL-2 for the development of Tregs in the thymus has been controversial due to the functional redundancy of IL7 and IL15. IL-$2^{-/-}$ and IL-$2R\alpha^{-/-}$ mice have only mildly reduced numbers of $FoxP3^+$ thymocytes, but in $CD132^{-/-}$ mice (common γ-chain deficiency (SCID)) these Tregs are practically absent, suggesting a critical role of common γ-chain associated signaling pathways involving STAT transcription factors. In agonist-driven TCR transgenic mouse models of Treg differentiation, however, it was shown that IL-2 and IL-2Rα expression are not required for the generation of Tregs [61]. The selection process of Tregs depends on the affinity of the TCR for a specific peptide. It was shown that in thymocytes expressing TCRs with high affinity for self-antigens that is close to the threshold affinity for negative selection, Treg differentiation is favored [62]. Although FoxP3 is the lineage specific transcription factor and its expression is essential for the regulatory function of Tregs, commitment to the Treg lineage in the thymus seems to be dependent on co-stimulatory CD28 signaling, rather than just the induction of FoxP3 expression [63]. It was shown that $AIRE^+$ TRA presenting mTECs, expressing abundant co-stimulatory molecules, promote the differentiation of TRA-specific Tregs

and that under conditions of low antigen concentrations, Treg differentiation is favored over negative selection, i.e. deletion [64-66]. Moreover, mTECs are a source for transforming growth factor (TGF)-β and express members of the tumor-necrosis factor receptor (TNFR) family, including OX40 (CD134) and GITR (CD357), which are also required for the induction of Treg differentiation. Any defect that results in a compromised Treg compartment can potentially induce severe multi-organ autoimmunity [67].

iv) Treg conversion in the periphery

Besides the generation of Tregs by *de novo* pathways in the thymus, they can also be differentiated under certain conditions from naïve $CD4^+$ T cell populations in the periphery [68]. Conversion of naïve $CD4^+$ T cells into induced Tregs (iTregs) is induced by stimulation with agonist peptides present at low concentrations in the absence of co-stimulation. Presence of TGF-β can also promote conversion into iTregs by stabilizing FoxP3 expression and inhibiting $CD4^+$ T cell proliferation. Retinoic acid produced by gut-resident DCs further contributes to iTreg conversion [69]. iTregs can express the same levels of FoxP3 and have equal suppressive capacity compared to thymus derived Tregs. The conversion of conventional $CD4^+$ T cells into iTregs contributes to antigen-specific tolerance in the mature T cell compartment and is likely to occur in response to gut associated non-self antigens [70]. The conversion of high-affinity self-reactive T cells into iTregs is suggested to represent another mechanism for induction of self-tolerance under physiological conditions. However, in inflammatory environments with abundant IL-6 and TGF-β, such potentially dangerous T cells may instead differentiate along the Th17 lineage and possibly induce autoimmunity when activated by self-antigen [71, 72].

iTregs are mainly found in mucosal tissue where they control immunity to food-antigens or components of the intestinal microbiota [73].

2. Systemic Lupus Erythematous

a) SLE in man

Systemic lupus erythematous (SLE) is the prototypical autoimmune disease characterized by loss of B cell tolerance leading to production of pathogenic autoantibodies and their formation of immune complexes with self-antigens. Human SLE presents as a variable systemic disease of unpredictable course characterized by phases of flares and remissions [74]. Almost all organ systems can be affected and symptoms manifest in a diverse array of mucocutaneous, musculoskeletal, pleuropulmonary, cardiovascular and hematologic alterations partially overlapping with other autoimmune diseases. Most prominent is the development of lupus nephritis resulting from deposition of circulating immune complexes in the kidneys causing high mortality in patients suffering from SLE. The susceptibility of individuals to develop SLE is significantly influenced by genetic predisposition and environmental factors. For SLE a gender bias can be observed with women being affected ten times more often than men. The concordance rate for SLE in monozygotic twins is between 20 - 40% in contrast to 2 - 5% for dizygotic twins and other siblings, arguing for a strong genetic influence on diseases susceptibility. Certain MHC-II alleles, e.g. HLA-DR2 and HLA-DR3, were shown to be associated with SLE and confer increased risk to develop the disease [75]. Genome wide association studies (GWAS) performed on SLE cohorts identified several single nucleotide polymorphisms (SNP) in genes of important biological pathways that are dysregulated in SLE patients [76]. To date more than 20 loci have been identified that

are strongly associated with the development of SLE. Typical susceptibility genes found in these loci are involved in immune complex processing and activation of the complement cascade (FCGR2 or C1Q), lymphocyte signaling and signal transduction (HLA-DR, BTK, PTPN22) or other toll-like receptor (TLR)- or interferon (IFN)-mediated innate immune pathways (IRAK1, IRF5, TREX1). The synergistic effect of multiple susceptibility genes in combination with environmental factors like UV-irradiation, drugs or viral infection can lead to a break of tolerance and subsequently to autoantibody production through the activation of autoreactive lymphocytes. Once tolerance is broken and autoantibodies are produced, especially to nucleic acids, T cell independent but TLR-mediated mechanisms sustain and amplify the inflammatory response by stimulating type-I IFN production. Defective clearance of apoptotic cell material promotes the development of SLE as demonstrated by the rare C1q deficiency. This defect is the only known monogenetic alteration leading to SLE in more then 90% of the patients carrying a C1q null mutation [77]. Effector cells (e.g. macrophages and neutrophils) recruited to the site of immune complex deposition meditate tissue damage through the release of reactive oxygen intermediates or inflammatory cytokines, including TNF-α and IFN-α. Dysregulated peripheral tolerance mechanisms represent another important aspect in the pathogenesis of lupus. In many SLE patients there is a quantitative or qualitative defect in regulatory T cells resulting from disturbed, IL-2 dependent, homeostatic proliferation leading to an imbalanced ratio of effector and regulatory T cells [78, 79].

Contributions from mouse models of lupus as well as deeper insights into genetic factors obtained from GWAS advanced the understanding of cellular and molecular mechanism of this autoimmune disease and led to an improved treatment of SLE. However,

additional specific therapies are still needed since lupus is a very heterogeneous disease. Traditional approaches for SLE therapy include general anti-inflammatory and immunosuppressive drugs like corticosteroids, cyclophosphamide and azathioprine. B cells are central to the pathogenesis of SLE, not only because of their production of autoantibodies, but also because B cells contribute to the activation of T cells through autoantigen presentation and co-stimulatory signaling, making them a good therapeutic target [80]. Rituximab, a chimeric mouse/human monoclonal antibody targeting CD20 on B cells, was shown to efficiently deplete B cells at all maturational stages and reduce disease symptoms in mice. However, in placebo-controlled phase II/III clinical trials in SLE, rituximab failed to meet the primary and secondary endpoints. A reason might be that rituximab does not deplete plasma cells, the primary source of antibody production [81].

Interfering with BAFF signaling is another promising therapeutic approach in SLE. B cells critically depend on BAFF as a survival factor from the immature stage until the plasma cell stage. Increased BAFF levels, as found in SLE patients, promote the survival of autoreactive B cells and lead to macrophage activation. Treatment with belimumab, a fully human monoclonal antibody against BAFF, decreased total numbers of B cells as well as serum Ig levels and anti-DNA specific IgG titers in two phase III studies leading to decreased SLE activity according to criteria of the SLE disease activity index [82, 83]. Belimumab binds the soluble form of BAFF (also called BLyS for B-lymphocyte stimulator) and inhibits signaling through BAFF-R and TACI.

Several other therapies targeting co-stimulatory pathways that mediate activation and differentiation in B cells and T cells are under development. In murine lupus models, the inhibition of T/B cell interaction by neutralizing anti-CD40L antibody showed a positive

effect through diminished germinal center reactions. In clinical trials however, anti-CD40L treatment was associated with increased thrombotic events [84, 85]. Abatacept, a fusion protein of the extracellular domain of CTLA-4 and IgG1 constant region, binds to B7 on APCs and inhibits co-stimulatory interaction with CD28 expressing T cells. Clinical trials with abatacept in rheumatoid arthritis or psoriasis demonstrated safety for human use and also in murine SLE models the treatment showed a positive effect on the disease [86]. Recently, a small retrospective study including 11 SLE patients with refractory disease indicated a beneficial clinical effect of abatacept although primary endpoints were not met [87]. The induced co-stimulator (ICOS) pathway represents another promising target for therapeutic intervention due to its central role in T cell dependent differentiation of B cells into memory and antibody secreting plasma cells. Notably, $CD4^+$ and $CD8^+$ T cells of SLE patients were shown to express elevated levels of ICOS on their surface.

b) Animal models of SLE

The use of mouse models has always been an important and indispensible pillar of preclinical research in order to advance the understanding of etiology and disease promoting mechanisms of autoimmune diseases and, of course, for the evaluation of potential therapeutic treatments. Systemic autoimmune diseases in humans can manifest by diverse symptoms with varying severity from patient to patient. By using only one mouse model it is impossible to recapitulate the diversity of human autoimmune symptoms. The main reason might be the genetic diversity of the human population in contrast to inbred laboratory mouse strains. Nevertheless, each mouse model has proven its value by unraveling the contribution of specific alterations in genes or pathways leading to the induction or promotion of autoimmunity. For SLE, the

prototypic systemic autoimmune disease, many mouse models exist and each of them shares a subset of lupus-like symptoms with the human disease [88]. The typical lupus-like symptoms include autoantibody production, lymphoid hyperactivity and glomerulonephritis.

i) (NZWxNZB)F1

The classical and oldest SLE mouse model is the F1 hybrid of the New Zealand Black (NZB) and New Zealand White (NZW) strain (NZB/W F1), belonging to the group of spontaneous mouse models of SLE [89]. The parental stains of NZB/W F1 show only limited autoimmunity which is severely enhanced in the F1 generation leading to lymphadenopathy, splenomegaly and elevated serum antinuclear antibodies including anti-DNA IgG (mainly IgG2 and IgG3). Deposition of immune complexes and the resulting failure in kidney function leads to death within 10 - 12 months. Although this model represents a broad spectrum of SLE symptoms, autoantibodies against RNA containing complexes that are typically found in human lupus are absent in NZB/W F1 mice. On the other hand, female NZB/W F1 develop a more severe SLE-like disease compared to male. This female bias in severity is also observed for human lupus. SLE susceptibility loci identified in the NZM2410 strain, a strain derived from backcrossing NZB/W F1 to NZW, were shown to induce SLE-like disease on non-autoimmune B6 background but only if all three loci (Sle1-3) are present [90]. Two important receptors involved in B cell signaling and activation (FcγR2β and CR2) are encoded within the Sle1 locus. Additionally, members of the signaling lymphocyte activation molecule (SLAM) family are also found within this locus. More recently, Sle1 has been associated with decreased IL-2 production leading to impaired Treg functionality in lupus prone mice [91] and also other polymorphism in genes mapped to the SLE susceptibility loci from

NZM2410 were also shown to correlate with increase susceptibility for lupus in man [92].

ii) MRL/lpr

The MRL/lpr strain is another commonly used mouse model for SLE-like autoimmunity and also belongs to the group of spontaneous SLE models. In these mice a spontaneous mutation occurred on chromosome 19 in the gene encoding the Fas receptor that results in non-functional transcripts due to alternative splicing of the Fas gene [93]. MRL/lpr mice develop fatal autoimmune syndrome similar to SLE with symptoms including lymphadenopathy due to accumulation of double-negative T cells, circulating autoantibodies against several self-antigens (ss/dsDNA, Smith-protein, rheumatoid factor) along with immune complex deposition and glomerulonephritis [94]. MRL/lpr mice do not show gender bias in regard to disease severity as observed in the NZB/W F1 model. A similar phenotype results from a single amino acid mutation (Leu→Phe) in the gene encoding FasL on chromosome 1. The gld (generalized lymphoproliferative disorder) mutation prevents interaction of FasL with the FAS receptor and results in defective Fas-mediated apoptosis and systemic autoimmunity [95]. In humans, defective Fas signaling causes the autoimmune lymphoproliferative syndrome (ALPS) [96].

iii) BXSB/Yaa

The BXSB/Yaa strain is characterized by high penetrance of SLE-like symptoms but, interestingly, with greater severity in males. Male BXSB/Yaa mice have a lifespan of roughly 5 months in contrast to females with about 14 months [97]. The lupus-like phenotype of BXSB/Yaa mice is caused by a translocation of the telomeric end of the X chromosome to the Y chromosome, the so-called Y-linked autoimmune accelerator (Yaa). Yaa leads to the duplication of several genes, among others the gene encoding for

TLR 7, a pattern recognition receptor for nucleic acids that can provide signal 2 for the activation of autoreactive B cells [98]. B6 mice with the Yaa translocation do not develop overt autoimmunity due to functional compensatory mechanisms on the B6 background. But in addition with the SLE susceptibility locus Sle1, B6/Yaa/Sle1 mice develop severe SLE-like autoimmunity. Another susceptibility locus identified in BXSB mice is Bsx2 on chromosome 1 that overlaps with Sle1 and seems to be a major contributor to disease development by encoding genes important in clearance of apoptotic cell material [99]. The genetic background has considerable influence on disease development, which can be nicely demonstrated in the BXSB/Yaa model. BXSB/Yaa mice on a H-$2^{d/d}$ background show less severe disease symptoms compared to congenic mice on a H-$2^{b/d}$ or H-$2^{b/b}$ BSXB background which display full-blown disease. In contrast, mice carrying Yaa on a B6 background (also H-$2^{b/b}$) do not show signs of autoimmunity indicating that other non-MHC-linked loci from the BXSB background have a great influence on the induction and the severity of the disease [100, 101].

c) Murine chronic Graft-versus-Host Disease as model for SLE

The induction of chronic Graft-versus-Host Disease (cGvHD) is a well-established and widely used model for murine lupus. The cGvHD model is based on MHC-disparate donor lymphocytes that, upon intravenous (i.v.) injection into otherwise non-autoimmune recipient strains, break host tolerance to autoantigens by stimulation of autoreactive B cells. For this, alloreactive donor $CD4^+$ T cells are required and sufficient [102, 103]. The MHC-disparity between donor and host strains has to be combined in a way that donor lymphocytes are activated upon recognition of foreign host MHC structures but the immune system of the recipient is tolerant to the donor allograft

allowing for persistent donor T cell engraftment and stimulation. There are various combinations of strains where a single injection of donor lymphocytes induces a robust SLE-like cGvHD. One combination is the transfer of either parental $CD4^+$ T cells into the semi-allogeneic F1 generation (p→F1) leading to lupus-like symptoms in mice. An acute course of GvHD can be observed in the p→F1 model when $CD8^+$ T cells are present within the donor inoculum and cells are transferred between strains mismatched for MHC-I and MHC-II proteins [104]. The developing acute Graft-versus-Host Disease (aGvHD) is characterized by an initial stimulatory phase followed by $CD8^+$ T cell mediated elimination of the host immune system within a few weeks after induction. This process is accompanied by severe weight loss. An exception to the above-mentioned requirements of the p→ F1 model for the induction of cGvHD is the injection of parental lymphocytes from the DBA/2 strain into BDF1 mice [105]. Despite the presence of both $CD4^+$ and $CD8^+$ T cells in the donor inoculum and the transfer into MHC-I and MHC-II mismatched recipients, chronic GvHD ensues. This model was shown to be valuable for the screening of agents capable to promote $CD8^+$ T cells responses and thereby "reverting" the chronic course of the disease into an rather acute one [106]. It was shown that IL-21 is an interesting candidate for the modulation of $CD8^+$ T cell activity in cGvHD settings. Administration of IL-21 to BDF1 mice undergoing cGvHD upon injection of DBA/2 lymphocytes resulted in reduced SLE-like symptoms due to enhanced CTL differentiation and a reduction of autoreactive B cells [107]. Similar results were obtained from aGvHD studies where IL-$21R^{-/-}$ donor lymphocytes from B6 IL-21R deficient mice were injected into BDF1 mice. Such mice develop milder cGvHD due to the absence of IL-21R signaling. Donor $CD8^+$ T cells do not efficiently differentiate into CTLs and additionally, the stimulation of host B cells by donor $CD4^+$ T cells is less efficient since these interaction depend on IL-21 signaling as well [108].

Induced models have the advantage over spontaneous models that disease initiating /tolerance breaking events leading to SLE-like symptoms can be better controlled and manipulated. Autoantibody production is detected in cGvHD models as early as 10 - 14 days after disease induction whereas in spontaneous models it takes several weeks to months after birth to develop such symptoms. Furthermore, as already described above, tolerance breaking mechanisms leading to disease development in spontaneous models are the result of multiple genetic factors. For induced cGvHD models the antigens that initially lead to beaking tolerance are encoded by MHC peptides. Another advantage of induced models is the disease-driving alloreactive donor T cell population, which is relatively easy to observe because of polyclonal activation and expansion; this is in contrast to the rather rare events of T cell tolerance breakdown, which occur in spontaneous models. Since many knock-out mice are available on the B6 background, the transfer of $CD8^+$ T cell depleted B6 lymphocytes represents an interesting and useful model system to investigate the influence of several genetic alterations on disease development.

3. Interleukin-2

a) IL-2 signaling and function

Interleukin-2 (IL-2), a 15 kDa four-alpha-helical bundle cytokine belonging to the type-I family of cytokines was one of the first cytokines discovered [109]. While activated CD4 T cells are the main source of IL-2, $CD8^+$ T cells, NK cells and DCs also secrete IL-2, but to a lesser extent. TCR signaling in the presence of co-stimulation leads to rapid (within hours) and transient (for approximately 2 days) production of IL-2. Transcriptional activation of the IL-2 gene is regulated by cooperative binding of AP-1, NFκB (p65/rel),

Oct-1 and NFAT transcription factors to the promoter/enhancer regions approximately 500bp upstream of the transcriptional start site of the IL-2 gene. Transcriptional silencing of the IL-2 gene is accomplished by a negative auto-regulatory feedback loop which depends on IL-2 signaling-induced Blimp-1 expression [110]. The important role of Blimp-1 in the regulation of IL-2 expression has been demonstrated by ectopic expression of Blimp-1 in activated T cells leading to inhibition of IL-2 production and in Blimp-1 deficient T cells where the lack of negative regulation results in augmented IL-2 production [111, 112]. Moreover, central memory T cells express low levels of Blimp-1 indicating a role for IL-2-induced Blimp-1 expression in the control of memory T cell differentiation. Additionally, low Blim-1 expression in memory T cells allows for rapid and augmented IL-2 expression upon stimulation [113]. Prolonged TCR stimulation together with IL-2 signaling induces the expression of Fas and FasL on T cells, mediating activation induced cell death in these cells [114].

Based on early *in vitro* studies, the biological importance of IL-2 was first described as a T cell proliferation factor essential for appropriate expansion of T cell populations upon activation. However, with the availability of the first knockout mice where IL-2 signaling was interrupted (IL-$2^{-/-}$ or IL-$2R^{-/-}$) it was shown that IL-2 signaling is crucial for maintenance of self-tolerance since these knockout mice developed severe autoimmune symptoms instead of an expected immunodeficiency as predicted from the original *in vitro* observations [115, 116]. The resulting autoimmunity is primarily caused by impaired Treg homeostasis and the associated defect in regulatory function, which critically depends on IL-2 [59].

Responsiveness to IL-2 requires the expression of either the dimeric, low-affinity IL-2 receptor (IL-2R$\beta\gamma_c$) or the trimeric, high-affinity IL-2 receptor (IL-2R$\alpha\beta\gamma_c$) [117]. The

low-affinity IL-2 receptor is composed of the common γ-chain (γ_c; CD132), which is also found in receptors for other members of the type-I cytokine family (e.g. IL-4, IL-7, IL-9, IL-15, IL-21) and the IL-2 receptor β-chain (IL-2Rβ; also known as CD122), which is shared only with the IL-15 receptor. In order to form the high-affinity IL-2 receptor, expression of IL-2 receptor α-chain (IL-2Rα; also known as CD25) is required. Association of the α-chain protein (CD25) to the dimeric, low-affinity IL-2 receptor increases the overall affinity of the receptor for IL-2 up to 100-fold with a dissociation constant of Kd 10^{-11}. CD25 only functions to establish high-affinity binding to IL-2, since it lacks a cytoplasmic tail it is not involved in downstream signal transduction. The binding of IL-2 to the trimeric IL-2 receptor is suggested to occur in a step-wise manner with IL-2 first binding to CD25 and subsequent recruitment of the two other receptor components to the complex [118]. Transduction of IL-2 signals critically depends on the β-chain and the common γ-chain subunit of the IL-2 receptor which are associated with JAK (1/3) proteins. Knockout mice deficient for either of these two IL-2 receptor subunits show severe immune-regulatory defects. Autoimmune symptoms have been observed in IL-2R β-chain deficient mice, while a severe combined immune deficiency (SCID) phenotype is present in IL-2R γ-chain knockout animals. [116, 119]. Engagement of the IL-2 receptor activates several downstream signaling pathways including STAT5 signaling, the PI3K-Akt-mTOR pathway and also the Ras-Raf-Mek-Erk axis (MAPK pathway); this promotes cell growth and survival as well as the expression of target genes like CD25 [120]. Upon binding of IL-2 to its receptor and initiation of signal transduction, the quaternary IL-2/IL-2R complex is rapidly internalized followed by degradation of IL-2, CD122 and CD132 whereas CD25 can be recycled to the cell surface [121]. IL-2 can also be presented in trans by CD25 expressing DCs, however, the biological importance of this mechanism is still not fully understood.

The expression of high- and low-affinity IL-2 receptors and the respective subunits varies significantly across different cells of the immune system. Naïve $CD4^+$ T cell express hardly any dimeric IL-2 receptor whereas naïve $CD8^+$ T cells or memory $CD4^+$ T cells express significantly but low amounts of dimeric IL-2 receptor. Memory $CD8^+$ T cells typically express high levels of dimeric IL-2 receptor making them responsive to exogenously administrated IL-2, but their sensitivity to endogenous IL-2 concentrations is relatively low [122]. Upon activation, T cells start to express transiently high levels of CD25 and CD122 rendering them highly sensitive to IL-2. Tregs constitutively express high levels of CD25 and are critically dependent on IL-2 produced by other T cells.

IL-2 signaling and the ensuing activation of STAT5 is also an important factor during differentiation of $CD4^+$ helper T cell subsets [123]. IL-2 signaling in Th1 cells induces the upregulation of IL-12Rβ2 on the cell surface along with increased intracellular levels of the transcription factor T-bet, resulting in increased IFN-γ production. In Th2 cells, enhanced expression of IL-4Rα and enhanced production of the subset specific cytokines IL-4, IL-5 and IL-13 is observed in response to IL-2. In regulatory T cells, which are unable to produce IL-2 by themselves and therefore depend on IL-2 from other cell sources, STAT5 signaling maintains expression of intracellular FoxP3 and high levels of CD25 on the surface [124]. In contrast, differentiation along the Th17 lineage is negatively regulated by IL-2 signaling. IL-2 induced STAT5 activation leads to downregulation of IL-6Rβ expression and subsequent decreased STAT3 signaling and RoRγt expression. Additionally, STAT5 competes with STAT3 for binding at regulatory regions in the IL-17 gene locus [123]. Follicular helper T (Tfh) cells are suggested to differentiate mainly from naïve $CD4^+$ T cells expressing low levels of CD25. Upon antigen-specific activation by DCs in secondary lymphatic organs, IL-2 signaling induces

expression of B cell lymphoma 6 protein (Bcl-6) and CXC chemokine receptor 5 (CXCR5) mediating the differentiation into Tfh cells and localization to the B cell follicle [125].

Beside IL-2's primary role in maintaining the Treg subset and "dominant tolerance", it is also critical for the generation of optimal $CD8^+$ T cell responses to infections. IL-2 directly affects several aspects of the immune response including the primary expansion of antigen-specific T cells, the contraction phase after the clearance of the pathogen and the generation of T cell memory. IL-$2^{-/-}$ mice show a 3-fold reduction in $CD8^+$ T cell expansion after acute viral infection and impaired viral clearance compared to IL-2 sufficient mice [126]. Studies with bone marrow chimeric mice containing a mixture of $CD25^{-/-}$ and normal $CD8^+$ T cells demonstrate the importance of IL-2 signaling during the primary immune response beyond optimal expansion of effector populations. Chimeric mice showed, similar to the results obtained from IL-$2^{-/-}$ mice, a reduction in initial expansion of the $CD8^+$ T cells in the $CD25^{-/-}$ population and upon secondary challenge an even more compromised memory response [127]. Notably, IL-2 signaling in the absence of CD25 during primary expansion results in impaired memory generation. These results indicate that IL-2 is a critical factor for the expansion of effector $CD8^+$ T cell populations and equally important for generation T cell memory.

The strength of IL-2 signaling that $CD4^+$ and $CD8^+$ antigen-specific T cells receive during priming is suggested to directly affect the memory/effector fate decision. High surface expression of CD25 and the resultant enhanced IL-2 signaling induces T cells to differentiate into short-lived effector T cells expressing higher levels of Blimp-1. These terminal effecter cells do not survive the contraction phase after the infection has been cleared. On the other hand, $CD25^{lo}$ T cells were shown to differentiate into central

memory phenotype T cells with increased survival and expression of typical memory markers like IL-7Rα, CD62L or CCR7 [128].

b) IL-2 therapy and IL-2 complexes

The use of IL-2 as a therapeutic agent especially for autoimmune diseases has not been successful due to complications linked to the pleiotropic effects of IL-2 on the immune system. Initially discovered and described as a growth factor for activated T cells *in vitro* (T cell growth factor, TCGF), IL-2 was soon used in clinics to treat carcinoma and melanoma patients. However, it was soon noticed that high doses of IL-2, which were necessary to archive a clinical response, are toxic and induce severe side effects, including pulmonary edema and general vascular leakage syndrome due to responsiveness of CD25 expressing endothelial cells [129, 130]. Another reason for the inefficiency of IL-2 therapies at that time (resulting in low response rate in many clinical trails) was probably due to the effect of IL-2 on Tregs, which might have suppressed the desired anti-tumor activity.

That IL-2 had a potential therapeutic benefit in autoimmune settings was shown in the 1990s by *in vivo* studies in MRL/lpr mice infected with an IL-2 producing recombinant vaccinia virus. However, IL-2 was only approved for immunotherapy of several carcinoma and metastatic melanoma by the US Food and Drug Administration (FDA). Despite the long established positive effect on autoimmunity, the first clinical trials in order to investigate the potential of IL-2-mediated Treg stimulation in autoimmune settings were not conducted before 2010 [131].

The pitfall of IL-2 in immunotherapy, besides its toxicity at clinically relevant doses and the short half-life time in circulation, is also the variable responsiveness of immune cells

to IL-2, especially T cells, due to the heterogeneous expression of IL-2 receptor components (CD25, CD122 and CD132). For a specific therapy, it would be preferable to selectively stimulate specific subsets of T cells with appropriate effector functions. This would require a specific expansion of tumor-specific $CD8^+$ T cells in the setting of cancer immunotherapy, or regulatory T cells in case of autoimmunity, inflammation or allograft immunity. Coupling of IL-2 to monoclonal antibodies (mAb) not only prolongs the half-life in the circulation and thus biological availability, but it was also shown to restrict the biological activity of IL-2 to specific populations of IL-2 receptor-expressing cells depending on the epitope-specificity of the anti-IL-2 mAb [122, 132, 133].

The mAb S4B6 and the IL-2R α chain bind the IL-2 molecule at the same site (amino acid 26 - 45 of murine IL-2) [134]. A conformational change in the IL-2 molecule induced through S4B6 binding increases its' affinity for the dimeric IL-2 receptor. The binding of S4B6 to IL-2 is suggested mimic the binding of CD25 to IL-2 since similar conformational changes in the IL-2 molecule are observed upon interaction with CD25 [135]. Thus, by preventing interaction between CD25 and IL-2, the S4B6/IL-2 complex "concentrates" its biological activity on cells expressing the low-affinity dimeric IL-2 receptor e.g. $CD122^{hi}$ memory phenotype $CD8^+$ T cells or NK cells. Initial studies showed that S4B6/IL-2 complexes substantially increase the absolute number of memory phenotype $CD8^+$ T cells and, to a lesser extent, NK cells in spleen and lymph nodes of mice after one week of daily injections [122]. Similar results were obtained with mAb JES6-5/IL-2 and mAb 602/hIL-2 complexes. In a mouse model of pulmonary melanoma, treatment with S4B6/IL-2 complexes improved the antitumor response without IL-2-mediated pulmonary edema at concentrations where normal IL-2 showed no effect [130].

Another anti-IL-2 mAb that modulates receptor interactions with IL-2 is JES6-1. JES6-1/IL-2 complexes restrict the biological activity to cells expressing high levels of CD25 resulting in a selective expansion of regulatory T cells when injected into mice [122]. When complexed with JES6-1, IL-2 epitopes for binding of CD122 and CD132 are blocked, thus only the interactions with CD25 can take place. Binding of CD25 to JES6-1/IL-2 complexes causes JES6-1 to dissociate from the complexes. This renders binding sites for CD122 and CD132 accessible again and formation of high-affinity IL-2 receptors can take place [135]. Transcriptional feedback loops further augment the selectivity of JES6-1/IL-2 complexes for cells expressing CD25 by increasing surface CD25 expression, which is not observed in response to S4B6/IL-2 signaling. *In vivo* administration of JES6-1/IL-2 complexes to non-immunized mice selectively expands Tregs although activated antigen-specific $CD8^+$ T cells respond to JES6-1/IL-2 treatment with a more pronounced expansion compared to S4B6/IL-2 treatment [136]. The therapeutic potential of JES6-1/IL-2 treatment was demonstrated in several mouse models of autoimmune disease including colitis and experimental autoimmune encephalomyelitis (EAE). Furthermore, JES6/IL-2 treatment induced tolerance to a pancreatic islet allograft across a full MHC-mismatch without preconditioning with immunosuppressive regimes; suggesting that JES6-1/IL-2 complexes might be useful in a clinical setting of organ transplantation [137, 138].

JES6-1/IL-2 complexes show superior specificity over S4B6/IL-2 complexes regarding the expansion of $CD4^+$ $CD25^+$ regulatory T cells and memory phenotype $CD8^+$ T cells. JES6-1/IL-2 complexes specifically expand the absolute numbers of Tregs whereas S4B6/IL-2 complexes expand both $CD8^+$ T cells and Tregs to a similar extent as JES6-1/IL-2 complexes [122]. Thus, in normal mice, Tregs respond similarly to S4B6/IL-2 and

JES6-1/IL-2 treatment with regard to proliferation and expansion. However, in spleens of 14 weeks old MRL/lpr mice that received IL-2/mAb complexes, only JES6/IL-2 complexes mediated Treg expansion [139].

Materials and Methods

Mice

(C57BL/6 x DBA/2)F1 (BDF1), DBA/2, (C57BL/6-Tg(TcraTcrb)1100Mjb x DBA/2)F1 (OT1-BDF1), (C57BL/6-Tg(TcraTcrb)425Cbn x DBA/2)F1 (OT2-BDF1) were bred in our animal facility. All mice were maintained under specific pathogen fee conditions in our animal facility. For experiments and analysis, age- and sex-matched mice between 8 and 12 weeks of age were used. Mice were sacrificed by CO_2 inhalation and organs were removed by standard procedures. Animal experiments were carried out within institutional guidelines (authorization number 1888 and 2434) from Cantonal Veterinarian Office, Basel).

Preparation of donor cells and induction of GvHD

For the preparation of donor cells, spleen and LN (cervical, axillary, brachial, inguinal and mesenteric) were removed from DBA/2 mice and gently passed through a 40 µm nylon mesh to obtain single cell suspensions in serum-free (SF) IMDM supplemented with 2% FCS (MP Biomedical, USA) and 0.5% Ciproxin (Bayer AG, CH). Spleen cell suspensions were treated with ACK buffer in order to lyse erythrocytes. Single cell suspensions were pooled, counted by Trypan blue exclusion and washed in serum-free DMEM (Sigma-Aldrich, USA) prior to injection. GvHD was induced by i.v. injection of $70x10^6$ DBA/2 lymphocytes in a volume of 200 µl SF-IMEM.

Depletion of donor $CD8^+$ T cells

In order to obtain donor cell suspensions depleted of $CD8^+$ T cells, DBA/2 mice were injected i.v. with 200 µl of 1 mg/ml YTS-156, a rat anti-mouse mAb specific for CD8β, four days before donor cell suspensions are prepared from these mice. YTS-156 was

purified from a hybridoma culture supernatant according to standard procedures. Efficiency of $CD8^+$ T cell depletion was confirmed by flow cytometry analysis using fluorescent-labeled anti-CD8α specific mAb (53-6.7).

Preparation of IL-2 complexes

For a single injection, 2.5 µg rIL-2 and 7.5 µg anti-IL-2 mAb JES6.1A12 (BioXCell, USA) or S4B6 (purified from hybridoma culture supernatants by standard procedures) were mixed to prepare the IL-2 complexes. After 30 min incubation at 37°C, the volume was adjusted to 200 µl with sterile PBS and injected i.p. into mice. In control mice cGvHD was left untreated.

Detection of autologous IgG anti-erythrocyte antibodies (anti-RBC)

For the detection of anti-RBC in the blood of cGvHD mice, Coombs test was performed as described elsewhere [140]. Briefly, 100µl heparinized blood was first diluted 1:20 in PBS containing 2% FCS and 0.1% of 1 M NaN_3. 25 µl of diluted blood was incubated with 50µl of 1:200 diluted fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch) for 30 min at 4°C. Cells were washed and bound antibody was detected using a FACSCalibur (BD Bioscience, USA) machine. Mice were scored positive when the median of fluorescence intensity was increased more than 2-fold compared to healthy controls.

Detection of anti-nuclear antibodies (ANA)

For detection of ANA in sera of cGvHD mice, 8µm snap-frozen sections obtained from kidneys of $RAG2^{-/-}$ mice were used. Sections were first incubated with 80µl sera from cGvHD mice at dilutions 1:20 to 1:5120 for 30 min at room temperature (RT) in the dark. After washing, bound ANA was detected by incubation with 80µl of a 1:200 diluted

FITC-labeled goat anti-mouse IgG antibody for 30 min at RT in the dark. The titer was determined by using a fluorescent microscope (Zeiss Axioscope) and was defined as the highest dilution that still gave a specific nuclear staining. Mice showing titers lower than 1:20 were considered as negative for ANA.

Measurement of proteinuria

Proteinuria was determined semi-quantitatively in weekly intervals using Albustix (Siemens Healthcare Diagnostics Inc., USA). Elevated protein levels in the urine are indicative of failure in kidney function [141]. Mice were scored positive when a concentration >3 mg/ml was indicated by color change of the Albustix.

Immunhistological analysis

Kidneys from proteinuria positive mice were embedded in OCT-compound (Sakura Finetek, NL) and snap-frozen on dry ice. 8 µm sections were prepared on glass slides, fixed in acetone for 10min and dried. For the detection of immune complex deposition in the glomeruli, sections were incubated with FITC-labeled goat anti-mouse IgG antibody for 30 min at RT. For the detection of complement deposition, kidney section from cGvHD mice were incubated for 30 min at RT in the dark with FITC-labeled anti-C3 mAb (a kind gift of Dr. S. Izui, University of Geneva) diluted 1:100 in FACS Buffer. Bound FITC-labeled mAb was detected using a fluorescent microscope.

Flow cytometry

For analysis by flow cytometry, lymphoid organs were removed and single cell suspensions was prepared by gently passing the organs through a 40 µm nylon mesh into SF-IMDM containing 2% FCS and 0.5% Ciproxin. In order to lyse erythrocytes, spleen cell suspensions were treated with ACK buffer for approximately 1 min. Stainings

were performed in a 96-well round bottom plate in a total volume of 100 µl containing 50 µl cell suspension (1-2x10^7 cells/ml) and 50 µl diluted antibody mix. Cells were incubated for 30 min at 4°C in the dark. Cells were washed with FACS buffer to remove unbound antibodies. When biotinylated antibodies were used, a second staining step (20 min, 4°C, in the dark) was performed for the binding of streptavidin-coupled fluorochromes. If applicable, cells were resuspended in FACS buffer containing 5 µg/ml propidium iodide (Sigma-Aldrich, USA). Intracellular stainings were performed according to standard procedure. In brief, subsequent to surface stainings, cells were fixed either with PBS containing 2% paraformaldehyde or with fix/perm buffer (eBioscience, USA) followed by intracellular stainings in FACS buffer containing 0.5% saponin (Sigma-Aldrich, USA) or in permeabilisation buffer (eBioscience, USA). Intracellular stainings were incubated for 30 min at 4°C in the dark followed by washing steps in FACS buffer to remove unbound antibodies. Flow cytometry was performed on a FACSCalibur or LSRFortessa machine (BD Bioscience, USA) and data was analyzed using FlowJo (Tree Star, USA) software.

PMA/ ionomycin stimulation

Single cell suspensions were stimulated during 4 hours using 1 µg/ml ionomycin (Sigma-Aldrich, USA) and 5 ng/ml phorbol-12-myristate-13-acetate (PMA) (Calbiochem, USA) in the presence of 10µg/ml brefeldin A (Sigma-Aldrich, USA). Cells were harvested and stained according to standard intracellular staining procedure (see above).

a) Media and Reagents

ACK buffer

PBS

0.15M NH_4Cl

1mM $KHCO_3$

0.1mM EDTA

0.22µm sterile filtered

SF-IMDM

176.6g IMDM powder (Gibco)

30.24g $NaHCO_3$ (Fluka)

100ml Penicillin/Streptomycin (Sigma-Aldrich)

100ml MEM non-essential amino acids (Sigma-Aldrich)

10ml 2-Mercaptoethanol (50mM) (Fluka)

30ml Primatone (10%) (Sigma-Aldrich)

Adjust to pH 7.0 with NaOH (Fluka)

Fill up to 10l with ddH_2O

0.22µm sterile filtered

FACS buffer

PBS

2% FCS

0.1% NaN_3

Antibodies

FITC- , phycoerythrin- (PE), allophycocyanin- (APC), Pacific Blue- (PB), Brilliant Violet- (BV), PE-Cy7-, PerCP-Cy5.5 or biotin-labeled mAb specific for CD4 (GK1.5), CD8α (53-6.7), CD8β (YTS-156.7.7), CD25 (PC 61), CD44 (IM7), H-2K^b (Y3), H-2K^d (19.191), TCRβc (H57-597), CD62L (Mel-14), CXCR5 (L138D7), PD-1 (RMP1-30), various Vβ-family members, Vα2 (B20.1), IFN-γ (XMG1.2) or FoxP3 (FJK-16s) were purchased from BD Bioscience, eBioscience or BioLegend or purified from hybridoma culture supernatant and labeled in our laboratory according to standard procedures. Antibodies were titrated and used at the lowest dilution that gave the best separation.

Results

1. Prophylactic administration of IL-2 complexes

These experiments were designed to investigate on the effects of IL-2 complexes (JES6/IL-2 or S4B6/IL-2) on murine SLE-like disease developing as a result of cGvHD. To study the *prophylactic* effect of IL-2 complexes we pretreated mice with IL-2 complexes before the disease was induced. The *therapeutic* effect of IL-2 complexes on SLE-like symptoms was studied by IL-2 complex treatment after 4 weeks of ongoing disease. A graphical overview of the administration protocols can be found in the supplementary material (**Fig. S1**).

To investigate the effect of *prophylactic* (p) administration of IL-2 complexes on the development of SLE-like symptoms in murine cGvHD, BDF1 were pretreated with IL-2 complexes (either S4B6/IL-2 or JES6/IL-2) on 3 consecutive days before disease induction. Each mouse was injected i.p. with the indicated IL-2 complexes composed of 2.5 µg rIL-2 and 7.5 µg anti-IL-2 mAb in 200 µl sterile PBS. cGvHD was induced by i.v. injection of $70x10^6$ DBA/2 lymphocytes from pooled preparations of splenocytes and LN cells. To assess the development of disease the presence of autoantibodies to autologous erythrocytes (anti-RBC) in the blood, the titers of anti-nuclear antibodies (ANA) in the serum and the incidence of proteinuria were measured over a period of 12 weeks. The following results represent pooled data of two independently conducted experiments for each prophylactic administration protocol. Groups contained in total 20 (pretreated) and 19 mice (untreated cGvHD) mice for the prophylactic treatment with JES6/IL-2 or 14 (pretreated) and 17 (untreated cGvHD) for the prophylactic treatment with S4B6/IL-2.

Prophylactic treatment with JES6/IL-2 complexes (pJES6/IL-2) was highly efficient in ameliorating the SLE-like symptoms of murine cGvHD in all measured parameters. As shown in **Fig. 1A**, prophylactic JES6/IL-2 treatment results in a reduced frequency of anti-RBC positive mice throughout the experiment. In the untreated cGvHD group the frequency of positive mice increased progressively, resulting in 58% (11/19) anti-RBC positive mice after 12 weeks. In contrast, the frequency of anti-RBC positive mice in the JES6/IL-2 prophylactically treated group was lower at all measured time points and only 20% (4/20) of JES6/IL-2 pretreated mice were positive for anti-RBC by 12 weeks.

Prophylactic JES6/IL-2 treatment showed a similar effect on the production of ANA (**Fig. 1B**). At all time points, serum IgG ANA titers (herein presented as dilution factor) were significantly lower in the pretreated group compared to the control group. After 4 weeks of cGvHD, the ANA titers of the prophylactically JES6/IL-2 treated group were on average 3.5-fold lower compared to the control group (mean: 404 (untreated cGvHD); 114 (pJES6/IL-2 treated)). Over time, ANA titers in the control group increased nearly 4-fold between weeks 4 and 12; however, ANA titers in the group prophylactically treated with JES6/IL-2 complexes remained constantly low (mean: 97.3 at 8 weeks and 126.3 at 12 weeks). By the end of the experiment, ANA titers in the JES6/IL-2 pretreated group were on average 10-fold lower compared to those of the controls (mean: 1412 (untreated cGvHD); 126.3 (pJES6/IL-2 treated)).

Mice prophylactically treated with JES6/IL-2 complexes showed a delayed onset and reduced incidence of proteinuria, which reflects the extent of immune complex induced glomerulonephritis (ICGN) (**Fig. 1C**). Whereas some mice in the control group already showed signs of proteinuria after 4 weeks of cGvHD induction, mice prophylactically treated with JES6/IL-2 showed the first signs of proteinuria only after 7 weeks of cGvHD.

At the end of the experiment, a significantly lower fraction of mice developed ICGN in the group pretreated with JES6/IL-2 complexes compared to the untreated control group (untreated cGvHD: 53% (10/19); pJES6/IL-2 treated: 20% (4/20)). The reduced kidney pathology was confirmed by immunohistological analysis of ICGN positive kidneys. Kidneys obtained from JES6/IL-2 pretreated mice (**Fig. 2B**) stained less intensively for the deposition of immune-complexes and complement when incubated with fluorescently labeled anti-IgG or anti-C3 antibodies.

In contrast, prophylactic treatment with the S4B6/IL-2 complexes (pS4B6/IL-2) induced stronger autoimmune symptoms and a more aggressive course of cGvHD. At all time points, a higher fraction of mice was positive for the production of anti-RBC antibodies in the group receiving the prophylactic S4B6/IL-2 treatment (**Fig. 1D**). After 4 weeks of cGvHD 64% (9/14) of mice, and after 12 weeks, 100% (14/14) of mice pretreated with S4B6/IL-2 were positive for anti-RBC antibodies. In the control group the frequency of positive mice progressively increased from 12% (2/17) at 4 weeks to 82% (14/17) by 12 weeks of cGvHD. In mice pretreated with S4B6/IL-2 complexes, enhanced ANA production was observed (**Fig. 1E**). Four weeks after disease induction serum ANA titers were increased 4.5-fold compared to control mice (mean: 1246 (pS4B6/IL-2 treated); 273 (untreated cGvHD). By 8 weeks of cGvHD, mean ANA titers in the control group increased to levels above those of the S4B6/IL-2 pretreated group, which had somewhat declined since the previous time point. However, there was no significant statistical difference in ANA titers between the two groups 8 weeks after disease induction. ANA titers measured after 12 weeks of ongoing cGvHD showed that mice in the control group still had high ANA titers in the serum (mean: 1251) whereas unexpectedly mice pretreated with S4B6/IL-2 complexes showed 10-fold decreased mean ANA titers

(mean: 121). The decrease in ANA titers observed at later time points likely results from the prolonged time of proteinuria in these mice. It is known that mice with proteinuria secrete not only large amounts of albumin by the urine but also immunoglobulins thus leading to decreased ANA titers in the serum.

In the group of mice pretreated with S4B6/IL-2 complexes, an accelerated development and full penetrance of ICGN was observed (**Fig. 1F**). Already after 9 weeks of cGvHD 100% mice in the S4B6/IL-2 pretreated group (14/14) developed ICGN, whereas in the control group less than 60% (10/17) of mice exhibited proteinuria by 12 weeks. The accelerated kinetics and higher incidence by which ICGN develops upon prophylactic S4B6/IL-2 treatment reflects the more aggressive course of disease induced by this treatment. Immunohistological analysis of ICGN kidneys from S4B6/IL-2 pretreated mice (**Fig. 2C**) showed a more intensive staining of IgG deposition compared to ICGN positive kidneys of untreated cGvHD mice (**Fig. 2A**). Moreover, staining for complement-cascade member C3 revealed a more distinct granular and sprinkled pattern in kidneys of S4B6/IL-2 pretreated mice compared to the staining pattern observed in kidneys from untreated cGvHD mice. This pattern of C3 staining was similar to the staining pattern for IgG deposition.

In summary, prophylactic treatment with JES6/IL-2 complexes leads to an amelioration of SLE-like symptoms whereas the prophylactic treatment with S4B6/IL-2 has an opposing effect and aggravates the course of cGvHD.

Figure 1

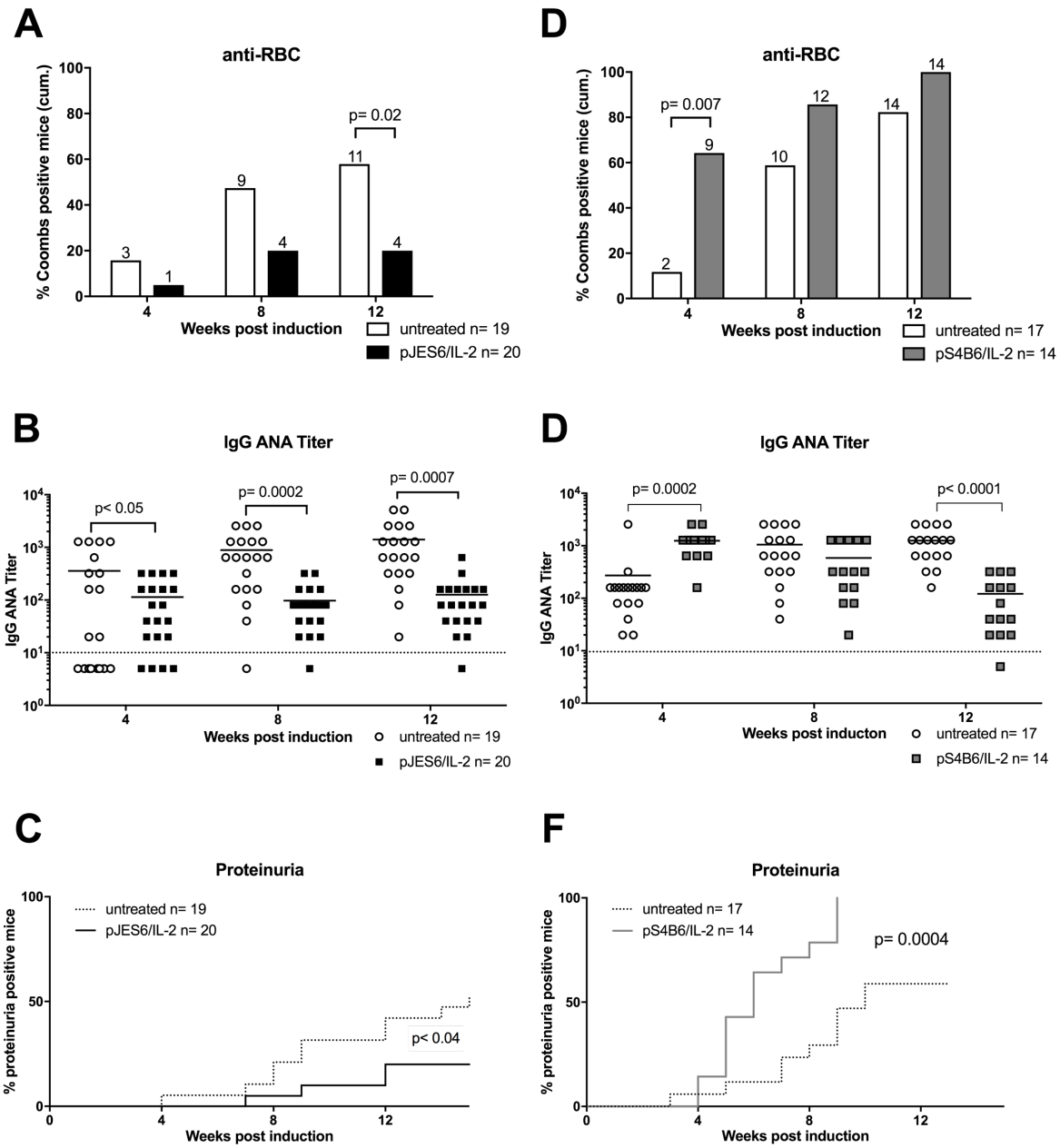

A
anti-RBC
% Coombs positive mice (cum.)
p= 0.02
3
1
9
4
11
4
Weeks post induction
untreated n= 19
pJES6/IL-2 n= 20
D
anti-RBC
% Coombs positive mice (cum.)
p= 0.007
2
9
10
12
14
14
Weeks post induction
untreated n= 17
pS4B6/IL-2 n= 14
B
IgG ANA Titer
p< 0.05
p= 0.0002
p= 0.0007
IgG ANA Titer
Weeks post induction
untreated n= 19
pJES6/IL-2 n= 20
D
IgG ANA Titer
p= 0.0002
p< 0.0001
IgG ANA Titer
Weeks post inducton
untreated n= 17
pS4B6/IL-2 n= 14
C
Proteinuria
untreated n= 19
pJES6/IL-2 n= 20
% proteinuria positive mice
p< 0.04
Weeks post induction
F
Proteinuria
untreated n= 17
pS4B6/IL-2 n= 14
p= 0.0004
% proteinuria positive mice
Weeks post induction

Figure 1: *Effect of prophylactic treatment with IL-2 complexes on autoimmune symptoms of mice undergoing cGvHD*

(A – C) The beneficial effect of prophylactic JES6/IL-2 treatment on BDF1 mice undergoing cGvHD (pJES6/IL-2: n= 20; untreated cGvHD: n= 19). **(D – F)** The adverse effect of prophylactic S4B6/IL-2 treatment on BDF1 mice undergoing cGvHD (pS4B6/IL-2: n= 14; untreated cGvHD: n= 17). **(A, D)** Cumulative frequencies of mice positive for anti-RBC autoantibodies determined at 4, 8 and 12 weeks of cGvHD. Numbers above the bars indicate positive mice. Open bars: untreated cGvHD; Filled bars: cGvHD prophylactically treated with JES6/IL-2 (black) or S4B6/IL-2 (grey) complexes. Statistical significance ($p< 0.05$) was calculated using a two-tailed Fisher's exact test and is indicated by the p-value. **(B, E)** IgG ANA titer in the serum of cGvHD mice determined 4, 8 and 12 weeks after disease induction. Horizontal bars indicate mean ANA titers in each group. Titers below the dotted line represent mice negative for IgG ANA. Deviations from initially used numbers of mice are indicated at the respective time point. Statistical significance ($p< 0.05$) was calculated using an unpaired student's t-test and is indicated by the p-value. Open circles: untreated cGvHD; filled squares: cGvHD prophylactically treated with JES6/IL-2 (black) or S4B6/IL-2 (grey) complexes **(C, F)** Frequencies of mice positive for proteinuria as determined by elevated albumin in the urine. Statistical significance ($p< 0.05$) was calculated using the Mantel-Cox test and is indicated by the p-value. Dotted line: untreated cGvHD; solid line: cGvHD prophylactically treated with JES6/IL-2 (black) or S4B6/IL-2 (grey) complexes.

Figure 2

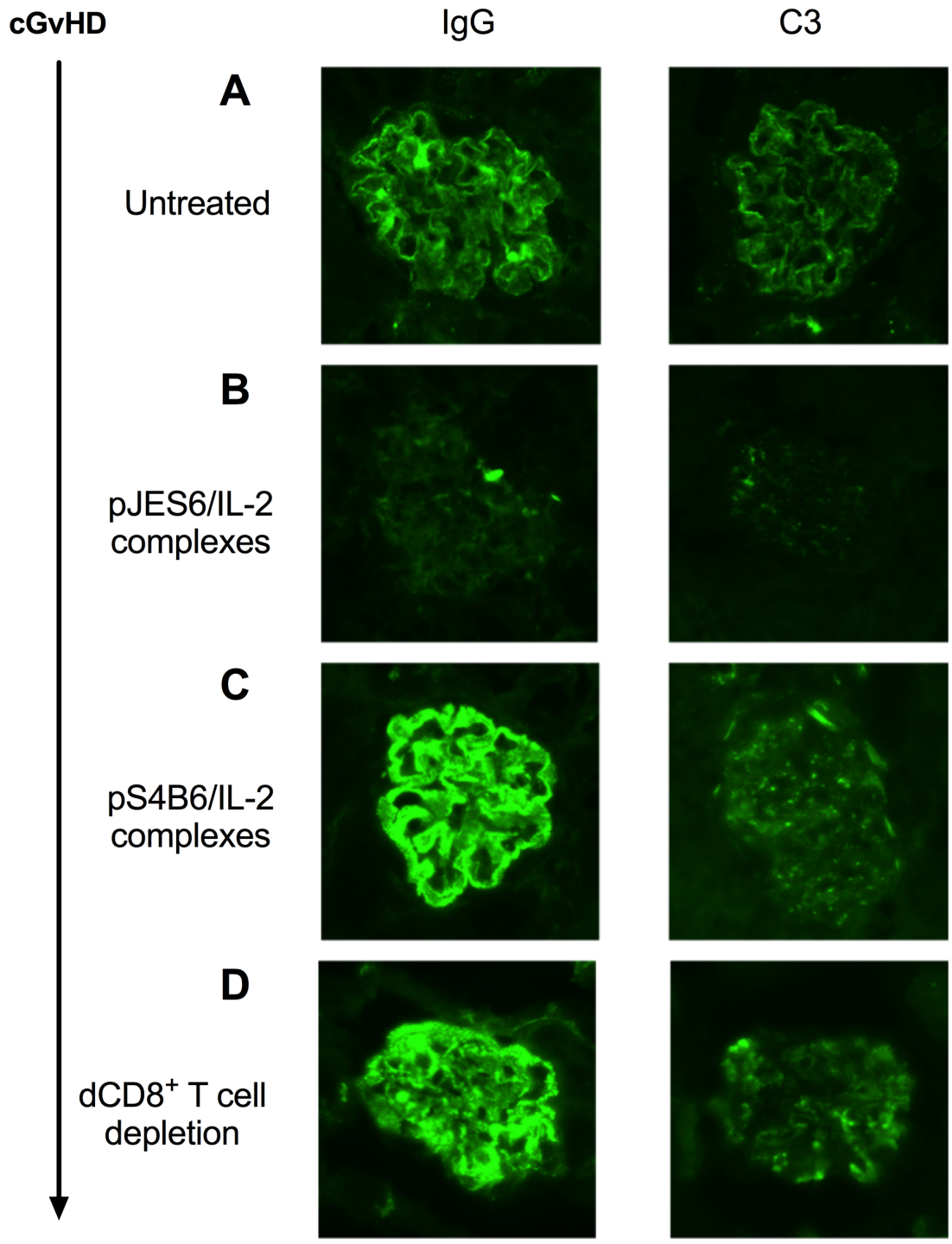

cGvHD
IgG
C3
A
Untreated
B
pJES6/IL-2 complexes
C
pS4B6/IL-2 complexes
D
dCD8+ T cell depletion

Figure 2: *Immunohistological staining of IgG and C3 deposition in glomeruli of proteinuria positive cGvHD mice*

Kidneys were obtained from mice positive for proteinuria for <1 week. Cryo sections were prepared and stained for IgG deposition (left panel) or C3 deposition (right panel). Stained sections were analyzed by using a Zeiss Axioscope mounted with a Nikon digital camera DXM 1200F in combination with imaging software (Nikon ACT-1). Photos show representative staining of kidneys from different experiments. **(A)** Untreated cGvHD in mice induced with DBA/2 lymphocytes. **(B)** cGvHD in mice as in (A) but prophylactically treated with JES6/IL-2. **(C)** cGvHD in mice as in (A) but prophylactically treated with S4B6/IL-2. **(D)** Untreated cGvHD mice whose disease was induced with DBA/2 lymphocytes depleted of $CD8^+$ T cells.

2. Cellular mechanisms underlying the opposing effects of prophylactic treatment with IL-2 complexes

To gain further insight into the cellular responses induced by the prophylactic administration of IL-2 complexes, multicolor flow cytometry was performed on splenocytes of mice prophylactically treated with JES6/IL-2 or S4B6/IL-2 complexes, two weeks following induction of cGvHD. For this experiment we used 4 mice in the control group and 5 mice in each of the groups pretreated with IL-2 complexes. All groups were induced and analyzed in parallel.

Mice pretreated with JES6/IL-2 complexes had a total (donor + host) splenic cellularity comparable to untreated cGvHD mice (**Fig. 3A**), however the absolute numbers of donor $CD4^+$ T cells recovered from the spleens of JES6/IL-2 complex treated mice were 8.8-fold reduced compared to those of the controls (11.8 ± 2.8 $x10^6$ (untreated cGvHD); 1.34 ± 0.82 $x10^6$ (pJES6/IL-2 treated) (**Fig. 3B**). Additionally, there were 13.4-fold fewer donor $CD4^+$ T cells in the spleens of pJES6/IL-2 treated mice displaying an activated ($CD44^{hi}$ / $CD62L^{lo}$) phenotype (9.43 ± 2.89 $x10^6$ (untreated cGvHD); 7.77 ± 0.44 $x10^5$ (pJES6/IL-2 treated) (**Fig. 3C**). Furthermore, the population of donor $CD4^+$ T cells with central memory (Tcm; $CD44^{hi}$ / $CD62L^{hi}$) phenotype was also significantly ($p= 0.0076$) reduced in the group prophylactically treated with JES6/IL-2 complexes (1.27 ± 0.47 $x10^6$ (untreated cGvHD); 0.36 ± 0.27 $x10^6$ (pJES6/IL-2 treated)) (**Fig. 3D**). There was no significant difference in the absolute numbers of donor Tfh cells ($CD4^+$ / $PD1^+$ / $CXCR5^+$) in pJES6/IL-2 treated compared to untreated cGvHD mice (**Fig. 3E**). In concordance with previous reports on JES6/IL-2 complexes, the host Tregs ($CD4^+$ / $FoxP3^+$) were

significantly ($p < 0.0001$) expanded 21.5-fold in mice pretreated with JES6/IL-2 (1.88 ± 0.93 $x10^5$ (untreated cGvHD); 3.87 ± 0.46 $x10^6$ (pJES6/IL-2 treated)) (**Fig. 3F**). The reduced engraftment of donor cells as well as their decreased activation status might be a consequence of the expanded host Treg compartment (20-fold increased compared to control group).

Mice prophylactically treated with the S4B6/IL-2 complexes showed significantly ($p=0.0016$) increased total cellularity of the spleens compared to untreated cGvHD mice (**Fig. 4A**). Spleens of mice prophylactically treated with S4B6/IL-2 complexes had a total cellularity of 1.77 ± 0.26 $x10^8$ cells whereas spleens of control mice had on average a total cellularity of 1.07 ± 0.12 $x10^8$ cells. Although the numbers of activated donor $CD4^+$ T cells in prophylactically S4B6/IL-2 treated mice (6.17 ± 1.20 $x10^6$) were reduced compared to untreated cGvHD animals (9.43 ± 2.89 $x10^6$), this difference was not statistically significant (**Fig. 4B**). In contrast, donor $CD4^+$ Tcm cells were expanded in mice treated prophylactically with S4B6/IL-2 complexes (1.27 ± 0.47 $x10^6$ (untreated cGvHD); 2.51± 0.42 $x10^6$ (pS4B6/IL-2 treated) (**Fig. 4D**). Moreover, in these mice, the number of donor Tfh cells was increased more than 5-fold (0.25 ± 0.12 $x10^6$ (untreated cGvHD); 1.4 ± 0.32 $x10^6$ (pS4B6/IL-2 treated) (**Fig 4E**). Unexpectedly, the magnitude of host Treg expansion in response to prophylactic treatment with S4B6/IL-2 complexes was 2-fold greater compared to the expansion induced by prophylactic JES6/IL-2 treatment (3.87 ±0.46 $x10^6$ (pJES6/IL-2 treated); 8.11 ± 1.54 $x10^6$ (pS4B6/IL-2 treated) (**Fig. 4F**). Despite the expanded host Treg numbers in mice prophylactically treated with S4B6/IL-2 complexes, no amelioration of autoimmune symptoms was observed in these mice. The increased numbers of donor $CD4^+$ Tcm and Tfh cells observed in mice

prophylactically treated with S4B6/IL-2 complexes might account for the more severe course of cGvHD in these animals.

Figure 3

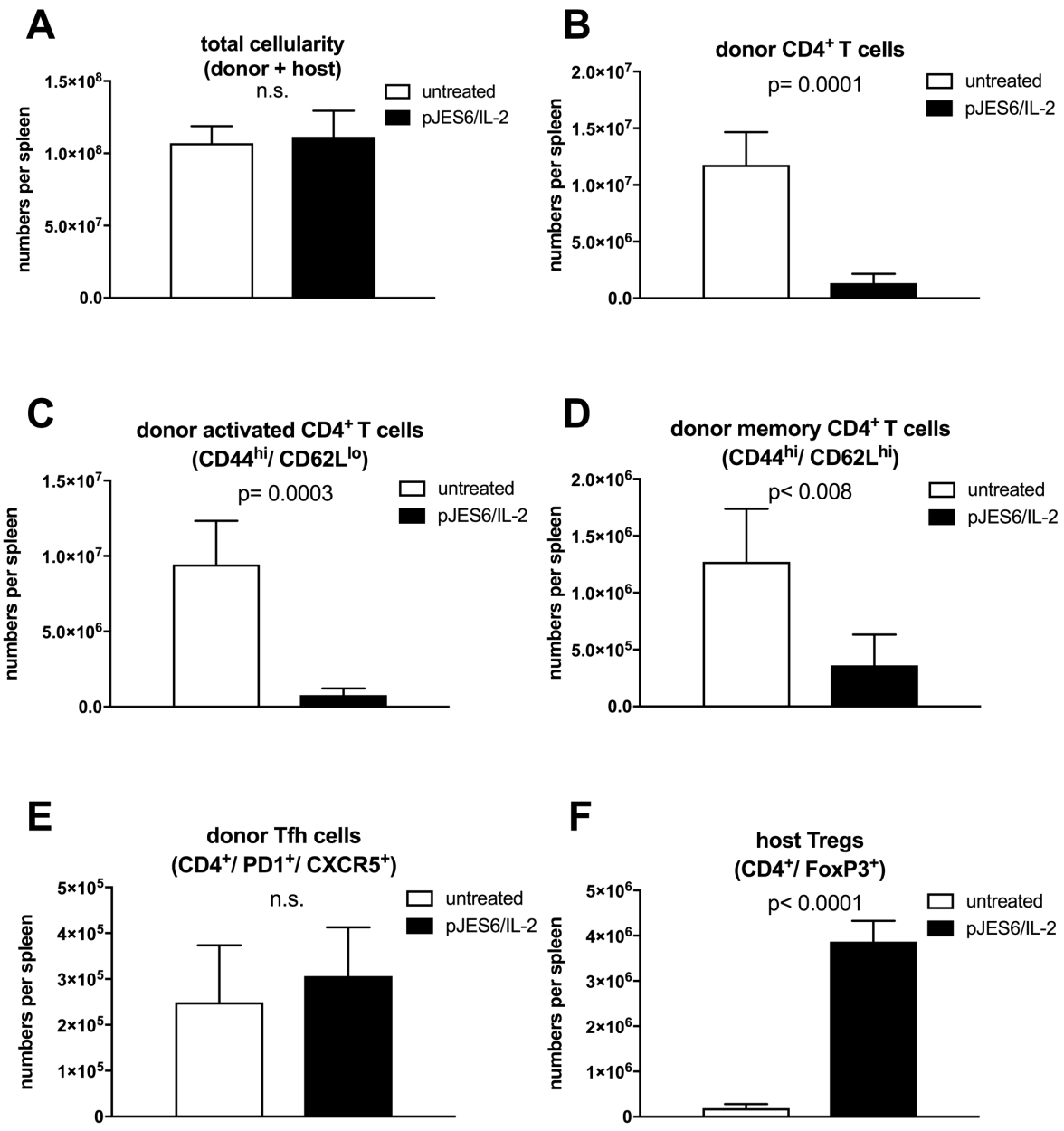

A
total cellularity
(donor + host)
n.s.
untreated
pJES6/IL-2
numbers per spleen
1.5×10^8
1.0×10^8
5.0×10^7
0.0
B
donor CD4+ T cells
p= 0.0001
untreated
pJES6/IL-2
numbers per spleen
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
C
donor activated CD4+ T cells
(CD44hi/ CD62Llo)
p= 0.0003
untreated
pJES6/IL-2
numbers per spleen
1.5×10^7
1.0×10^7
5.0×10^6
0.0
D
donor memory CD4+ T cells
(CD44hi/ CD62Lhi)
p< 0.008
untreated
pJES6/IL-2
numbers per spleen
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
E
donor Tfh cells
(CD4+/ PD1+/ CXCR5+)
n.s.
untreated
pJES6/IL-2
numbers per spleen
5×10^5
4×10^5
3×10^5
2×10^5
1×10^5
0
F
host Tregs
(CD4+/ FoxP3+)
p< 0.0001
untreated
pJES6/IL-2
numbers per spleen
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0

Figure 3: *T cell populations in splenocytes from cGvHD mice prophylactically treated with JES6/IL-2 complexes*

cGvHD mice were analyzed in parallel two weeks after disease induction. Mean number of total cellularity **(A)** as well as different donor **(B – E)** and host **(F)** T cell subsets per spleen are shown. **(A)** Mean number of total splenocytes (both donor and host origin). **(B)** Mean number of engrafted donor $CD4^+$ T cells. **(C)** Mean number of activated donor $CD4^+$ T cells expressing $CD44^{hi}$. **(D)** Mean number of donor $CD4^+$ T cells with central memory phenotype co-expressing $CD44^{hi}$ and $CD62L^{hi}$. **(E)** Mean number of donor $CD4^+$ T cells with follicular helper T cell (Tfh) phenotype co-expressing CXCR5 and PD1. **(F)** Mean number of host Tregs expressing CD4 and FoxP3. White bars: untreated cGvHD (n= 4); Black bars: cGvHD prophylactically treated with JES6/IL-2 (n= 5). Statistical significance ($p< 0.05$) was calculated using an unpaired student's t-test and is indicated by the p-value. Data are represented as mean values ± SD.

Figure 4

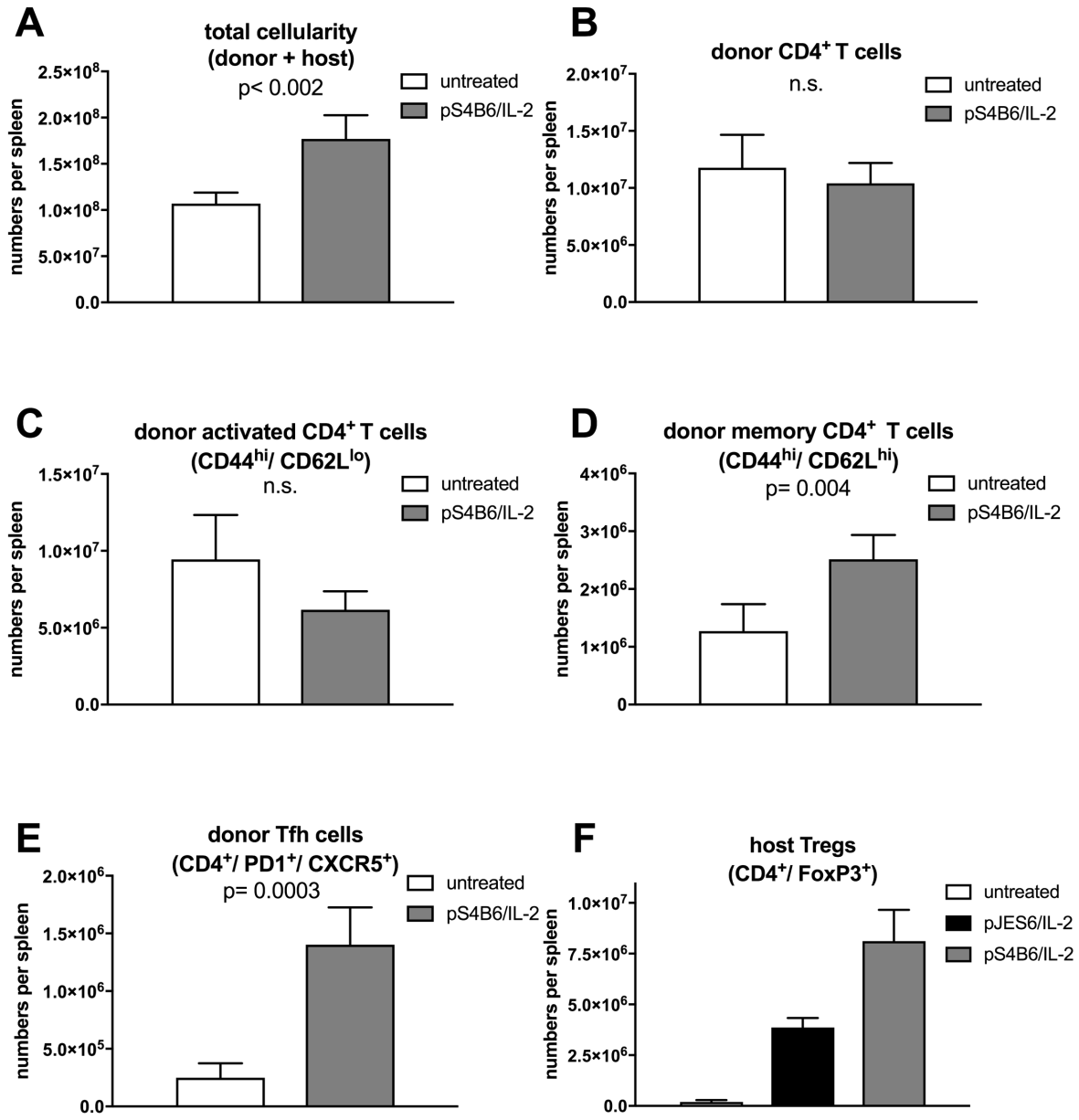

A
total cellularity
(donor + host)
p< 0.002
numbers per spleen
2.5×10^8
2.0×10^8
1.5×10^8
1.0×10^8
5.0×10^7
0.0
untreated
pS4B6/IL-2
B
donor CD4+ T cells
n.s.
numbers per spleen
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
untreated
pS4B6/IL-2
C
donor activated CD4+ T cells
(CD44hi/ CD62Llo)
n.s.
numbers per spleen
1.5×10^7
1.0×10^7
5.0×10^6
0.0
untreated
pS4B6/IL-2
D
donor memory CD4+ T cells
(CD44hi/ CD62Lhi)
p= 0.004
numbers per spleen
4×10^6
3×10^6
2×10^6
1×10^6
0
untreated
pS4B6/IL-2
E
donor Tfh cells
(CD4+/ PD1+/ CXCR5+)
p= 0.0003
numbers per spleen
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0.0
untreated
pS4B6/IL-2
F
host Tregs
(CD4+/ FoxP3+)
numbers per spleen
1.0×10^7
7.5×10^6
5.0×10^6
2.5×10^6
0.0
untreated
pJES6/IL-2
pS4B6/IL-2

Figure 4: *T cell populations in splenocytes from cGvHD mice prophylactically treated with S4B6/IL-2 complexes*

Mean number of total cellularity **(A)** as well as different donor **(B – E)** and host **(F)** T cell subsets per spleen are shown. **(A)** Mean number of total splenocytes (donor and host origin). **(B)** Mean number of engrafted donor $CD4^+$ T cells. **(C)** Mean number of activated donor $CD4^+$ T cells expressing $CD44^{hi}$. **(D)** Mean number of donor $CD4^+$ T cells with central memory phenotype co-expressing $CD44^{hi}$ and $CD62L^{hi}$. **(E)** Mean number of donor $CD4^+$ T cells with follicular helper T cell (Tfh) phenotype co-expressing CXCR5 and PD1. **(F)** Mean number of host Tregs expressing CD4 and FoxP3. White bars: untreated cGvHD (n= 4); grey bars: cGvHD prophylactically treated with S4B6/IL-2 (n= 5); black bars: cGvHD mice prophylactically treated with JES6/IL-2 (n= 5). Statistical significance ($p< 0.05$) was calculated using an unpaired student's t-test and is indicated by the p-value. Data are represented as mean values ± SD.

3. Therapeutic administration of IL-2 complexes

We also investigated the effect of IL-2 complexes on SLE-like symptoms during ongoing cGvHD disease. To do so, we administrated the same IL-2 complexes (either JES6/IL-2 or S4B6/IL-2) *therapeutically* (t) on 3 consecutive days starting 4 weeks after the induction of cGvHD. The following results represent pooled data of 2 independently conducted experiments for each therapeutic administration protocol. Groups contained in total 20 (therapy) and 20 (untreated cGvHD) mice for therapeutic treatment with JES6/IL-2 or 20 (therapy) and 17 (untreated cGvHD) mice for the therapeutic treatment with S4B6/IL-2.

Therapeutic treatment with JES6/IL-2 complexes had no significant effect on the extent of the measured autoimmune symptoms. There was a slight reduction of the frequency of anti-RBC autoantibody positive mice indicating a mild effect of therapeutic JES6/IL-2 treatment (**Fig. 5A**). The frequency of anti-RBC positive mice was lower in the treated group throughout the experiment. After 12 weeks, 45% (9/20) of mice therapeutically treated with JES6/IL-2 and 60% (12/20) of the control mice were positive for anti-RBC autoantibodies. However, ANA titers were not significantly different between the two groups at any time point (week 4 - 12: 740.6 ± 174.9 (untreated GvHD); 596.6 ± 136.4 (tJES6/IL-2 treated)) (**Fig. 5B**). The kinetics of ICGN development appeared to be a slightly accelerated in the JES6/IL-2 therapy group between week 6 and 8 but by the end of the experiment the frequencies of mice positive for proteinuria were comparable in both groups (untreated cGvHD: 55% (11/20); tJES6/IL-2 treated: 60% (12/20)) (**Fig. 5C**).

Upon therapeutic treatment with S4B6/IL-2 complexes, all autoimmune parameters were reduced compared to untreated cGvHD mice. As shown in **Fig. 5D**, the frequencies of anti-RBC positive mice treated therapeutically with S4B6/IL-2 complexes remained low throughout the observation time and only 22% (4/18) of these mice had anti-RBC autoantibodies by 12 weeks following the cGvHD induction. In the untreated cGvHD group, the fraction of mice positive for anti-RBC autoantibodies progressively increase from 10% (2/20) by 4 weeks to 70% (14/20) after 12 weeks of cGvHD.

As shown in **Fig. 5E**, therapeutic treatment with S4B6/IL-2 complexes resulted in significantly lower IgG ANA titers by 8 weeks (mean titer: 853 (untreated cGvHD); 286.7 (tS4B6/IL-2 treated); p= 0.019) and 12 weeks (mean titer: 1192 (untreated cGvHD); 530 (tS4B6/IL-2 treated); p= 0.025). The decreased production of autoantibodies and the generally suppressed autoimmunity was also reflected by the significantly (p= 0.019) reduced incidence of ICGN as shown in **Fig. 5F**. In contrast to the untreated cGvHD group, where 70% (14/20) of mice had proteinuria, less than 30% (5/17) of the mice therapeutically treated with S4B6/IL-2 complexes were proteinuria positive by the end of the experiment.

Taken together, therapeutic administration of JES6/IL-2 complexes had no significant effect on the development of murine lupus although a mild reduction in the frequency of anti-RBC autoantibody positive mice was observed. On the other hand, therapeutic administration of S4B6/IL-2 complexes induced a significant amelioration in all measured autoimmune parameters.

Figure 5

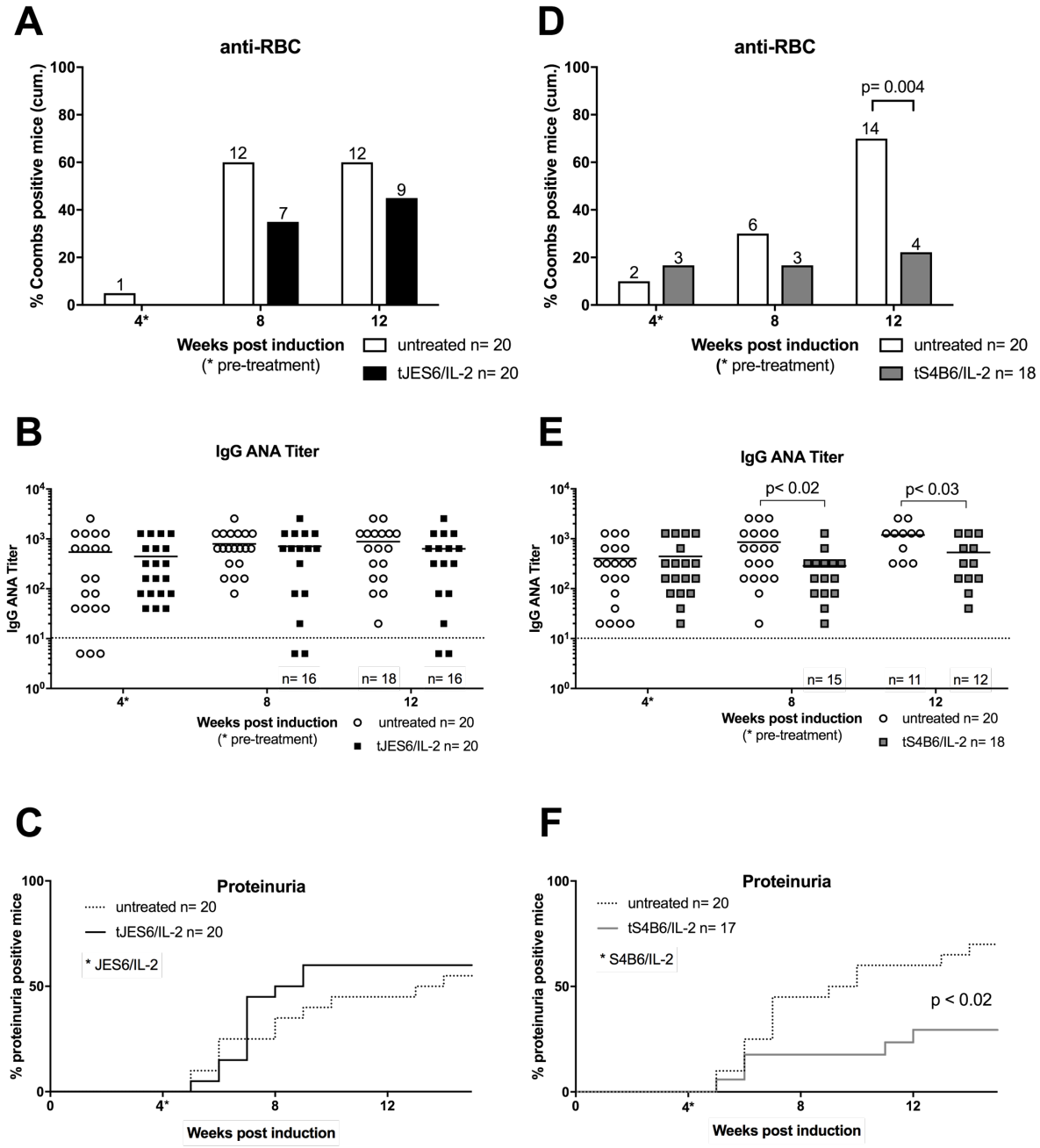

A
anti-RBC
% Coombs positive mice (cum.)
100
80
60
40
20
0
1
12
7
12
9
4*
8
12
Weeks post induction
(* pre-treatment)
untreated n= 20
tJES6/IL-2 n= 20
D
anti-RBC
% Coombs positive mice (cum.)
p= 0.004
2
3
6
3
14
4
4*
8
12
Weeks post induction
(* pre-treatment)
untreated n= 20
tS4B6/IL-2 n= 18
B
IgG ANA Titer
IgG ANA Titer
10^4
10^3
10^2
10^1
10^0
n= 16
n= 18
n= 16
4*
8
12
Weeks post induction
(* pre-treatment)
untreated n= 20
tJES6/IL-2 n= 20
E
IgG ANA Titer
IgG ANA Titer
p< 0.02
p< 0.03
n= 15
n= 11
n= 12
4*
8
12
Weeks post induction
(* pre-treatment)
untreated n= 20
tS4B6/IL-2 n= 18
C
Proteinuria
% proteinuria positive mice
100
50
0
untreated n= 20
tJES6/IL-2 n= 20
* JES6/IL-2
0
4*
8
12
Weeks post induction
F
Proteinuria
% proteinuria positive mice
100
50
0
untreated n= 20
tS4B6/IL-2 n= 17
* S4B6/IL-2
p < 0.02
0
4*
8
12
Weeks post induction

Figure 5: *Effect of therapeutic treatment with IL-2 complexes on autoimmune symptoms of mice undergoing cGvHD*

(A – C) The efficiency of therapeutic JES6/IL-2 treatment of cGvHD is confined to the production of anti-RBC (tJES6/IL-2: n= 20; untreated cGvHD: n= 19). **(D – F)** The beneficial effects of therapeutic S4B6/IL-2 treatment of cGvHD (tS4B6/IL-2: n= 18; untreated cGvHD: n= 20). **(A, D)** Cumulative frequencies of mice positive for anti-RBC autoantibodies determined at 4, 8 and 12 weeks of cGvHD. Numbers above the bars indicate positive mice. White bars: untreated cGvHD; filled bars: cGvHD therapeutically treated with JES6/IL-2 (black) or S4B6/IL-2 (grey) complexes. Statistical significance ($p< 0.05$) was calculated using a two-tailed Fisher's exact test and is indicated by the p-value. **(B, E)** IgG ANA titer in the serum of cGvHD mice determined 4, 8 and 12 weeks after disease induction. Horizontal bars indicate mean ANA titers in each group. Titers below the dotted line represent mice negative for IgG ANA. Deviations from initially used numbers of mice are indicated at the respective time point. Statistical significance ($p< 0.05$) was calculated using an unpaired student's t-test and is indicated by the p-value. Open circles: untreated cGvHD; filled squares: cGvHD therapeutically treated with JES6/IL-2 (black) or S4B6/IL-2 (grey) complexes. **(C, F)** Frequencies of mice positive for proteinuria as determined by elevated albumin in the urine. Statistical significance ($p< 0.05$) was calculated using the Mantel-Cox test and is indicated by the p-value. Dotted line: untreated cGvHD; solid line: cGvHD prophylactically treated with JES6/IL-2 (black) or S4B6/IL-2 (grey) complexes.

4. The contribution of donor CD8+ T cells to the pathogenesis of murine cGvHD

CD8+ T cells are suggested to play an important role in the development of SLE and murine cGvHD [142]. The potent capacity of S4B6/IL-2 complexes to expand CD8+ T cells and the observation of a beneficial clinical effect of therapeutic S4B6/IL-2 treatment prompted us to further examine the involvement of the CD8+ T cell compartment in mice undergoing cGvHD.

Analysis of IFN-γ production showed that CD8+ T cells from both, donor and host were highly activated irrespective of the time point of the analysis after cGvHD induction (**Fig 6A**). On average more than 80% (82.7 ± 11.5%) of donor CD8+ T cells produced IFN-γ after *in vitro* stimulation with PMA/ionomycin, whereas the frequency of IFN-γ producing cells in the host population was around 40% (41.2 ± 9.6%). These results further support a contribution of CD8+ T cells in the course of cGvHD. To demonstrate a mechanistic involvement of CD8+ T cells and to test the particular contribution of donor and host CD8+ T cells in the pathogenesis of cGvHD we monitored the development of SLE-like symptoms in the absence of donor CD8+ T cells. For this, CD8+ T cells were depleted from donor DBA/2 mice by i.v. injection of 200 µg YTS-156 (a rat anti-mouse CD8β mAb) four days in advance of harvesting DBA/2 (donor) lymphocytes for cGvHD induction. The high efficiency of *in vivo* CD8+ T cell depletion (>99%) of donor DBA/2 mice was confirmed by flow cytometry analysis of the lymphocyte preparations used to induce the disease (data not shown).

The results shown in **Fig. 6B - D** represent pooled data from 2 independently conducted experiments. Twenty mice per group were used to induce the disease as previously

described with either $CD8^+$ T cell depleted (dCD8-) or containing (dCD8+) DBA/2 lymphocytes. The data demonstrate that donor $CD8^+$ T cells have substantial effects on the disease symptoms and that a much more severe and accelerated disease develops in the absence of this population. 90% (18/20) of the BDF1 mice induced with $CD8^+$ T cell depleted donor lymphocytes (dCD8- group) developed anti-RBC antibodies already by 3 weeks of cGvHD whereas in the control cGvHD group (dCD8+ group), 70% (14/20) of mice developed anti-RBC antibodies but only by 12 weeks after disease induction (**Fig. 6B**).

ANA titers in the two groups were not significantly different during the first 6 weeks of cGvHD (week 3 – 6: 144.2 ± 96.38 (dCD8+); 137.6 ± 22.98 (dCD8-)). Between week 6 and 9, ANA titers in mice induced with DBA/2 cells depleted of $CD8^+$ T cells decreased whereas the titers of the control group (DBA/2 cells containing $CD8^+$ T cells) increased progressively (**Fig. 6C**). After 9 weeks of cGvHD, ANA titers of the control group were more than 10-fold higher compared to the group induced with donor cells depleted of $CD8^+$ T cells. The decrease in ANA titers among cGvHD mice induced with DBA/2 cells lacking $CD8^+$ T cells might be the consequence of the prolonged time and high incidence of proteinuria and the significant amount of immunoglobulin secreted into the urine.

As shown in **Fig. 6D**, mice in the donor $CD8^+$ T cell depleted group (dCD8-) showed accelerated development of ICGN with full penetrance after 9 weeks. The development of proteinuria in the control group (CD8+) was limited with only 20% (4/20) of mice exhibiting proteinuria by the end of the experiment. It should be pointed out that control groups of other experiments (induced with DBA/2 lymphocytes containing $CD8^+$ T cells) had on average 30 - 40% proteinuria positive mice after 9 weeks. Nevertheless, depleting $CD8^+$ T cells from the donor population accelerates proteinuria. The kinetics

by which ICGN (proteinuria) develops in cGvHD induced in absence of donor $CD8^+$ T cell resembles those observed in cGvHD mice prophylactically treated with S4B6/IL-2 complexes. Consistent with this observation, the analysis of kidneys of ICGN positive mice from the dCD8- group (**Fig. 2D**) showed enhanced deposition of IgG antibodies and complement-component C3 compared to controls.

Taken together, donor $CD8^+$ T cells are important modulators of cGvHD with the capacity to slow the development of SLE-like symptoms of cGvHD.

5. The beneficial effect of therapeutic S4B6/IL-2 treatment depends on donor CD8$^+$ T cells

The recognition of the role of donor CD8$^+$ T cells in limiting the severity of cGvHD symptoms leads to the question whether the observed effect of therapeutic S4B6/IL-2 administration depends on the presence of donor CD8$^+$ T cells. To test this, cGvHD was induced by transfer of donor CD8$^+$ T cell depleted DBA/2 lymphocytes into parental BDF1 mice. After 4 weeks of ongoing disease, one group of mice was treated with S4B6/IL-2 complexes on three consecutive days. The experiment was repeated twice with a total of 19 mice treated with S4B6/IL-2 complexes and 19 control mice left untreated.

The therapeutic effect of S4B6/IL-2 complexes on the development of proteinuria clearly depends on the presence of donor CD8$^+$ T cells (compare **Fig. 6D** and **Fig. 6G**). However, the depletion of donor CD8$^+$ T cells in this cGvHD model did not markedly affect production of autoantibodies. As shown in **Fig. 6E**, the frequencies of anti-RBC positive mice were comparably high in both groups before and after the treatment. Moreover, ANA titers did not change significantly over time in both groups before and after the treatment (**Fig. 6F**).

Proteinuria measurements might indicate a mild effect of therapeutic S4B6/IL-2 treatment on the kinetics of ICGN development but the difference was not statistically significant. After 9 weeks 79% (15/19) of S4B6/IL-2 treated mice and all (19/19) control mice were positive for proteinuria (**Fig. 6G**).

These results provide strong evidence that donor $CD8^+$ T cells are an important population mediating the therapeutic effect of S4B6/IL-2 complexes on the development of SLE-like symptoms in cGvHD mice.

Figure 6

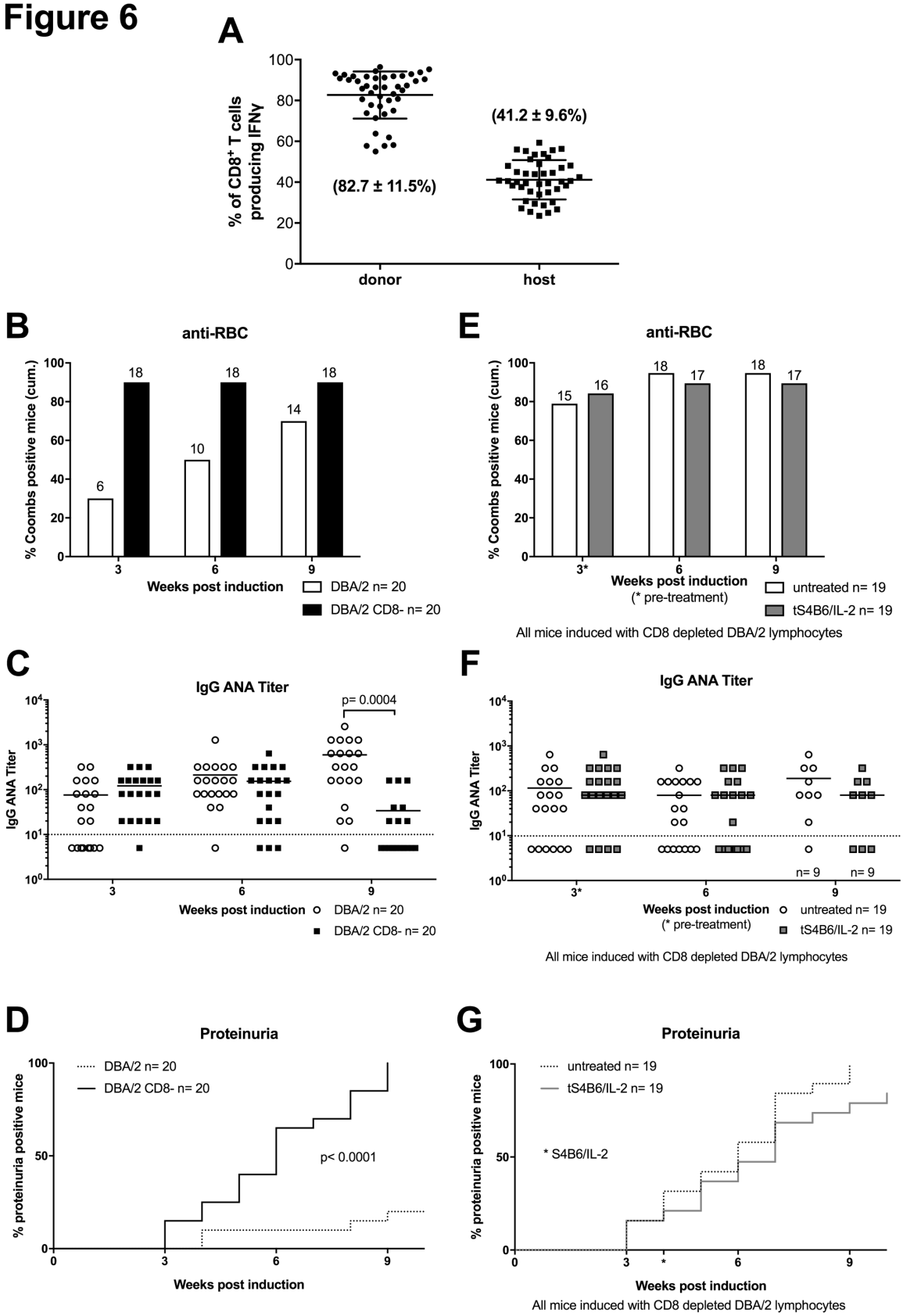

A
% of CD8+ T cells producing IFNγ
(82.7 ± 11.5%)
(41.2 ± 9.6%)
donor
host
B
anti-RBC
% Coombs positive mice (cum.)
Weeks post induction
DBA/2 n= 20
DBA/2 CD8- n= 20
E
anti-RBC
% Coombs positive mice (cum.)
Weeks post induction
(* pre-treatment)
untreated n= 19
tS4B6/IL-2 n= 19
All mice induced with CD8 depleted DBA/2 lymphocytes
C
IgG ANA Titer
p= 0.0004
Weeks post induction
DBA/2 n= 20
DBA/2 CD8- n= 20
F
IgG ANA Titer
n= 9
Weeks post induction
(* pre-treatment)
untreated n= 19
tS4B6/IL-2 n= 19
All mice induced with CD8 depleted DBA/2 lymphocytes
D
Proteinuria
DBA/2 n= 20
DBA/2 CD8- n= 20
p< 0.0001
% proteinuria positive mice
Weeks post induction
G
Proteinuria
untreated n= 19
tS4B6/IL-2 n= 19
* S4B6/IL-2
% proteinuria positive mice
Weeks post induction
All mice induced with CD8 depleted DBA/2 lymphocytes

Figure 6: *Donor CD8⁺ T cells are important modulators of cGvHD and mediate the beneficial effect of therapeutic S4B6/IL-2 therapy*

(A) Frequencies of IFN-γ producing CD8⁺ T cells of donor (circles) and host (squares) origin in splenocytes of untreated cGvHD mice analyzed at various time points between 2 and 12 weeks after disease induction. **(B - D)** A more severe cGvHD develops when induced with DBA/2 lymphocytes depleted of CD8⁺ T cells (DBA/2 CD8-) (DBA/2: n= 20; DBA/2 CD8-: n= 20). **(E – G)** No effect on cGvHD severity with S4B6/IL-2 therapy in the absence of donor CD8⁺ T cells (untreated cGvHD: n= 19; tS4B6/IL-2: n= 19). **(B, E)** Cumulative frequencies of mice positive for anti-RBC autoantibodies determined at 3, 6 and 9 weeks of cGvHD. Numbers above the bars indicate positive mice. Statistical significance ($p < 0.05$) was calculated using a two-tailed Fisher's exact test and is indicated by the p-value. **(B)** White bars: cGvHD mice induced with DBA/2 lymphocytes; Black bars: cGvHD induced with DBA/2 lymphocytes depleted of CD8⁺ T cells. **(e)** White bars: cGvHD induced with DBA/2 lymphocytes depleted of CD8⁺ T cells. **(C, F)** IgG ANA titer in the serum of cGvHD mice determined 3, 6 and 9 weeks after disease induction. Horizontal bars indicate mean of ANA titers in each group. Titers below the dotted line represent mice negative for IgG ANA. Deviations from initially used numbers of mice are indicated at the respective time point. Statistical significance ($p < 0.05$) was calculated using an unpaired student's t-test and is indicated by the p-value. **(C)** Open circles: cGvHD induced with DBA/2 lymphocytes; filled squares: cGvHD induced with DBA/2 lymphocytes depleted of CD8⁺ T cells. **(F)** Open circles: Untreated cGvHD mice induced with DBA/2 lymphocytes depleted of CD8⁺ T cells; filled squares: cGvHD mice induced with DBA/2 lymphocytes depleted of CD8⁺ T cells and therapeutically treated with S4B6/IL-2 complexes. **(D, G)** Frequencies of mice positive for proteinuria as determined by elevated albumin in the urine. Statistical significance ($p < 0.05$) was calculated using the Mantel-Cox test and is indicated by the p-value. **(D)** Dotted line: cGvHD induced with DBA/2 lymphocytes; solid line: cGvHD induced with DBA/2 lymphocytes depleted of CD8⁺ T cells. **(G)** Dotted line: cGvHD induced with DBA/2 lymphocytes depleted of CD8⁺ T cells; solid line: cGvHD induced with DBA/2 lymphocytes depleted of CD8⁺ T cells and therapeutically treated with S4B6/IL-2 complexes.

6. GvHD in TCR transgenic BDF1 mice

The contribution of host T cells to the pathogenesis of GvHD is still obscure [143, 144]. The finding that prophylactic treatment with IL-2 complexes, as described above, markedly influences the development of cGvHD is consistent with the idea that the host T cell compartment plays a role in the pathogenesis of this disease. In order to investigate this, we generated BDF1 mice with a restricted T cell repertoire by crossing B6 mice expressing the MHC-I (K^b) restricted OT-I TCR transgene to DBA/2 mice to generate OT1-BDF1 animals. Similarly, B6 mice expressing the MHC-II (I-A^b) restricted OT-II TCR transgene were crossed with DBA/2 animals to generate OT2-BDF1 mice. Due to the dominant expression of the transgenic TCR and the extensive but not complete suppression of host TCR gene rearrangement, these animals have a reduced T cell receptor repertoire.

As shown in **Fig. 7A**, the total absolute cellularity of spleens from normal non-transgenic BDF1 mice and OT1-BDF1 mice was comparable (81.1 ±23.1 x10^6 (BDF1); 70.2 ± 7.40 x10^6 (OT1-BDF1)) whereas spleens from OT2-BDF1 mice exhibited a modest increase in cellularity and contained 108.5 ± 9.0 x10^6 cells. However, the T cell compartment of both TCR transgenic BDF1 strains was heavily comprised in $CD4^+$ and $CD8^+$ T cell numbers compared to non-transgenic BDF1 mice (**Fig. 7B, C**). In spleens of OT1-BDF1 mice, $CD4^+$ (1.17 ± 0.17 x10^6) and $CD8^+$ (1.58 ± 0.06 x10^6) T cells represented only 10% and 23% of absolute $CD4^+$ and $CD8^+$ T cells normally found in BDF1 spleens (11.71 ± 2.54 x10^6 ($CD4^+$); 6.80 ± 1.02 x10^6 ($CD8^+$)). In OT2-BDF1 mice, numbers of $CD4^+$ (4.18 ± 0.31 x10^6) and $CD8^+$ (4.62 ± 0.56 x10^6) T cells were less compromised compared to OT1-BDF1 mice and represented 36% of $CD4^+$ and 68% of $CD8^+$ T cells usually found in spleens of normal BDF1 mice. Additionally, the Treg compartment in OT1-BDF1 spleens (0.53 ±

0.12 x10^6) was only 39% of that in normal BDF1 mice (1.35 ± 0.45 x10^6) (**Fig. 7D**). Absolute numbers of $CD4^+$ $FoxP3^+$ Tregs in OT2-BDF1 spleens (1.3 ± 0.5 x10^6) were slightly increased compared to normal BDF1 mice, however this difference was not statistically significant (**Fig. 7D**). Analysis of the TCR repertoire for expression of transgenic receptors (Vα2 and Vβ5) or TCRs with endogenously encoded Vβ-families, as shown in **Fig. 8**, revealed a highly restricted TCR repertoire in $CD4^+$ and $CD8^+$ T cells of both TCR transgenic BDF1 strains. A considerable fraction of splenic $CD4^+$ T cells (41.4 ± 3.0%) and virtually all $CD8^+$ T cells (98.8 ± 0.1%) in OT1-BDF1 mice expressed TCRs composed of Vβ5 on the surface. The frequency of T cells expressing other Vβ-families expressed in the $CD8^+$ T cell compartment was on average less than 1%. In contrast, the $CD4^+$ T cell compartment of OT1-BDF1 mice showed a higher proportion of cells expressing endogenous Vβ-families (**Fig. 8A**). Furthermore, 28.2 ± 2.4% of $CD4^+$ T cells and 94.0 ± 1.2% of $CD8^+$ T cells expressed both transgenic TCR chains ($V\alpha2^+/V\beta5^+$) in OT1-BDF1 mice (**Fig. 8C**). In normal BDF1 mice, only a minor fraction of $CD4^+$ T cells (0.15 ± 0.05%) and $CD8^+$ T cells (1.1 ± 0.22%) was positive for Vβ5 expression and $V\alpha2^+/V\beta5^+$ T cells are virtually absent. Taken together, these data show that the TCR repertoire in TCR transgenic BDF1 mice expresses a limited diversity of TCRs.

As shown in **Fig. 8A**, a higher fraction of OT2-BDF1 $CD4^+$ T cells express a $V\beta5^+$ TCR (54.2 ± 3.0%) compared to $CD4^+$ T cells in OT1-BDF1 mice (41.4 ± 3.0%). In $CD8^+$ T cells of OT2-BDF1 mice, the most frequently used Vβ-family was again Vβ5 (55.5 ± 3.0%), but additionally other Vβ-families were well represented (**Fig. 8B**). The frequency of cells expressing both $V\alpha2^+$ and $V\beta5^+$ TCR chains among $CD4^+$ and $CD8^+$ T cells in OT2-BDF1 mice was 44.8 ± 4.7% and 48.4 ± 3.7%, respectively.

In summary, OT1-BDF1 und OT2-BDF1 represent two TCR transgenic mouse strains exhibiting a variable degree of T cell deficiency and TCR repertoire restriction in both, the $CD4^+$ and the $CD8^+$ T cell compartments. Moreover, the Treg compartment in OT1-BDF1 mice is both, severely reduced in numbers and highly restricted in TCR repertoire diversity, whereas the Treg compartment in OT2-BDF1 mice is not reduced in numbers compared to normal BDF1 mice but it nevertheless expresses a highly restricted TCR repertoire.

Figure 7

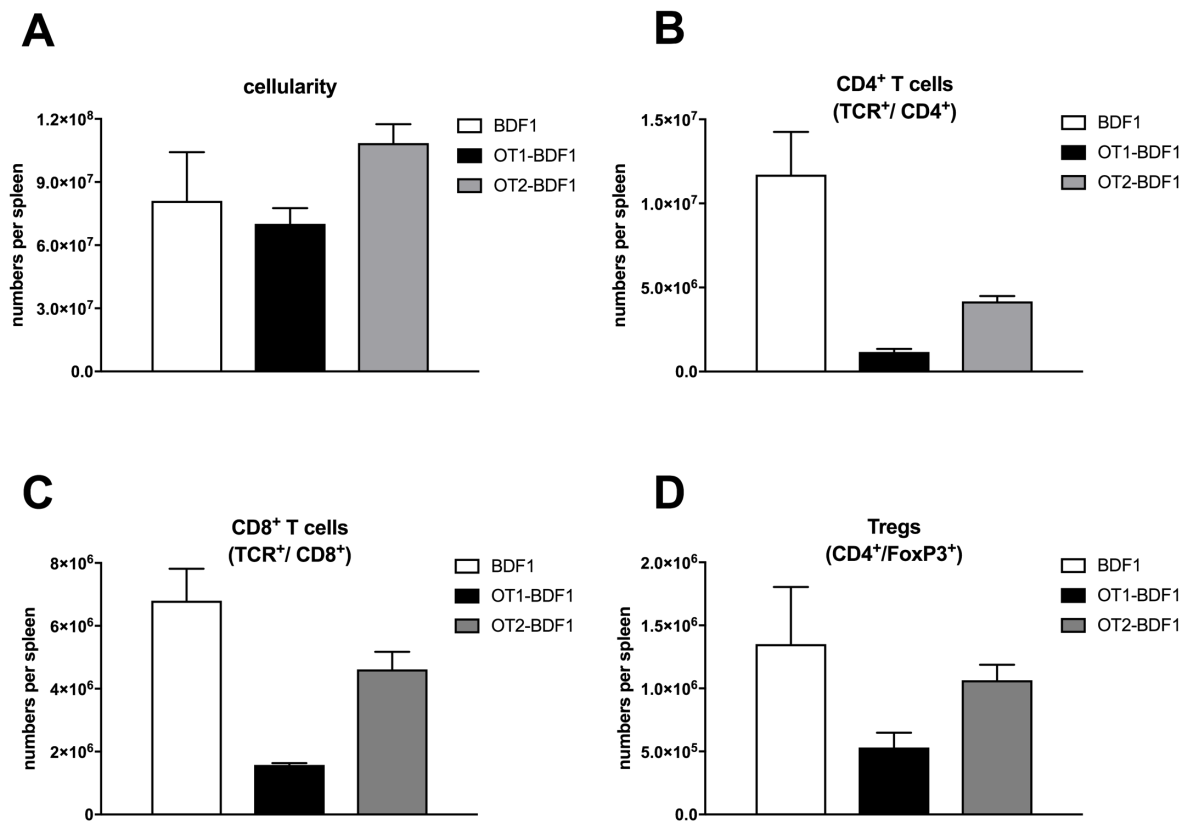


Figure 7: *Altered T cell compartments in OT1-BDF1 and OT2-BDF1 mice compared to normal BDF1 mice*

(A) Absolute cellularity of spleens. **(B)** Absolute numbers of CD4+ T cells. **(C)** Absolute numbers of CD8+ T cells. **(D)** Absolute numbers of Tregs expressing CD4 and FoxP3. BDF1: white bars, n= 4; OT1-BDF1: black bars, n= 4; OT2-BDF1: grey bars, n= 4. Data are presented as mean values ± SD.

Figure 8

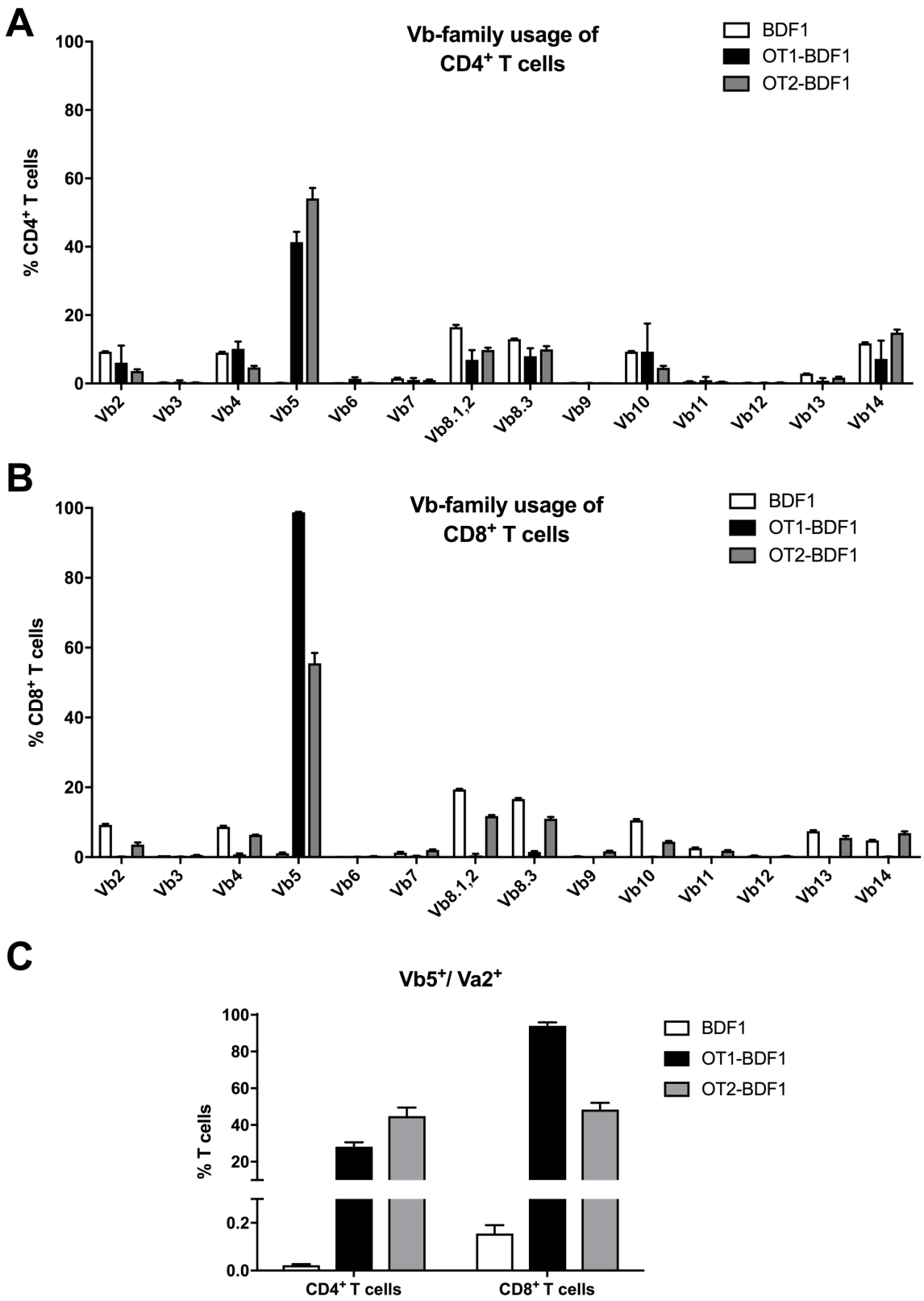

A
Vb-family usage of
CD4+ T cells
BDF1
OT1-BDF1
OT2-BDF1
% CD4+ T cells
100
80
60
40
20
0
Vb2
Vb3
Vb4
Vb5
Vb6
Vb7
Vb8.1,2
Vb8.3
Vb9
Vb10
Vb11
Vb12
Vb13
Vb14
B
Vb-family usage of
CD8+ T cells
BDF1
OT1-BDF1
OT2-BDF1
% CD8+ T cells
100
80
60
40
20
0
Vb2
Vb3
Vb4
Vb5
Vb6
Vb7
Vb8.1,2
Vb8.3
Vb9
Vb10
Vb11
Vb12
Vb13
Vb14
C
Vb5+/ Va2+
BDF1
OT1-BDF1
OT2-BDF1
% T cells
100
80
60
40
20
0.2
0.0
CD4+ T cells
CD8+ T cells

Figure 8: *Highly restricted TCR repertoire in OT1-BDF1 and OT2-BDF1 mice*

(A) Frequencies of various Vβ-families expressed by splenic $CD4^+$ T cell. **(B)** Frequencies of various Vβ-families expressed by splenic $CD8^+$ T cell. **(C)** Frequencies of $CD4^+$ and $CD8^+$ T cells expressing both transgene-encoded TCR α- and β-chains ($V\beta5^+/V\ \alpha2^+$). BDF1: white bars, n= 4; OT1-BDF1: black bars, n= 4; OT2-BDF1: grey bars, n= 4. Data are presented as mean values ± SD.

7. DBA/2 lymphocytes induce acute GvHD in TCR transgenic BDF1 mice

To examine the influence of a TCR restricted host T cell repertoire on the developing symptoms of GvHD, OT1-BDF1 (n= 13) and OT2-BDF1 (n= 7) mice were injected i.v. with $70x10^6$ DBA/2 lymphocytes.

Instead of the anticipated chronic course of GvHD (cGvHD) that usually results from the transfer of DBA/2 lymphocytes into normal BDF1 mice, we observed an acute form of GvHD (aGvHD) in TCR transgenic BDF1 host mice. This was accompanied by the characteristic disease symptoms including progressive loss of body weight and the destruction of the host lympho-hematopoietic system by donor $CD8^+$ CTLs. The symptoms were severe enough that the mice were sacrificed within weeks following disease induction.

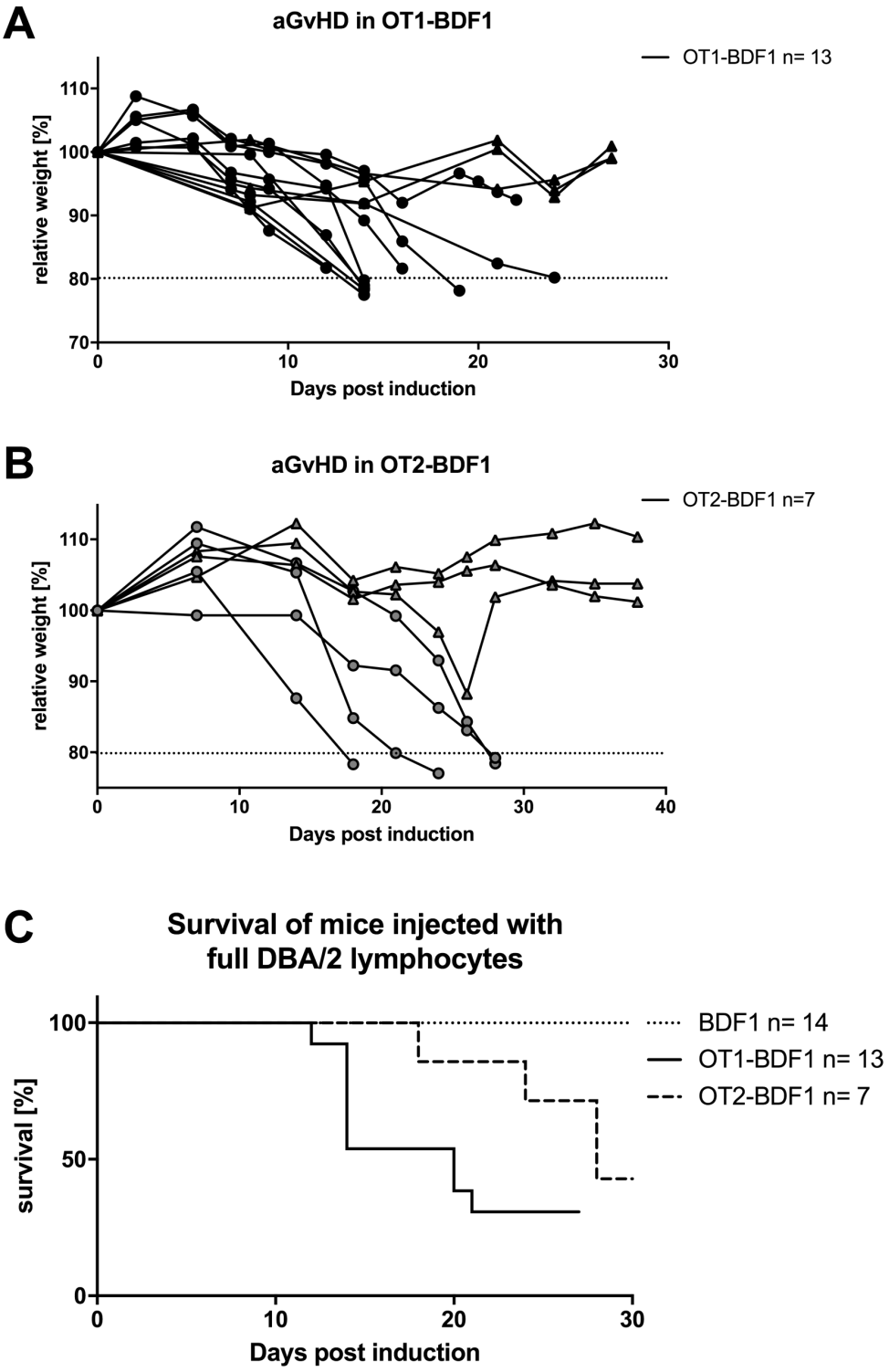
Fig. 9 shows the relative body weights (body weight compared to the weight at day 0 of disease induction) of OT1-BDF1 (**Fig. 9A**) and OT2-BDF1 mice (**Fig. 9B**) over a period of 30 and 40 days, respectively. The majority of OT1-BDF1 mice (10/13) were sacrificed after 2 to 3 weeks (**Fig. 9A**; filled circles; mean survival: 16.3 days) due to a reduction of initial body weight of more than 20%. Spleens (**Fig. 9E – G**) and bone marrow (**Fig. 9H**) of long-term survivors that survived the phase of lympho-hematopoietic depletion and almost regained their initial body weight (**Fig. 9A**; filled triangles) were analyzed by flow cytometry in order to determine the extent of reconstitution by donor DBA/2 ($H\text{-}2^{d/d}$) cells. Whereas more than 99% of all $CD4^+$, $CD8^+$ and $CD19^+$ cells in the spleens and bone marrow expressed K^d on their surface, only about 4 - 9% were positive for host derived K^b molecules. These results show that in these OT1-BDF1 mice almost complete

reconstitution by donor hematopoietic stem cells had occurred. It was previously shown that reconstitution by donor-derived splenic hematopoietic stem cells, contained in the donor inoculum used for disease induction, can rescue mice from developing a lethal form of aGvHD [145].

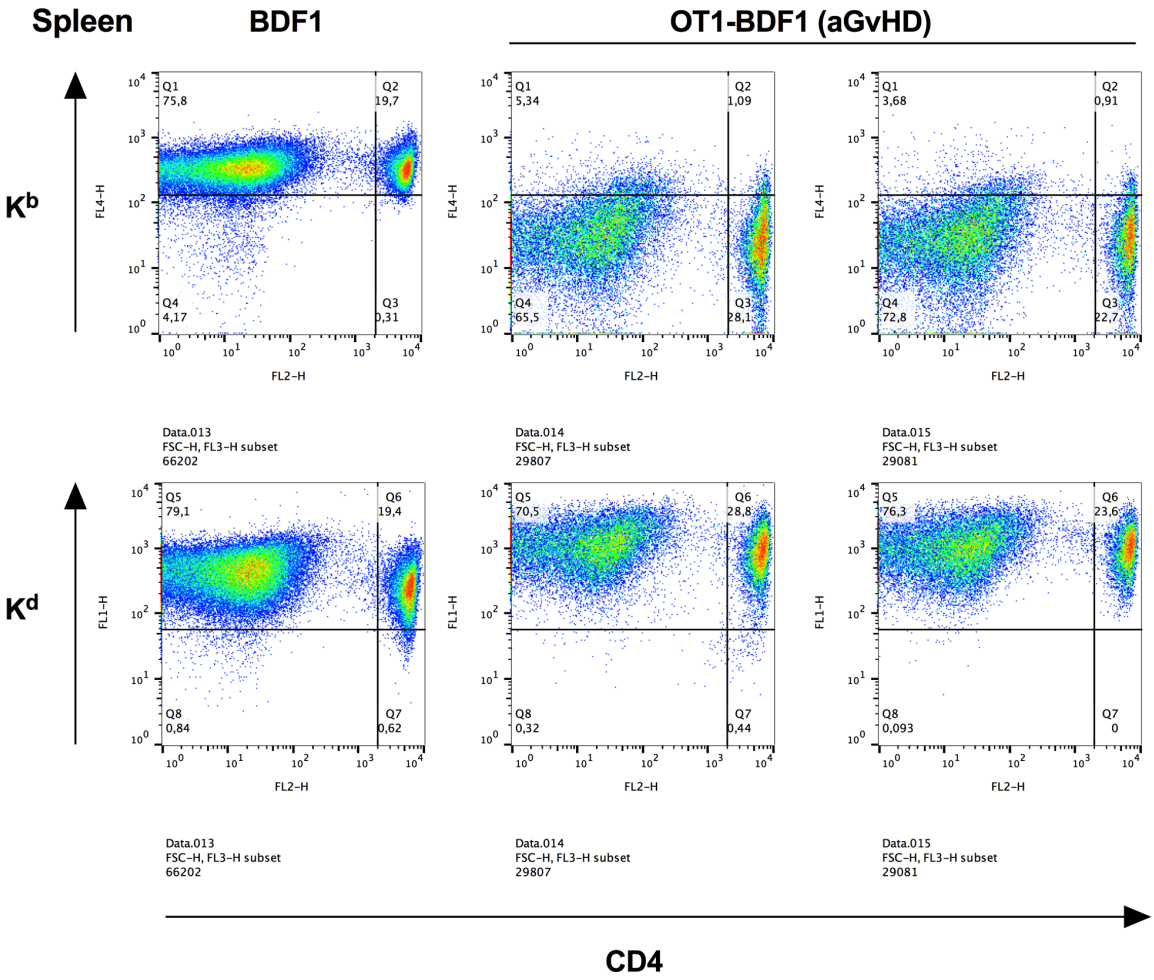
The transfer of DBA/2 lymphocytes into OT2-BDF1 mice (**Fig. 9B**) induced the same but milder aGvHD symptoms observed from the transfer of DBA/2 lymphocytes into OT1-BDF1 mice. The mice rapidly lost body weight and 57% (4/7; filled circles) had to be sacrificed within 3 to 4 weeks after disease induction. The mean survival time of OT2-BDF1 (24.5 days) was longer compared to OT1-BDF1 mice (16.3 days) (**Fig.9C**). A small number of OT1-BDF1 mice surviving the initial phase of aGvHD and the accompanying depletion of host lympho-hematopoietic system (43% (3/7); filled triangles) recovered from their weight loss and showed reconstitution by donor hematopoietic stem cells (data not shown). This is similar to what is observed in long-term survivors of aGvHD in OT1-BDF1 mice.

These results show that kinetics and symptoms resulting from GvHD are strongly influenced by host T cells and the diversity of the host TCR repertoire. The severity of the developing aGvHD symptoms correlates with a limited diversity of the TCR repertoire and the reduced size of the Treg compartment resulting in a more severe disease in OT1-BDF1 compared to OT2-BDF1 mice (**Fig. 9C**).

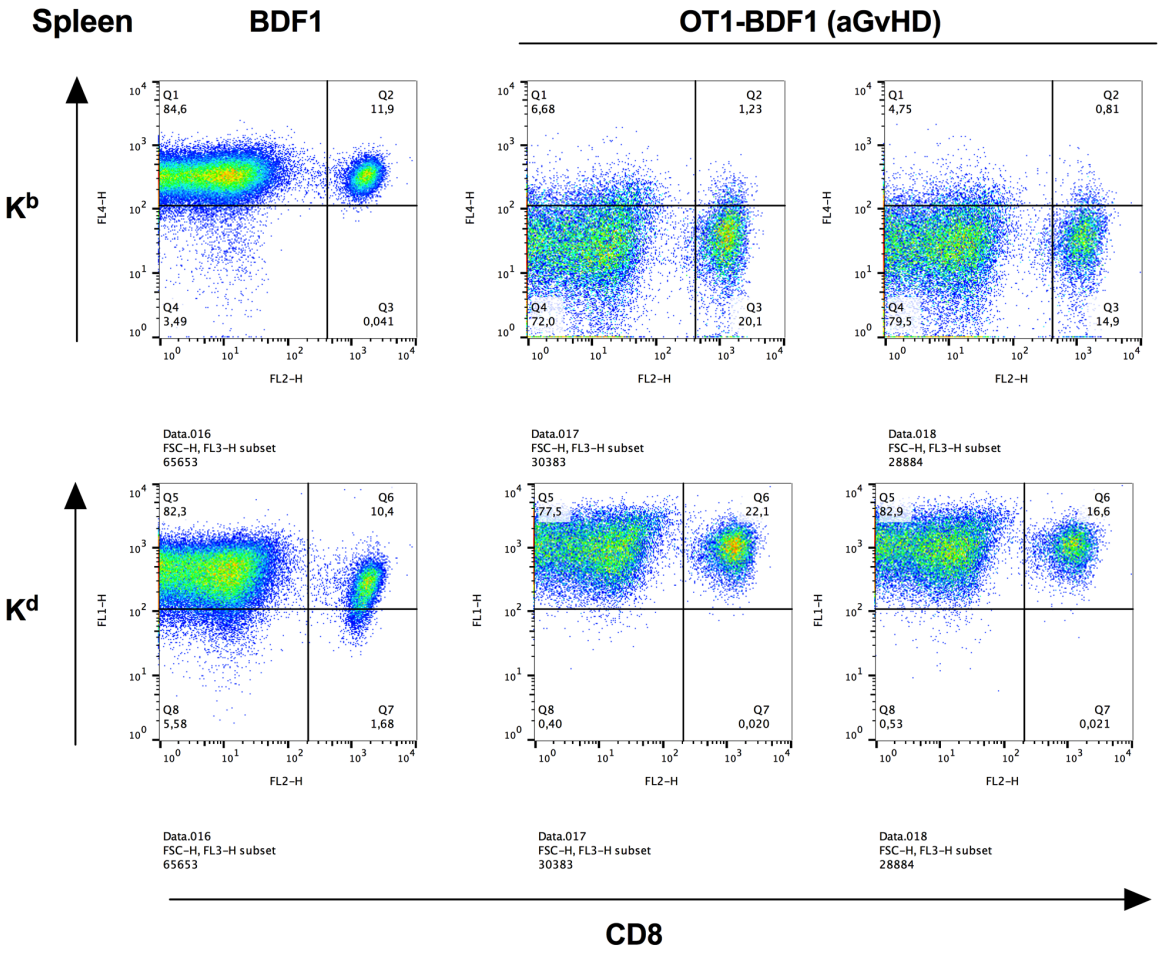
Figure 9


A
aGvHD in OT1-BDF1
OT1-BDF1 n= 13
relative weight [%]
110
100
90
80
70
0
10
20
30
Days post induction
B
aGvHD in OT2-BDF1
OT2-BDF1 n=7
relative weight [%]
110
100
90
80
0
10
20
30
40
Days post induction
C
Survival of mice injected with
full DBA/2 lymphocytes
BDF1 n= 14
OT1-BDF1 n= 13
OT2-BDF1 n= 7
survival [%]
100
50
0
0
10
20
30
Days post induction

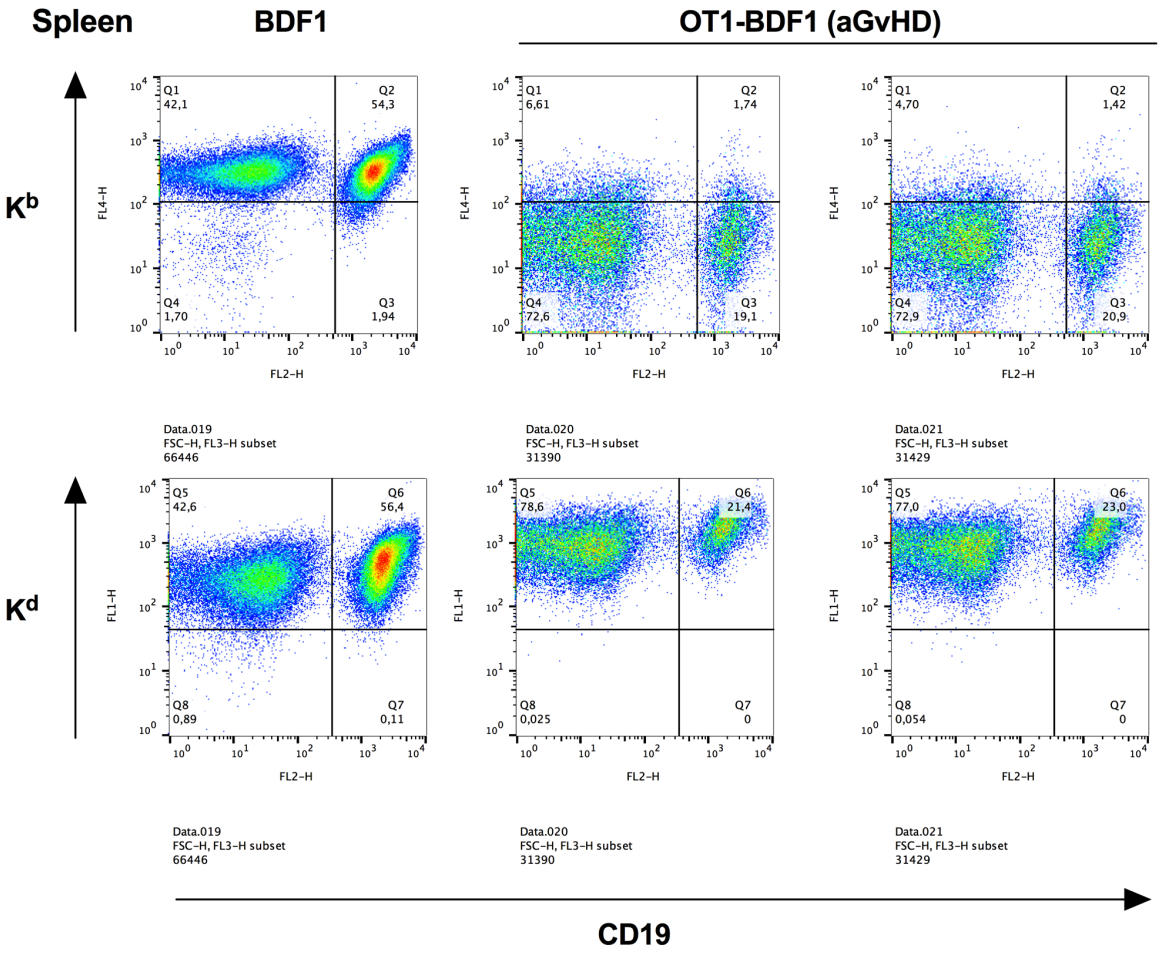
D


Spleen
BDF1
OT1-BDF1 (aGvHD)
Kb
Kd
CD4
FL4-H
FL1-H
FL2-H
Q1 75,8
Q2 19,7
Q4 4,17
Q3 0,31
Q1 5,34
Q2 1,09
Q4 65,5
Q3 28,1
Q1 3,68
Q2 0,91
Q4 72,8
Q3 22,7
Data.013
FSC-H, FL3-H subset
66202
Data.014
FSC-H, FL3-H subset
29807
Data.015
FSC-H, FL3-H subset
29081
Q5 79,1
Q6 19,4
Q8 0,84
Q7 0,62
Q5 70,5
Q6 28,8
Q8 0,32
Q7 0,44
Q5 76,3
Q6 23,6
Q8 0,093
Q7 0

E


Spleen
BDF1
OT1-BDF1 (aGvHD)
Kb
Kd
CD8
FL4-H
FL1-H
FL2-H
Q1 84,6
Q2 11,9
Q4 3,49
Q3 0,041
Q1 6,68
Q2 1,23
Q4 72,0
Q3 20,1
Q1 4,75
Q2 0,81
Q4 79,5
Q3 14,9
Q5 82,3
Q6 10,4
Q8 5,58
Q7 1,68
Q5 77,5
Q6 22,1
Q8 0,40
Q7 0,020
Q5 82,9
Q6 16,6
Q8 0,53
Q7 0,021
Data.016
FSC-H, FL3-H subset
65653
Data.017
FSC-H, FL3-H subset
30383
Data.018
FSC-H, FL3-H subset
28884

F


Spleen
BDF1
OT1-BDF1 (aGvHD)
Kb
FL4-H
FL2-H
Q1 42,1
Q2 54,3
Q4 1,70
Q3 1,94
Data.019
FSC-H, FL3-H subset
66446
Q1 6,61
Q2 1,74
Q4 72,6
Q3 19,1
Data.020
FSC-H, FL3-H subset
31390
Q1 4,70
Q2 1,42
Q4 72,9
Q3 20,9
Data.021
FSC-H, FL3-H subset
31429
Kd
FL1-H
FL2-H
Q5 42,6
Q6 56,4
Q8 0,89
Q7 0,11
Data.019
FSC-H, FL3-H subset
66446
Q5 78,6
Q6 21,4
Q8 0,025
Q7 0
Data.020
FSC-H, FL3-H subset
31390
Q5 77,0
Q6 23,0
Q8 0,054
Q7 0
Data.021
FSC-H, FL3-H subset
31429
CD19

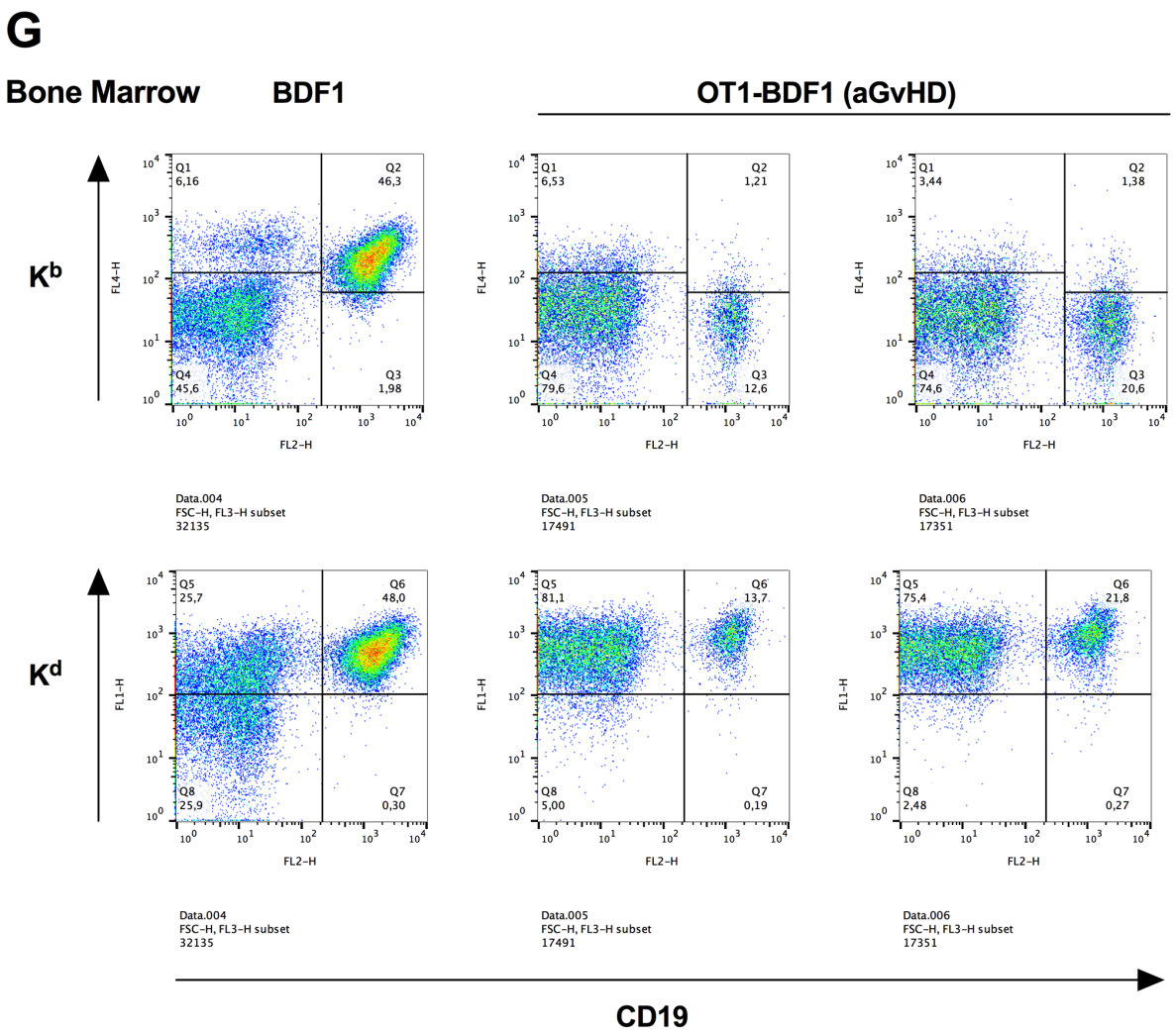


Figure 9: *Injection of full DBA/2 lymphocytes into OT1-BDF1 and OT2-BDF1 mice induces acute GvHD*

(A) Relative body weight of OT1-BDF1 mice (n=13) over a period of 30 days. Black circles (n= 10): mice developing lethal aGvHD had to be sacrificed due to reduction of initial body weight of more than 20%. Black triangles (n= 3): Long-term survivors of aGvHD showing reconstitution by DBA/2 HSC. **(B)** Relative body weight of OT2-BDF1 (n=7) mice over a period of 40 days. Grey circles (n= 4): mice developing lethal aGvHD had to be sacrificed due to reduction of initial body weight of more than 20%. Grey triangles (n= 3): Survivors of aGvHD showing reconstitution by DBA/2 HSC. **(C)** Survival of OT1-BDF1 (solid line), OT2-BDF1 (dashed line) and BDF1 mice (dotted line) after the injection of full DBA/2 lymphocytes. **(D – G)** Analysis of spleens and bone marrow of OT1-BDF1 mice surviving the phase of aGvHD for the expression of H-2K^b (y-axis, upper panel) and H-2K^d (y-axis, lower panel) and CD4, CD8 and CD19 (x-axis).

8. DBA/2 lymphocytes depleted of CD8+ T cells induce chronic GvHD in TCR transgenic BDF1 mice

It was shown previously that donor CD8+ T cells are required for the induction of aGvHD, In order to test whether depletion of CD8+ T cells prevents the induction of aGvHD in TCR transgenic hosts, OT1-BDF1 and OT2-BDF1 mice were induced with DBA/2 donor cells depleted of CD8+ T cells. Injection of CD8+ T cell depleted donor lymphocytes into OT1-BDF1 (n= 13) or OT2-BDF1 (n= 6) transgenic hosts induced cGvHD with characteristic production of autoantibodies and the occurrence of proteinuria (**Fig. 10A - C**). Three weeks following disease induction, all (13/13) OT1-BDF1 mice produced significant amounts of anti-RBC autoantibodies (**Fig. 10A**). Mean IgG ANA titers in the serum of OT1-BDF1 mice undergoing cGvHD (week 3 – 6: 122.8 ± 26.54) were comparable to those of normal BDF1 mice when disease was induced with CD8+ T cell depleted DBA/2 lymphocytes (**Fig. 10B** (compare to **Fig. 6C** and **Fig. 6F**)). The most striking observation in cGvHD OT1-BDF1 mice was the fast kinetics and early onset by which proteinuria emerged (**Fig. 10C**). By 7 days of cGvHD, significantly elevated concentrations of albumin could be detected in the urine of almost all OT1-BDF1 mice (7/8); after 2 weeks all mice were proteinuria positive in this group. The extremely early disease onset suggests that proteinuria in these mice may not be mediated by the typical deposition of immune complexes in the kidney. This was confirmed by staining for IgG deposition in kidney sections prepared from proteinuria positive OT1-BDF1 mice. There was no detectable deposition of IgG immune complex in the glomeruli of those mice (data not shown). Thus, other mechanisms apart from renal deposition of immune complex causing SLE-like glomerulonephritis might have to be

considered for the failure of kidney function in these mice. Due to the early onset of proteinuria in OT1-BDF1 mice, the experiments were terminated after 6 weeks.

When DBA/2 lymphocytes depleted of $CD8^+$ T cells were injected into OT2-BDF1 host mice, cGvHD developed that was comparable to the disease in OT1-BDF1 mice in terms of anti-RBC and ANA production (**Fig. 10C - E**). All OT2-BDF1 mice (6/6) produced anti-RBC autoantibodies within 3 weeks after disease induction (**Fig. 10D**). IgG ANA titers in OT2-BDF1 mice during the first 6 weeks of cGvHD (220 ± 28.28) were comparable to those in OT1-BDF1 (122.8 ± 26.54). After 9 weeks, ANA titers in OT2-BDF1 mice again declined, likely due to the prolonged time of proteinuria. In contrast to the proteinuria in OT1-BDF1 mice, proteinuria in OT2-BDF1 was delayed. The proportion of proteinuria positive OT2-BDF1 mice progressively increased starting 3 weeks after disease induction and reached full penetrance after 6 weeks of cGvHD (**Fig. 10C**). The time point of proteinuria onset in cGvHD OT2-BDF1 mice is similar to that observed in normal BDF1 mice induced with $CD8^+$ T cell depleted donor lymphocytes. However, the accelerated kinetics that lead to full penetrance of ICGN almost 3 weeks earlier indicate that DBA/2 donor lymphocytes depleted of $CD8^+$ T cells induce a more severe cGvHD in hosts with a restricted TCR repertoire compared to normal hosts.

Corresponding to the development of aGvHD in these mice, we observed that also cGvHD in these TCR repertoire restricted mice evolves with variable degrees of severity. Using kidney involvement as major criteria for the severity of SLE-like cGvHD, a markedly more severe disease develops in OT1-BDF1 compared to OT2-BDF1 mice following transfer of DBA/2 cells depleted of $CD8^+$ T cells.

The fact that OT1-BDF1 and OT2-BDF1 mice differ in their number of Tregs, is consistent with the idea that Tregs with the capacity to control the activation of donor $CD8^+$ T cells may play a role in this model. The fact that fairly normal numbers of Tregs in OT2-BDF1 mice could not prevent the induction of acute GvHD upon injection of DBA/2 lymphocytes containing all T cell subsets indicates that Tregs with a reduced TCR diversity might fail to control the activation of donor $CD8^+$ T cells. How the host TCR repertoire influences the suppression of donor $CD8^+$ T cells requires further investigation.

Figure 10

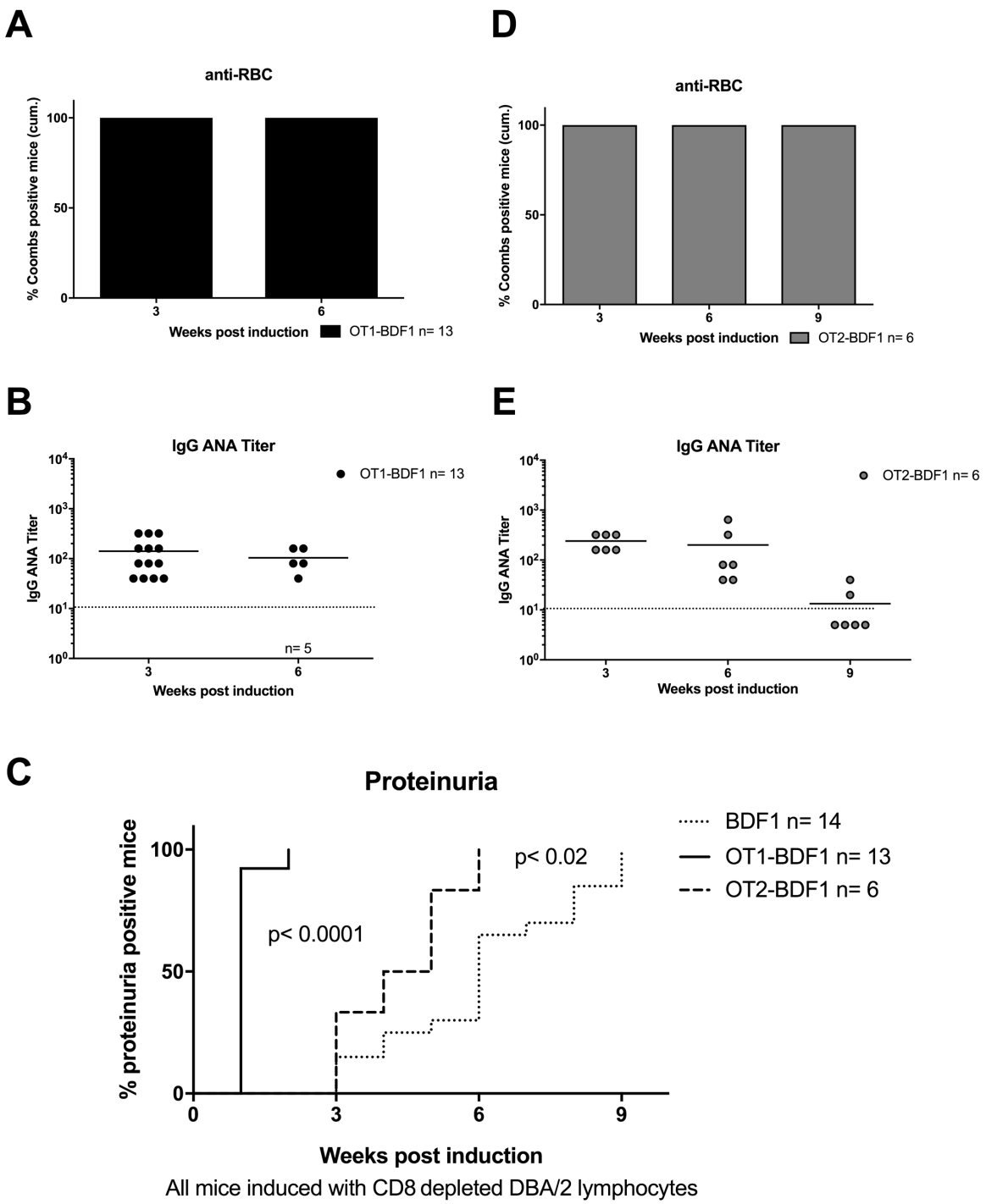

A
anti-RBC
% Coombs positive mice (cum.)
100
50
0
3
6
Weeks post induction
OT1-BDF1 n= 13
D
anti-RBC
% Coombs positive mice (cum.)
100
50
0
3
6
9
Weeks post induction
OT2-BDF1 n= 6
B
IgG ANA Titer
OT1-BDF1 n= 13
IgG ANA Titer
10^4
10^3
10^2
10^1
10^0
n= 5
3
6
Weeks post induction
E
IgG ANA Titer
OT2-BDF1 n= 6
IgG ANA Titer
10^4
10^3
10^2
10^1
10^0
3
6
9
Weeks post induction
C
Proteinuria
BDF1 n= 14
OT1-BDF1 n= 13
OT2-BDF1 n= 6
p< 0.02
p< 0.0001
% proteinuria positive mice
100
50
0
0
3
6
9
Weeks post induction
All mice induced with CD8 depleted DBA/2 lymphocytes

Figure 10: *Injection of DBA/2 lymphocytes depleted of CD8⁺ T cells into OT1-BDF1 and OT2-BDF1 mice induces chronic GvHD*

(A, D) Frequencies of mice positive for anti-RBC autoantibodies determined at 3, 6 (and 9) weeks of cGvHD (cumulative). Black bars: OT1-BDF1 (n= 13); grey bars: OT2-BDF1 (n= 6). **(B, E)** IgG ANA titers in the serum of OT1-BDF1 (black circles) and OT2-BDF1 mice (grey circles) determined 3, 6 (and 9) weeks after disease induction. Horizontal bars indicate the mean of ANA titers. Titers below the dotted line represent mice negative for IgG ANA. Deviations from initially used numbers of mice are indicated at the respective time point. **(C)** Frequencies of mice positive for proteinuria as determined by elevated albumin concentrations in the urine. Statistical significance ($p < 0.05$) was calculated using the Mantel-Cox test and is indicated by the p-value. Solid line: OT1-BDF1; dashed line: OT2-BDF1; dotted line: BDF1.

Discussion

Murine cGvHD results from the injection of DBA/2 lymphocytes into BDF1 mice, generating autoimmune symptoms, which closely resemble those present in SLE patients. There is still no universally effective therapy for this class of systemic autoimmune diseases. Previously, it was shown that IL-2 could ameliorate autoimmunity in MRL mice [131]. However, due to the severe side effects IL-2 was not considered for therapeutic purposes. Some years ago it was demonstrated that adverse side effects of this cytokine could be prevented when IL-2 is administered together with an anti-IL-2 mAb, in the form of an immune complex [133]. Moreover, it was shown that depending on the anti-IL-2 mAb used for the formation of such IL-2 complexes different T cell subsets could be stimulated. Thus, administration of JES6/IL-2 complexes results in the expansion of IL-2R$\alpha\beta\gamma_c^+$ regulatory T cells whereas S4B6/IL-2 complexes preferentially induce the expansion of $CD8^+$ T cells with high levels of IL-2R$\beta\gamma_c$ on the surface [122].

Recently, the potency of these immune complexes in immunotherapy was shown in several mouse models. Thus, JES6/IL-2 complexes induced resistance to EAE, a murine model for multiple sclerosis, and induced long-term tolerance to pancreatic islet allografts in transplantation settings [137]. On the other hand, S4B6/IL-2 complexes were shown to be highly efficient in cancer immunotherapy in a murine model of melanoma [130]. However, studies are lacking which examine on the use of IL-2 complexes in an autoimmune disease like SLE, which is characterized by a particularly strong autoantibody response and consequent pathology.

Thus, we investigated the prophylactic and therapeutic potential of JES6/IL-2 and S4B6/IL-2 complexes on the development of a SLE–like cGvHD in BDF1 mice induced by the transfer of DBA/2 lymphocytes.

Prophylactic treatment with the different IL-2 complexes showed opposing effects on the symptoms of developing cGvHD. The amelioration of disease upon JES6/IL-2 treatment was accompanied by significantly decreased numbers of donor $CD4^+$ T cells that had engrafted in the spleens of recipient mice 2 weeks after transfer. Donor $CD4^+$ T cells are central to the pathogenesis of murine cGvHD since they provide help to autoreactive B cells in order to produce disease driving autoantibodies. Most likely, disease amelioration is a consequence of the expanded host Treg compartment with increased suppressive capacity at the time of disease induction. It is well established that Tregs are capable to regulate various immune responses and prevent autoimmunity. Host Treg numbers in JES6/IL-2 pretreated cGvHD mice were still elevated 2 weeks after the last injection of IL-2 complexes. The fact that Treg numbers in healthy mice usually return to baseline within approximately 10 days after the treatment with IL-2 complexes [137] supports the idea that cGvHD associated events (e.g. elevated endogenous IL-2 levels) maintain the increase Treg numbers in these mice.

In contrast, prophylactic treatment with S4B6/IL-2 complexes resulted in a more severe form of cGvHD. In these mice, we found significantly increased numbers of donor $CD4^+$ T cells with a central memory phenotype, identified by their high expression of CD44 and CD62L on the surface ($CD44^{hi}$ / $CD62L^{hi}$). Memory T cells have a lower activation threshold and exhibit enhanced effector function upon re-stimulation compared to their less differentiated counterparts. Besides the increased numbers of memory $CD4^+$ T cells, S4B6/IL-2 pretreated mice also contain significantly more $CD4^+$ Tfh cells of donor origin,

expressing PD1 and CXCR5 two weeks after disease induction compared to untreated cGvHD mice. Tfh cells are involved in germinal center reaction and promote the differentiation of antibody secreting cells through IL-21 secretion along with co-stimulatory signaling. These findings suggest that the exacerbated disease in mice pretreated with S4B6/IL-2 complexes might be primarily driven by the markedly expanded numbers of differentiated donor $CD4^+$ T cells providing enhanced stimulation to host B cells resulting in elevated production of pathogenic autoantibodies.

The finding of a more severe autoimmunity in cGvHD mice pretreated with S4B6/IL-2 complexes despite a significantly increased Treg compartment after 2 weeks of ongoing disease is intriguing. The literature about S4B6/IL-2 complexes and the underlying mechanism usually describes a potent expansion of $CD8^+$ T cell populations as well as NK cells. The mechanism is explained by the binding of S4B6 mAb to the epitope of the IL-2 molecule required for the interaction with high affinity component of the IL-2 receptor, CD25 [134, 135]. Thereby, more IL-2 is available for cells expressing low-affinity receptors for IL-2 (IL-2R$\beta\gamma_c$). Nevertheless, S4B6/IL-2 complexes obviously have a considerable effect on $CD4^+$ / $CD25^+$ T cells as shown by Boyman at el. in the original description of these IL-2 complexes [122]. There, S4B6/IL-2 complexes were at least equally efficient in the expansion of the $CD4^+$ / $CD25^+$ (assumed to be Tregs) compartment compared to JES6/IL-2 complexes. Considering these results, it seems plausible that, at the time of disease induction, the Treg compartment in recipient mice was similarly expanded in terms of absolute numbers in response to S4B6/IL-2 and JES6/IL-2 treatment. However, only the Treg compartment expanded upon JES6/IL-2 treatment efficiently suppressed the activation and differentiation of donor $CD4^+$ T cells resulting in reduced autoimmunity. This suggests a qualitative difference in the

suppressive capacity of Tregs expanded by S4B6/IL-2 or JES6/IL-2 complexes. Possibly, this might result from differences in IL-2R signaling and activation of downstream pathways. Whether the Treg TCR repertoire differs between JES6/IL-2 and S4B6/IL-2 treated mice and whether these differences might impact the suppressive capacity needs to be further investigated.

Another open question regarding the exacerbated disease induced in S4B6/IL-2 pretreated mice is the pronounced differentiation of donor $CD4^+$ T cells into Tfh cells. We found this subset significantly increased only in mice pretreated with S4B6/IL-2 complexes. These findings might suggest a direct effect of S4B6/IL-2 complexes on the differentiation of activated donor $CD4^+$ T cells into various effector subsets. However, it was shown previously that already 4 hours after administration, the biological activity of S4B6/IL-2 complexes to stimulate $CD8^+$ T cells is substantially reduced and after 24 hours the stimulatory effect was essentially gone [122]. Whether this also applies for the stimulation of $CD4^+$ T cells has not been reported. In addition, naïve $CD4^+$ T cells express relatively low levels of IL-2 receptor on their surface and are less responsive to IL-2 compared to their $CD8^+$ counterparts. Taken together, these findings rather point towards an indirect effect of S4B6/IL-2 complexes on the differentiation of donor $CD4^+$ T cells into cells of the follicular helper subset. Perhaps, the differentiation of donor cells into follicular helper T cells is a secondary effect of prophylactic S4B6/IL-2 treatment on other cells of the host immune system (e.g. DCs).

We also examined the effect of these two IL-2 complexes in treating ongoing cGvHD. The therapeutic administration of JES6/IL-2 showed no significant effect on the symptoms of cGvHD. Probably, after 4 weeks, the cGvHD has already progressed for too long so that the established effector mechanisms were no longer suppressible by JES6/IL-2

stimulated Tregs. It might also well be envisaged that a stimulatory effect of JES6/IL-2 complexes on previously activated donor $CD4^+$ T cells exceeds the suppressive capacity of the expanded host Treg compartment.

$CD8^+$ T cells from donor DBA/2 mice are most likely the population accountable for the positive effect of therapeutic administration of S4B6/IL-2 complexes. This assumption is supported by the following observations: First, in the absence of donor $CD8^+$ T cells a more severe cGvHD develops, and second, host $CD8^+$ T cells alone are unable to ameliorate disease symptoms after stimulation with S4B6/IL-2 complexes.

Our findings confirm the important influence of donor $CD8^+$ T cells and emphasize paradoxical features for this population in the development of cGvHD in BDF1 mice. On one hand, in our cGvHD model, donor $CD8^+$ T cells seem to be insufficiently stimulated to mount a strong anti-host response that would result in aGvHD. On the other hand, they are critical for mechanisms leading to an amelioration of cGvHD symptoms upon S4B6/IL-2 therapy. Possibly, therapeutic S4B6/IL-2 treatment promotes the stimulation of donor $CD8^+$ T cells above a threshold sufficient to induce low-level anti-host responses. This would resemble a mild form of aGvHD where host cells (e.g. autoreactive B cells) expressing $H\text{-}2^b$ antigens are targeted by S4B6/IL-2 activated donor $CD8^+$ T cells, leading to an amelioration of disease symptoms. Data supporting this hypothesis is provided by Nguyen et al., showing that stimulation of donor or host $CD8^+$ T cells with IL-21 results in anti-host responses that lead to an amelioration of cGvHD induced by DBA/2 lymphocytes in BDF1 mice [107]. Since there is no disparity in MHC antigens expressed by $CD8^+$ T cells and B cell of the host, other antigenic determinants would be required to make autoreactive B cell a potential target of S4B6/IL-2 stimulated host $CD8^+$ T cells [146].

In conclusion, the use of IL-2 complexes for the treatment of autoimmune disease seems to be critically dependent on the stage of disease. While JES6/IL-2 complexes could mediate disease amelioration only when administrated prophylactically, their therapeutic potential might be critically limited to patients with a very early diagnosis of SLE in the clinics. Thus, together with improved diagnostic tools, the administration of JES6/IL-2 complexes might represent a more specific approach to prevent the development of severe lupus with fatal organ damage. On the other hand, the therapeutic administration of JES6/IL-2 complexes showed no positive clinical effect on the cGvHD symptoms in our experimental model. Due to the strong alloresponses driving the pathology of cGvHD it can't be excluded that therapeutic approaches with JES6/IL-2 complex might be efficient in other lupus model or even human SLE. From experiments examining the therapeutic treatment with S4B6/IL-2 complexes as well as from the depletion experiments we can conclude that, mechanisms leading to disease amelioration critically depend on alloresponses mediated by donor $CD8^+$ T cells. Since no donor $CD8^+$ T cells exist in human SLE and activation of host $CD8^+$ T cells correlates with disease severity due to increased production and availability of self-antigens, a clinical application of S4B6/IL-2 complexes as a therapeutic approach for SLE might be questionable. Based on our results, it seems likely that S4B6/IL-2 complexes impose a risk to augment autoimmune diseases through enhanced stimulation of CD8+ T cells in the host.

GvHD results from the injection of parental lymphocytes into F1 mice. Moreover, DBA/2 lymphocytes injected into BDF1 mice induce cGvHD that resembles SLE in man. Our findings from the prophylactic treatment of cGvHD with IL-2 complexes indicate that host T cells have an influence on the development on this disease. To further establish the role played by host T cells on developing symptoms we generated OT1-BDF1 and OT2-BDF1 mice. These mice have a highly restricted TCR repertoire in both, their $CD4^+$ and $CD8^+$ T cell compartments. Additionally, Treg numbers are substantially reduced in OT1-BDF1 mice compared to OT2-BDF1 and normal BDF1 mice. Thus, these mice represent a good model system to demonstrate the impact of altered host T cell compartments on the development of GvHD.

In the second part of this thesis it was investigated whether alterations in the diversity of the host TCR repertoire has an impact on GvHD resulting from injection of DBA/2 lymphocytes into OT1-BDF1 and OT2-BDF1.

The induction of acute GvHD in T cell repertoire limited, TCR transgenic BDF1 mice was a rather unexpected event that has not been previously described in the GvHD literature. These findings suggest a critical role of host T cells in the inhibition of aGvHD in normal BDF1 mice. In normal BDF1 host mice, donor $CD8^+$ T cells are efficiently controlled leading to cGvHD instead of aGvHD. It seems likely that “full” Treg compartment in the BDF1 host mediates the suppression of donor $CD8^+$ T cells and prevents the development of aGvHD. The observation that a milder form of aGvHD develops in OT2-BDF1 mice provides further evidence for the control of donor $CD8^+$ T cells by host Tregs. The Treg compartment in OT2-BDF1 is significantly larger compared to OT1-BDF1 mice. It’s conceivable that this larger Treg compartment protected OT2-BDF1 mice from the more severe form of aGvHD observed in OT1-BDF1 animals. However, although the Treg

compartment in OT2-BDF1 mice is comparable to normal BDF1 mice in terms of cell numbers the OT2-BDF1 Tregs are unable to efficiently control donor $CD8^+$ T cells and completely inhibit the development of aGvHD. It might be envisaged that is a consequence of the restricted TCR repertoire of $CD4^+$ T cells in OT2-BDF1 mice.

Another reason for the stronger aGvHD in OT1-BDF1 mice might be elevated levels of IL-2 resulting from the lack of IL-2 consumption by Tregs. It was previously shown that deprivation of IL-2 by Tregs is an important mechanism to promote the suppression of $CD8^+$ T cells [147].

The chronic form of GvHD resulting from the injection of $CD8^+$ T cell-depleted DBA/2 lymphocytes into TCR repertoire restricted (i.e. TCR transgenic) BDF1 mice further confirms the requirement for $CD8^+$ T cells in the donor inoculum for the induction aGvHD. When cGvHD was induced in the absence of donor $CD8^+$ T cells, OT1-BDF1 and OT2-BDF1 mice developed stronger disease symptoms especially in regard to kidney pathology compared to normal BDF1 mice. Interestingly, similar to the situation in aGvHD, the cGvHD in OT1-BDF1 mice was also associated with stronger disease symptoms compared to OT2-BDF1. The strikingly rapid development of proteinuria in OT1-BDF1 mice undergoing cGvHD likely reflects their severely impaired Treg compartment. Besides the highly restricted TCR repertoire in OT1-BDF1 mice, the Treg compartment is considerably reduced in absolute numbers in comparison to OT2-BDF1 and normal BDF1 mice.

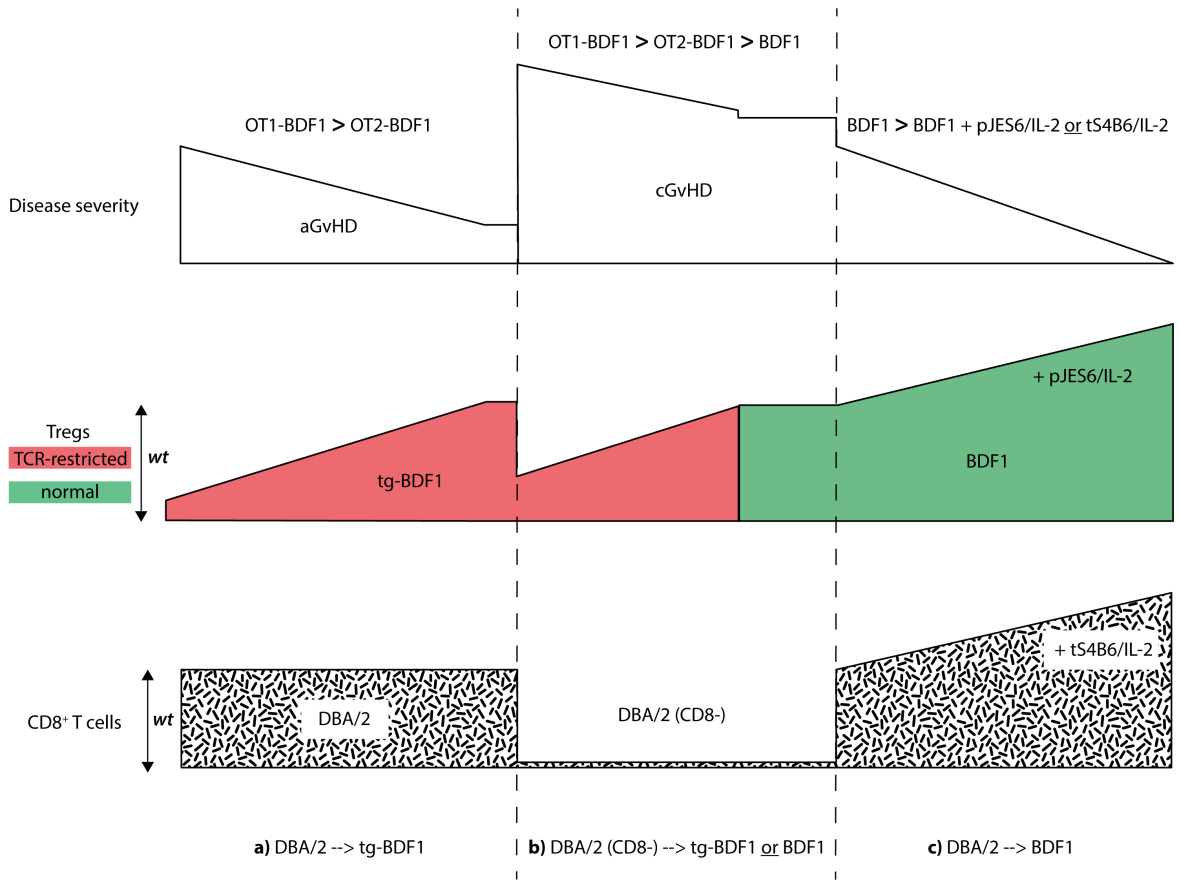
Injection of 10 $x10^6$ DBA/2 lymphocytes depleted of $CD8^+$ T cells (suboptimal dose for the induction of cGvHD in normal recipients) induce low-level cGvHD without the abnormal fast development of proteinuria in OT1-BDF1 mice whereas in OT2-BDF1 and

normal BDF1 mice this dose is not sufficient to induce autoimmune symptoms (preliminary results). These findings further support the notion that the Treg compartment in OT1-BDF1 and OT2-BDF1 mice varies in the capacity to suppress graft-versus-host reactions.

In conclusion, the presented results strongly argue for an important role of host T cells, especially Tregs, in the control of the alloresponses, which induce the development of GvHD.

In summary Graft-versus-Host Diseases are caused by a complex interplay of donor and host cell populations. Which populations dominate and drive these cellular interactions determine the pathology, symptoms and outcome of the disease in an individual patient. Immunologists working on therapies for this set of systemic diseases need to keep these interactions and population dynamics in mind.

The impact of host Tregs and donor CD8⁺ T cells on the developing GvHD is depicted in the following graphical summary:



a) DBA/2 → tg-BDF1

The injection of DBA/2 lymphocytes into TCR restricted BDF1 (tg-BDF1) mice induces aGvHD. The disease is ameliorated in OT2-BDF1 mice having increased numbers of TCR restricted Tregs. Normal numbers (wt) of TCR restricted Tregs do not prevent aGvHD.

b) DBA/2 (CD8-) → tg-BDF1 or BDF1

The injection of DBA/2 lymphocytes depleted of CD8⁺ T cells (DBA/2(CD8-)) induces exacerbated cGvHD in tg-BDF1 and normal BDF1 mice. The disease is ameliorated in OT2-BDF1 mice that have increased numbers of tg-Tregs. The presence of normal Tregs further ameliorates disease symptoms. GvHD in **b)** is characterized by full penetrance of proteinuria within 9 weeks.

c) DBA/2 → BDF1 + pJES6/IL-2 or tS4B6/IL-2

In normal BDF1 mice, the presence of donor CD8⁺ T cells leads to an amelioration of cGvHD. Disease symptoms resulting from this GvHD can be ameliorated by prophylactic JES6/IL-2 treatment (+ pJES6/IL-2) or by therapeutic S4B6/IL-2 treatment (+ tS4B6/IL-2). GvHD in **c)** is characterized by 30 - 40% proteinuria positive mice after 9 weeks. Indicated IL-2 complexes ameliorate GvHD by expansion of the indicated T cell subsets.

* Level of disease severity resulting from GvHD in OT1-BDF1, OT2-BDF1 and normal BDF1 is indicated on top of each section.

** Double-headed arrows indicate size of the depicted T cell populations in wild-type (wt), untreated BDF1 or DBA/2 mice.

References

1. Melchers, F. and A.R. Rolink, *B cell tolerance--how to make it and how to break it.* Curr Top Microbiol Immunol, 2006. **305**: p. 1-23.
2. Nemazee, D.A. and K. Burki, *Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes.* Nature, 1989. **337**(6207): p. 562-6.
3. Gay, D., et al., *Receptor Editing - an Approach by Autoreactive B-Cells to Escape Tolerance.* Journal of Experimental Medicine, 1993. **177**(4): p. 999-1008.
4. Tiegs, S.L., D.M. Russell, and D. Nemazee, *Receptor Editing in Self-Reactive Bone-Marrow B-Cells.* Journal of Experimental Medicine, 1993. **177**(4): p. 1009-1020.
5. Radic, M.Z., et al., *B-Lymphocytes May Escape Tolerance by Revising Their Antigen Receptors.* Journal of Experimental Medicine, 1993. **177**(4): p. 1165-1173.
6. Goodnow, C.C., et al., *Altered Immunoglobulin Expression and Functional Silencing of Self-Reactive Lymphocytes-B in Transgenic Mice.* Nature, 1988. **334**(6184): p. 676-682.
7. Goodnow, C.C., R. Brink, and E. Adams, *Breakdown of self-tolerance in anergic B lymphocytes.* Nature, 1991. **352**(6335): p. 532-6.
8. Goodnow, C.C., et al., *Induction of self-tolerance in mature peripheral B lymphocytes.* Nature, 1989. **342**(6248): p. 385-91.
9. Phan, T.G., et al., *B cell receptor-independent stimuli trigger immunoglobulin (Ig) class switch recombination and production of IgG autoantibodies by anergic self-reactive B cells.* Journal of Experimental Medicine, 2003. **197**(7): p. 845-860.
10. Cyster, J.G. and C.C. Goodnow, *Antigen-induced exclusion from follicles and anergy are separate and complementary processes that influence peripheral B cell fate.* Immunity, 1995. **3**(6): p. 691-701.
11. Rolink, A.G., T. Radaszkiewicz, and F. Melchers, *The autoantigen-binding B cell repertoires of normal and of chronically graft-versus-host-diseased mice.* J Exp Med, 1987. **165**(6): p. 1675-87.
12. Russell, D.M., et al., *Peripheral deletion of self-reactive B cells.* Nature, 1991. **354**(6351): p. 308-11.
13. Akkaraju, S., K. Canaan, and C.C. Goodnow, *Self-reactive B cells are not eliminated or inactivated by autoantigen expressed on thyroid epithelial cells.* Journal of Experimental Medicine, 1997. **186**(12): p. 2005-2012.
14. Kench, J.A., D.M. Russell, and D. Nemazee, *Efficient peripheral clonal elimination of B lymphocytes in MRL/lpr mice bearing autoantibody transgenes.* J Exp Med, 1998. **188**(5): p. 909-17.
15. Rolink, A.G., J. Andersson, and F. Melchers, *Molecular mechanisms guiding late stages of B-cell development.* Immunol Rev, 2004. **197**: p. 41-50.
16. Rolink, A.G., et al., *BAFF is a survival and maturation factor for mouse B cells.* European Journal of Immunology, 2002. **32**(7): p. 2004-2010.
17. Mackay, F., et al., *Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations.* Journal of Experimental Medicine, 1999. **190**(11): p. 1697-1710.
18. Gross, J.A., et al., *TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease: Impaired B cell maturation in mice lacking BLyS.* Immunity, 2001. **15**(2): p. 289-302.

19. Rolink, A.G. and F. Melchers, *BAFFIed B cells survive and thrive: roles of BAFF in B-cell development.* Current Opinion in Immunology, 2002. **14**(2): p. 266-275.
20. Thien, M., et al., *Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches.* Immunity, 2004. **20**(6): p. 785-98.
21. Mathis, D. and C. Benoist, *Aire.* Annual Review of Immunology, 2009. **27**: p. 287-312.
22. Bennett, C.L., et al., *The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3.* Nature Genetics, 2001. **27**(1): p. 20-21.
23. Daniels, M.A., et al., *Thymic selection threshold defined by compartmentalization of Ras/MAPK signalling.* Nature, 2006. **444**(7120): p. 724-729.
24. Abramson, J. and G. Anderson, *Thymic Epithelial Cells.* Annu Rev Immunol, 2017. **35**: p. 85-118.
25. Klein, L., et al., *Antigen presentation in the thymus for positive selection and central tolerance induction.* Nature Reviews Immunology, 2009. **9**(12): p. 833-844.
26. Berg, L.J., et al., *Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand.* Cell, 1989. **58**(6): p. 1035-46.
27. Zinkernagel, R.M. and P.C. Doherty, *Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system.* Nature, 1974. **248**(5450): p. 701-2.
28. Pullen, A.M., P. Marrack, and J.W. Kappler, *The T-Cell Repertoire Is Heavily Influenced by Tolerance to Polymorphic Self-Antigens.* Nature, 1988. **335**(6193): p. 796-801.
29. Barton, G.M. and A.Y. Rudensky, *Requirement for diverse, low-abundance peptides in positive selection of T cells.* Science, 1999. **283**(5398): p. 67-70.
30. Hogquist, K.A., et al., *Identification of a naturally occurring ligand for thymic positive selection.* Immunity, 1997. **6**(4): p. 389-399.
31. Hogquist, K.A., et al., *T-Cell Receptor Antagonist Peptides Induce Positive Selection.* Cell, 1994. **76**(1): p. 17-27.
32. Palmer, E. and D. Naeher, *Affinity threshold for thymic selection through a T-cell receptor-co-receptor zipper.* Nat Rev Immunol, 2009. **9**(3): p. 207-13.
33. Naeher, D., et al., *A constant affinity threshold for T cell tolerance.* Journal of Experimental Medicine, 2007. **204**(11): p. 2553-2559.
34. Murata, S., Y. Takahama, and K. Tanaka, *Thymoproteasome: probable role in generating positively selecting peptides.* Current Opinion in Immunology, 2008. **20**(2): p. 192-196.
35. Nakagawa, T., et al., *Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus.* Science, 1998. **280**(5362): p. 450-3.
36. Zijlstra, M., et al., *Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells.* Nature, 1990. **344**(6268): p. 742-6.
37. Hernandez-Hoyos, G., et al., *Lck activity controls CD4/CD8 T cell lineage commitment.* Immunity, 2000. **12**(3): p. 313-322.
38. Scott, B., et al., *The generation of mature T cells requires interaction of the alpha beta T-cell receptor with major histocompatibility antigens.* Nature, 1989. **338**(6216): p. 591-3.
39. Borgulya, P., et al., *Exclusion and Inclusion of Alpha-T-Cell and Beta-T-Cell Receptor Alleles.* Cell, 1992. **69**(3): p. 529-537.

40. Brandle, D., et al., *Engagement of the T-cell receptor during positive selection in the thymus down-regulates RAG-1 expression.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9529-33.
41. Surh, C.D. and J. Sprent, *T-Cell Apoptosis Detected in-Situ during Positive and Negative Selection in the Thymus.* Nature, 1994. **372**(6501): p. 100-103.
42. Wu, L. and K. Shortman, *Heterogeneity of thymic dendritic cells.* Seminars in Immunology, 2005. **17**(4): p. 304-312.
43. Li, J.C., et al., *Thymus-homing peripheral dendritic cells constitute two of the three major subsets of dendritic cells in the steady-state thymus.* Journal of Experimental Medicine, 2009. **206**(3): p. 607-622.
44. Klein, L. and B. Kyewski, *"Promiscuous" expression of tissue antigens in the thymus: a key to T-cell tolerance and autoimmunity?* Journal of Molecular Medicine-Jmm, 2000. **78**(9): p. 483-494.
45. Werlen, G., et al., *Signaling life and death in the thymus: Timing is everything.* Science, 2003. **299**(5614): p. 1859-1863.
46. Hogquist, K.A., *Signal strength in thymic selection and lineage commitment.* Curr Opin Immunol, 2001. **13**(2): p. 225-31.
47. Kishimoto, H. and J. Sprent, *Several different cell surface molecules control negative selection of medullary thymocytes.* Journal of Experimental Medicine, 1999. **190**(1): p. 65-73.
48. Punt, J.A., et al., *Negative Selection of Cd4(+)Cd8(+) Thymocytes by T-Cell Receptor-Induced Apoptosis Requires a Costimulatory Signal That Can Be Provided by Cd28.* Journal of Experimental Medicine, 1994. **179**(2): p. 709-713.
49. Bevan, M.J., *In a radiation chimaera, host H-2 antigens determine immune responsiveness of donor cytotoxic cells.* Nature, 1977. **269**(5627): p. 417-8.
50. Sha, W.C., et al., *Positive and Negative Selection of an Antigen Receptor on T-Cells in Transgenic Mice.* Nature, 1988. **336**(6194): p. 73-76.
51. Kisielow, P., et al., *Positive selection of antigen-specific T cells in thymus by restricting MHC molecules.* Nature, 1988. **335**(6192): p. 730-3.
52. McGargill, M.A., J.M. Derbinski, and K.A. Hogquist, *Receptor editing in developing T cells.* Nature Immunology, 2000. **1**(4): p. 336-341.
53. Kisielow, P., et al., *Tolerance in T-Cell-Receptor Transgenic Mice Involves Deletion of Nonmature Cd4+8+ Thymocytes.* Nature, 1988. **333**(6175): p. 742-746.
54. Hogquist, K.A., M.A. Gavin, and M.J. Bevan, *Positive Selection of Cd8+ T-Cells Induced by Major Histocompatibility Complex Binding Peptides in Fetal Thymic Organ-Culture.* Journal of Experimental Medicine, 1993. **177**(5): p. 1469-1473.
55. Derbinski, J., et al., *Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self.* Nature Immunology, 2001. **2**(11): p. 1032-1039.
56. Anderson, M.S., et al., *Projection of an immunological self shadow within the thymus by the aire protein.* Science, 2002. **298**(5597): p. 1395-1401.
57. Fontenot, J.D., et al., *Regulatory T cell lineage specification by the forkhead transcription factor FoxP3.* Immunity, 2005. **22**(3): p. 329-341.
58. Sakaguchi, S., *Naturally arising CD4(+) regulatory T cells for immunologic self-tolerance and negative control of immune responses.* Annual Review of Immunology, 2004. **22**: p. 531-562.
59. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.

60. Itoh, M., et al., *Thymus and autoimmunity: Production of CD25(+)CD4(+) naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance.* Journal of Immunology, 1999. **162**(9): p. 5317-5326.
61. D'Cruz, L.M. and L. Klein, *Development and function of agonist-induced CD25(+)Foxp3(+) regulatory T cells in the absence of interleukin 2 signaling.* Nature Immunology, 2005. **6**(11): p. 1152-1159.
62. Hinterberger, M., et al., *Autonomous role of medullary thymic epithelial cells in central CD4(+) T cell tolerance.* Nature Immunology, 2010. **11**(6): p. 512-U80.
63. Tai, X.G., et al., *CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2.* Nature Immunology, 2005. **6**(2): p. 152-162.
64. Aschenbrenner, K., et al., *Selection of Foxp3(+) regulatory T cells specific for self antigen expressed and presented by Aire(+) medullary thymic epithelial cells.* Nature Immunology, 2007. **8**(4): p. 351-358.
65. Swee, L.K., et al., *The amount of self- antigen determines the effector function of murine T cells escaping negative selection.* European Journal of Immunology, 2014. **44**(5): p. 1299-1312.
66. Malhotra, D., et al., *Tolerance is established in polyclonal CD4(+) T cells by distinct mechanisms, according to self-peptide expression patterns.* Nature Immunology, 2016. **17**(2): p. 187-195.
67. Asano, M., et al., *Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation.* Journal of Experimental Medicine, 1996. **184**(2): p. 387-396.
68. Chen, W.J., et al., *Conversion of peripheral CD4(+)CD25(-) naive T cells to CD4(+)CD25(+) regulatory T cells by TGF-beta induction of transcription factor Foxp3.* Journal of Experimental Medicine, 2003. **198**(12): p. 1875-1886.
69. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid.* Journal of Experimental Medicine, 2007. **204**(8): p. 1775-1785.
70. Kretschmer, K., et al., *Inducing and expanding regulatory T cell populations by foreign antigen.* Nature Immunology, 2005. **6**(12): p. 1219-1227.
71. Ivanov, II, et al., *The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells.* Cell, 2006. **126**(6): p. 1121-33.
72. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
73. Lathrop, S.K., et al., *Peripheral education of the immune system by colonic commensal microbiota.* Nature, 2011. **478**(7368): p. 250-U142.
74. Kotzin, B.L., *Systemic lupus erythematosus.* Cell, 1996. **85**(3): p. 303-306.
75. Gladman, D.D., et al., *Increased Frequency of Hla-Drw2 in Sle.* Lancet, 1979. **2**(8148): p. 902-902.
76. Harley, I.T.W., et al., *Genetic susceptibility to SLE: new insights from fine mapping and genome-wide association studies.* Nature Reviews Genetics, 2009. **10**(5): p. 285-290.
77. Bowness, P., et al., *Hereditary C1q Deficiency and Systemic Lupus-Erythematosus.* Quarterly Journal of Medicine, 1994. **87**(8): p. 455-464.
78. Miyara, M., et al., *Human FoxP3(+) regulatory T cells in systemic autoimmune diseases.* Autoimmunity Reviews, 2011. **10**(12): p. 744-755.

79. Alcocervarela, J. and D. Alarconsegovia, *Decreased Production of and Response to Interleukin-2 by Cultured Lymphocytes from Patients with Systemic Lupus-Erythematosus.* Journal of Clinical Investigation, 1982. **69**(6): p. 1388-1392.
80. Shlomchik, M.J., J.E. Craft, and M.J. Mamula, *From T to B and back again: positive feedback in systemic autoimmune disease.* Nature Reviews Immunology, 2001. **1**(2): p. 147-153.
81. Merrill, J.T., et al., *Efficacy and Safety of Rituximab in Moderately-to-Severely Active Systemic Lupus Erythematosus The Randomized, Double-Blind, Phase II/III Systemic Lupus Erythematosus Evaluation of Rituximab Trial.* Arthritis and Rheumatism, 2010. **62**(1): p. 222-233.
82. Furie, R., et al., *A phase III, randomized, placebo-controlled study of belimumab, a monoclonal antibody that inhibits B lymphocyte stimulator, in patients with systemic lupus erythematosus.* Arthritis and Rheumatism, 2011. **63**(12): p. 3918-3930.
83. Navarra, S.V., et al., *Efficacy and safety of belimumab in patients with active systemic lupus erythematosus: a randomised, placebo-controlled, phase 3 trial.* Lancet, 2011. **377**(9767): p. 721-731.
84. Wang, X.B., et al., *Mechanism of action of combined short-term CTLA4Ig and Anti-CD40 ligand in murine systemic lupus erythematosus.* Journal of Immunology, 2002. **168**(4): p. 2046-2053.
85. Boumpas, D.T., et al., *A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis.* Arthritis and Rheumatism, 2003. **48**(3): p. 719-727.
86. Daikh, D.I. and D. Wofsy, *Cutting edge: Reversal of murine lupus nephritis with CTLA4Ig and cyclophosphamide.* Journal of Immunology, 2001. **166**(5): p. 2913-2916.
87. Danion, F., et al., *Efficacy of abatacept in systemic lupus erythematosus: a retrospective analysis of 11 patients with refractory disease.* Lupus, 2016. **25**(13): p. 1440-1447.
88. Perry, D., et al., *Murine Models of Systemic Lupus Erythematosus.* Journal of Biomedicine and Biotechnology, 2011. **2011**: p. 1-19.
89. Andrews, B.S., et al., *Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains.* Journal of Experimental Medicine, 1978. **148**(5): p. 1198-1215.
90. Wakeland, E.K., et al., *Genetic dissection of systemic lupus erythematosus.* Current Opinion in Immunology, 1999. **11**(6): p. 701-707.
91. Cuda, C.M., et al., *Murine lupus susceptibility locus Slela controls regulatory T cell number and function through multiple mechanisms.* Journal of Immunology, 2007. **179**(11): p. 7439-7447.
92. Crampton, S.P., P.A. Morawski, and S. Bolland, *Linking susceptibility genes and pathogenesis mechanisms using mouse models of systemic lupus erythematosus.* Disease Model & Mechanisms, 2014. **7**(9): p. 1033-1046.
93. Cohen, P.L. and R.A. Eisenberg, *Lpr and Gld - Single Gene Models of Systemic Autoimmunity and Lymphoproliferative Disease.* Annual Review of Immunology, 1991. **9**: p. 243-269.
94. Watson, M.L., et al., *Genetic-Analysis of Mrl-Lpr Mice - Relationship of the Fas Apoptosis Gene to Disease Manifestations and Renal Disease-Modifying Loci.* Journal of Experimental Medicine, 1992. **176**(6): p. 1645-1656.

95. Lynch, D.H., et al., *The Mouse Fas-Ligand Gene Is Mutated in Gld Mice and Is Part of a Tnf Family Gene-Cluster.* Immunity, 1994. **1**(2): p. 131-136.
96. Straus, S.E., et al., *An inherited disorder of lymphocyte apoptosis: The autoimmune lymphoproliferative syndrome.* Annals of Internal Medicine, 1999. **130**(7): p. 591-601.
97. Maibaum, M.A., et al., *Lupus susceptibility loci map within regions of BXSB derived from the SB/Le parental strain.* Immunogenetics, 2000. **51**(4-5): p. 370-372.
98. Subramanian, S., et al., *A Tlr7 translocation accelerates systemic autoimmunity in murine lupus.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(26): p. 9970-9975.
99. Munoz, L.E., et al., *The role of defective clearance of apoptotic cells in systemic autoimmunity.* Nature Reviews Rheumatology, 2010. **6**(5): p. 280-289.
100. Merino, R., et al., *H-2-Linked Control of the Yaa Gene-Induced Acceleration of Lupus-Like Autoimmune-Disease in Bxsb Mice.* European Journal of Immunology, 1992. **22**(2): p. 295-299.
101. Izui, S., et al., *The Y chromosome from autoimmune BXSB/MpJ mice induces a lupus-like syndrome in (NZW x C57BL/6)F1 male mice, but not in C57BL/6 male mice.* Eur J Immunol, 1988. **18**(6): p. 911-5.
102. Rolink, A.G. and E. Gleichmann, *Allosuppressor- and allohelper-T cells in acute and chronic graft-vs.-host (GVH) disease. III. Different Lyt subsets of donor T cells induce different pathological syndromes.* Journal of Experimental Medicine, 1983. **158**(2): p. 546-558.
103. Rolink, A.G., S.T. Pals, and E. Gleichmann, *Allosuppressor and allohelper T cells in acute and chronic graft-vs.-host disease. II. F1 recipients carrying mutations at H-2K and/or I-A.* J Exp Med, 1983. **157**(2): p. 755-71.
104. Pals, S.T., T. Radaszkiewicz, and E. Gleichmann, *Allosuppressor- and allohelper-T cells in acute and chronic graft-vs-host disease. IV. Activation of donor allosuppressor cells is confined to acute GVHD.* J Immunol, 1984. **132**(4): p. 1669-78.
105. Van Elven, E.H., et al., *Capacity of genetically different T lymphocytes to induce lethal graft-versus-host disease correlates with their capacity to generate suppression but not with their capacity to generate anti-F1 killer cells. A non-H-2 locus determines the inability to induce lethal graft-versus-host disease.* J Exp Med, 1981. **153**(6): p. 1474-88.
106. Eisenberg, R.A. and C.S. Via, *T cells, murine chronic graft-versus-host disease and autoimmunity.* J Autoimmun, 2012. **39**(3): p. 240-7.
107. Nguyen, V., et al., *CTL-Promoting Effects of IL-21 Counteract Murine Lupus in the Parent -> F1 Graft-versus-Host Disease Model.* Journal of Immunology, 2016. **196**(4): p. 1529-1540.
108. Nguyen, V., et al., *IL-21 Promotes Lupus-like Disease in Chronic Graft-versus-Host Disease through Both CD4 T Cell- and B Cell-Intrinsic Mechanisms.* Journal of Immunology, 2012. **189**(2): p. 1081-1093.
109. Taniguchi, T., et al., *Structure and expression of a cloned cDNA for human interleukin-2.* Nature, 1983. **302**(5906): p. 305-10.
110. Gong, D.P. and T.R. Malek, *Cytokine-dependent blimp-1 expression in activated T cells inhibits IL-2 production.* Journal of Immunology, 2007. **178**(1): p. 242-252.
111. Martins, G.A., et al., *Transcriptional repressor Blimp-1 regulates T cell homeostasis and function.* Nature Immunology, 2006. **7**(5): p. 457-465.

112. Kallies, A., et al., *Transcriptional repressor Blimp-1 is essential for T cell homeostasis and self-tolerance.* Nature Immunology, 2006. **7**(5): p. 466-474.
113. Rutishauser, R.L., et al., *Transcriptional Repressor Blimp-1 Promotes CD8(+) T Cell Terminal Differentiation and Represses the Acquisition of Central Memory T Cell Properties.* Immunity, 2009. **31**(2): p. 296-308.
114. Lenardo, M.J., *Interleukin-2 Programs Mouse Alpha-Beta-Lymphocytes-T for Apoptosis.* Nature, 1991. **353**(6347): p. 858-861.
115. Sadlack, B., et al., *Generalized Autoimmune-Disease in Interleukin-2-Deficient Mice Is Triggered by an Uncontrolled Activation and Proliferation of Cd4(+) T-Cells.* European Journal of Immunology, 1995. **25**(11): p. 3053-3059.
116. Suzuki, H., et al., *Deregulated T-Cell Activation and Autoimmunity in Mice Lacking Interleukin-2 Receptor-Beta.* Science, 1995. **268**(5216): p. 1472-1476.
117. Minami, Y., et al., *The Il-2 Receptor Complex - Its Structure, Function, and Target Genes.* Annual Review of Immunology, 1993. **11**: p. 245-268.
118. Rickert, M., et al., *The structure of interleukin-2 complexed with its alpha receptor.* Science, 2005. **308**(5727): p. 1477-1480.
119. Bosma, G.C., R.P. Custer, and M.J. Bosma, *A Severe Combined Immunodeficiency Mutation in the Mouse.* Nature, 1983. **301**(5900): p. 527-530.
120. Gaffen, S.L., *Signaling domains of the interleukin 2 receptor.* Cytokine, 2001. **14**(2): p. 63-77.
121. Hemar, A., et al., *Endocytosis of Interleukin-2 Receptors in Human T-Lymphocytes - Distinct Intracellular-Localization and Fate of the Receptor Alpha-Chain, Beta-Chain, and Gamma-Chain.* Journal of Cell Biology, 1995. **129**(1): p. 55-64.
122. Boyman, O., et al., *Selective stimulation of T cell subsets with antibody-cytokine immune complexes.* Science, 2006. **311**(5769): p. 1924-1927.
123. Liao, W., et al., *Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages.* Nature Immunology, 2011. **12**(6): p. 551-U247.
124. Fontenot, J.D., et al., *A function for interleukin 2 in Foxp3-expressing regulatory T cells.* Nature Immunology, 2005. **6**(11): p. 1142-1151.
125. Choi, Y.S., et al., *ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6.* Immunity, 2011. **34**(6): p. 932-946.
126. Kundig, T.M., et al., *Immune-Responses in Interleukin-2 Deficient Mice.* Science, 1993. **262**(5136): p. 1059-1061.
127. Williams, M.A., A.J. Tyznik, and M.J. Bevan, *Interleukin-2 signals during priming are required for secondary expansion of CD8(+) memory T cells.* Nature, 2006. **441**(7095): p. 890-893.
128. Obar, J.J., et al., *CD4(+) T cell regulation of CD25 expression controls development of short-lived effector CD8(+) T cells in primary and secondary responses.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(1): p. 193-198.
129. McDermott, D.F. and M.B. Atkins, *Application of IL-2 and other cytokines in renal cancer.* Expert Opinion on Biological Therapy, 2004. **4**(4): p. 455-468.
130. Krieg, C., et al., *Improved IL-2 immunotherapy by selective stimulation of IL-2 receptors on lymphocytes and endothelial cells.* Proceedings of the National Academy of Science of the United States of America, 2010. **107**(26): p. 11906-11911.

131. Gutierrez-Ramos, J.C., et al., *Recovery from autoimmunity of MRL/lpr mice after infection with an interleukin-2/vaccinia recombinant virus.* Nature, 1990. **346**(6281): p. 271-274.
132. Boyman, O., C.D. Surh, and J. Sprent, *Potential use of IL-2/anti-IL-2 antibody immune complexes for the treatment of cancer and autoimmune disease.* Expert Opinion on Biological Therapy, 2006. **6**(12): p. 1323-1331.
133. Rosalia, R.A., et al., *Use of enhanced interleukin-2 formulations for improved immunotherapy against cancer.* Current Opinion in Chemical Biology, 2014. **23**: p. 39-46.
134. Letourneau, S., et al., *IL-2/anti-IL-2 antibody complexes show strong biological activity by avoiding interaction with IL-2 receptor alpha subunit CD25.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(5): p. 2171-2176.
135. Spangler, J.B., et al., *Antibodies to Interleukin-2 Elicit Selective T Cell Subset Potentiation through Distinct Conformational Mechanisms.* Immunity, 2015. **42**(5): p. 815-825.
136. Tomala, J., et al., *In Vivo Expansion of Activated Naive CD8(+) T Cells and NK Cells Driven by Complexes of IL-2 and Anti-IL-2 Monoclonal Antibody As Novel Approach of Cancer Immunotherapy.* Journal of Immunology, 2009. **183**(8): p. 4904-4912.
137. Webster, K.E., et al., *In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression.* Journal of Experimental Medicine, 2009. **206**(4): p. 751-760.
138. Boyman, O., et al., *Selectively expanding subsets of T cells in mice by injection of interleukin-2/antibody complexes: implications for transplantation tolerance.* Transplantation Proceedings, 2012. **44**(4): p. 1032-1034.
139. Mizui, M., et al., *IL-2 Protects Lupus-Prone Mice from Multiple End-Organ Damage by Limiting CD4(-)CD8(-) IL-17-Producing T Cells.* Journal of Immunology, 2014. **193**(5): p. 2168-2177.
140. Gleichmann, E., E.H. Vanelven, and J.P.W. Vanderveen, *A Systemic Lupus-Erythematosus (Sle)-Like Disease in Mice Induced by Abnormal T-B-Cell Cooperation - Preferential Formation of Autoantibodies Characteristic of Sle.* European Journal of Immunology, 1982. **12**(2): p. 152-159.
141. Rolink, A.G., H. Gleichmann, and E. Gleichmann, *Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. VII. Immune-complex glomerulonephritis.* Journal of Immunology, 1983. **130**(1): p. 209-215.
142. Puliaeva, I., R. Puliaev, and C.S. Via, *Therapeutic potential of CD8+ cytotoxic T lymphocytes in SLE.* Autoimmun Rev, 2009. **8**(3): p. 219-23.
143. Chen, F., et al., *The role of host (endogenous) T cells in chronic graft-versus-host autoimmune disease.* J Immunol, 1998. **161**(11): p. 5880-5.
144. Choudhury, A., et al., *The role of host CD4 T cells in the pathogenesis of the chronic graft-versus-host model of systemic lupus erythematosus.* Journal of Immunology, 2005. **174**(12): p. 7600-7609.
145. Rozendaal, L., et al., *Protection from Lethal Graft-Vs-Host Disease by Donor Stem-Cell Repopulation.* European Journal of Immunology, 1992. **22**(2): p. 575-579.
146. Fan, G.C. and R.R. Singh, *Vaccination with minigenes encoding V-H-derived major histo compatibility complex class I-binding Epitopes activates cytotoxic T cells that ablate autoantibody-producing B cells and inhibit lupus.* Journal of Experimental Medicine, 2002. **196**(6): p. 731-741.

147. Chinen, T., et al., *An essential role for the IL-2 receptor in T-reg cell function.* Nature Immunology, 2016. **17**(11): p. 1322-1333.

V. Supplementary Material

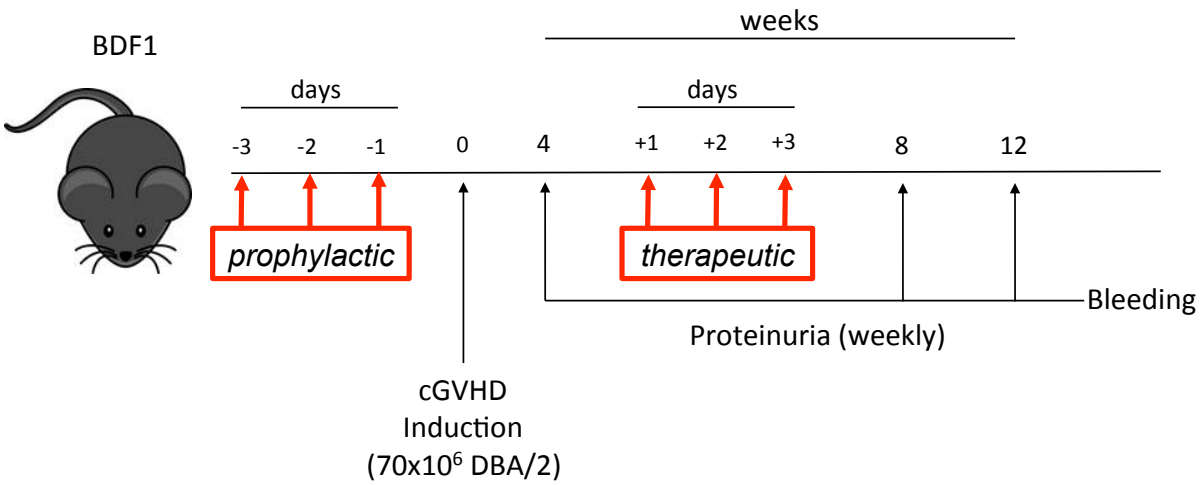


Figure S1: *Protocol for either prophylactic or therapeutic administration of IL-2 complexes*

VI. Appendix

Two Distinct Pathways in Mice Generate Antinuclear Antigen-Reactive B cell Repertoires

Martin Faderl[1,2,†], Fabian Klein[1,†], Oliver F. Wirz[1,3,†], Stefan Heiler[1], Llucia Albertí-Servera[1], Corinne Engdahl[1], Jan Andersson[1,*] and Antonius Rolink[1]

[†]These authors contributed equally to this work

[1]Developmental and Molecular Immunology, Department of Biomedicine, University of Basel, Switzerland

[2]Present address, Experimental Pathology, University of Bern, Switzerland

[3]Present address, Swiss Institute of Allergy and Asthma Research, Davos, Switzerland

frontiers in Immunology

ORIGINAL RESEARCH
published: 22 January 2018
doi: 10.3389/fimmu.2018.00016

Two Distinct Pathways in Mice Generate Antinuclear Antigen-Reactive B Cell Repertoires

Martin Faderl†‡, Fabian Klein‡, Oliver F. Wirz†‡, Stefan Heiler, Llucia Alberti-Servera, Corinne Engdahl, Jan Andersson and Antonius Rolink*

Developmental and Molecular Immunology, Department of Biomedicine, University of Basel, Basel, Switzerland

The escape of anti-self B cells from tolerance mechanisms like clonal deletion, receptor editing, and anergy results in the production of autoantibodies, which is a hallmark of many autoimmune disorders. In this study, we demonstrate that both germline sequences and somatic mutations contribute to autospecificity of B cell clones. For this issue, we investigated the development of antinuclear autoantibodies (ANAs) and their repertoire in two different mouse models. First, in aging mice that were shown to gain several autoimmune features over time including ANAs. Second, in mice undergoing a chronic graft-versus-host disease (GVHD), thereby developing systemic lupus erythematosus-like symptoms. Detailed repertoire analysis revealed that somatic hypermutations (SHM) were present in all Vh and practically all Vl regions of ANAs generated in these two models. The ANA B cell repertoire in aging mice was restricted, dominated by clonally related Vh1-26/Vk4-74 antibodies. In the collection of GVHD-derived ANAs, the repertoire was less restricted, but the usage of the Vh1-26/Vk4-74 combination was still apparent. Germline conversion showed that the SHM in the 4-74 light chain are deterministic for autoreactivity. Detailed analysis revealed that antinuclear reactivity of these antibodies could be induced by a single amino acid substitution in the CDR1 of the Vk4-74. In both aging B6 and young GVHD mice, conversion of the somatic mutations in the Vh and Vl regions of non Vh1-26/Vk4-74 using antibodies showed that B cells with a germline-encoded V gene could also contribute to the ANA-reactive B cell repertoire. These findings indicate that two distinct pathways generate ANA-producing B cells in both model systems. In one pathway, they are generated by Vh1-26/Vk4-74 expressing B cells in the course of immune responses to an antigen that is neither a nuclear antigen nor any other self-antigen. In the other pathway, ANA-producing B cells are derived from progenitors in the bone marrow that express B cell receptors (BCRs), which bind to nuclear antigens and that escape tolerance induction, possibly as a result of crosslinking of their BCRs by multivalent determinants of nuclear antigens.

Keywords: antinuclear antibodies, autoantibodies, monoclonal antibodies, mouse model, systemic lupus erythematosus-like disease, somatic hypermutation

OPEN ACCESS

Edited by:
Karsten Kretschmer, Technische Universität Dresden, Germany

Reviewed by:
Siegfried Weiss, National Research Centre for Biotechnology, Germany
Claudia Berek, Charité Universitätsmedizin Berlin, Germany

****Correspondence:***
Jan Andersson
jan.andersson@unibas.ch

†Present address:
Martin Faderl, Experimental Pathology, University of Bern, Bern, Switzerland; Oliver F. Wirz, Swiss Institute of Allergy and Asthma Research, Davos, Switzerland

‡These authors have contributed equally to this work.

Specialty section:
This article was submitted to Immunological Tolerance and Regulation, a section of the journal Frontiers in Immunology

Received: *05 October 2017*
Accepted: *04 January 2018*
Published: *22 January 2018*

Citation:
Faderl M, Klein F, Wirz OF, Heiler S, Albertí-Servera L, Engdahl C, Andersson J and Rolink A (2018) Two Distinct Pathways in Mice Generate Antinuclear Antigen-Reactive B Cell Repertoires. Front. Immunol. 9:16. doi: 10.3389/fimmu.2018.00016

INTRODUCTION

A hallmark of the autoimmune disease systemic lupus erythematosus (SLE) is the presence of antinuclear autoantibodies (ANAs) in the serum (1–6). These antibodies are directed against histones, DNA, histone–DNA complexes, and various ribonuclear complexes (anti-SM, anti-Ro, and anti-La) (3–6) and may be found in immune complexes that play an important role in the pathogenesis of SLE. The disease occurs more frequently in females than males (ratio 10:1) with a peak incidence at 45–65 years. Based on the findings that ANA producing B cells have undergone Ig class switching and carry large numbers of somatic mutations, it is very likely that ANAs arise from B cells participating in T cell dependent antigen responses (3–6).

Studies using mouse models spontaneously developing an SLE-like disease have improved our knowledge of the etiology of this disease (7–9). In particular, these studies have highlighted the complex genetic contribution to the development of the disease as well as the important role of somatic mutations of antibody genes in the formation of autoantibodies (7–13).

The generation of a self-tolerant B cell repertoire is critically dependent upon the processes of clonal deletion, receptor editing, and anergy (14–19). Exactly how B cells escape central tolerance is, however, still not completely understood. Ample evidence has been provided indicating that non-autoreactive B cells can become autoreactive through somatic mutations in their variable heavy (Vh) and light (Vl) chain regions (6, 10, 13, 20). Equally, B cells using germline-encoded Vh and Vl regions escaping central tolerance induction in the bone marrow could also generate autoreactive B cells (21).

Recently we showed that almost all aging (8–12 months old) C57BL/6 (B6) mice develop several features characteristic of autoimmunity. This included germinal center formations in the spleen, kidney depositions of IgM, lymphocyte infiltrates in the salivary glands, as well as the production of high titers of IgG ANAs. Furthermore, this IgG ANA generation was shown to be T cell dependent (22). However, aging B6 mice do not develop real signs of disease. Here, we compare the ANA B cell repertoire of such aging B6 mice with that of (B6 × B6.*H-2*^{bm12})F_1 mice undergoing a chronic graft-versus-host disease (cGVHD) and thus developing an SLE-like disease and death (23). Results indicate that the ANA B cell repertoire of aging B6 mice is more restricted than that of mice undergoing GVHD and is only partially overlapping. Moreover, we show that the ANA producing B cells in aging mice and in GVHD mice are derived from progenitors expressing B cell receptors (BCRs) either recognizing or not recognizing nuclear antigens. These findings indicate that ANA producing B cells in both aging mice and in GVHD mice are generated by two pathways: by defective tolerance induction in the bone marrow or by hypermutation in the V-regions of B cells responding to a foreign antigen.

MATERIALS AND METHODS

Mice and Induction of cGVHD

C57BL/6 and (C57BL/6 × B6(C)-*H2-Ab*^{bm12}/KhEgJ)F_1 mice were bred under specific pathogen free conditions in our animal unit. A cGVHD was induced by i.v. injection of 8×10^7 spleen plus lymph node cells from B6 mice into 8–10 weeks old B6 × bm12 mice, following established protocols (23).

Generation of Hybridomas

Spleen cells derived from aging B6 mice (aged 8–12 months) or B6 × bm12 mice undergoing a cGVHD were fused to the Sp2/0-Ag14 fusion partner following standard protocols. In brief, 2×10^7 Sp2/0 cells were used for the PEG1500 (Roche Diagnostics)-mediated fusion of all lymphoid cells prepared from an entire spleen. The fused cells were plated into 25 flat-bottom 96-well plates containing 200 µl HAT-medium (2%FBS; GIBCO, 2% IL6, in house, 1× HAT supplement, Sigma) per well and incubated at 37°C in 10% CO_2 in air. After 10–12 days, supernatants were tested for IgG production by ELISA and for antinuclear reactivity by immunofluorescence (see below). Cells from IgG ANA positive wells were thereafter sub-cloned at limiting dilution. We routinely obtained fusion frequencies between 10^{-3} and 5×10^{-3}. Thus, for each mouse, we have screened between 5,000 and 20,000 hybrids for IgG ANA production. Since the vast majority of B cell hybrids produce IgM, we tested for the success of sub-cloning by performing simultaneous ELISA for IgM and IgG (see below) in supernatants of growing clones. The IgG containing supernatants were re-tested for ANA reactivity, before being further processed for Ig V-gene analyses (see below).

Determination of IgG Sub-Class and Anti-Histone/DNA/Sm/SS-B/La Reactivity

Determination of IgG sub-class, L-chain, and detection of anti-DNA antibodies was done by standard ELISA. For determination of anti-histone, anti-Sm or anti-SS-B/La ELISA plates were coated with 2.5 µg/ml of the respective antigens in PBS (all purchased from Immunovision). Alkaline phosphatase labeled goat anti-mouse IgG, goat anti-mouse L-chain or goat anti-human IgG (Southern Biotech) was used for detection. The ELISA was performed as previously described (24–26).

Antinuclear Autoantibody Determination

Kidney cryosections from Rag2$^{-/-}$ mice of homozygous matings were incubated with supernatants or purified antibodies as described in Ref. (27). For detection, either FITC labeled goat anti-mouse IgG (Jackson ImmunoResearch) or FITC labeled rabbit anti-human IgG (Jackson ImmunoResearch) were used.

Vh and Vl Sequencing Analysis

RNA from ANA positive hybridomas was extracted using TRI Reagent (Sigma) followed by cDNA synthesis (GoScript Reverse Transcriptase) according to the manufacturer's protocol and using primers as specified in Table S5 in Supplementary Material. Amplification of the heavy and light chain V-regions was performed using Vent polymerase (New England Biolabs) and, subsequently, the Vh and Vl regions were ligated into the pJet1.2 blunt end cloning vector (Thermo Fisher Scientific). For sequencing, plasmids were sent to Microsynth (Balgach, Switzerland).

Resulting sequences were inspected using DNASTAR and aligned to the germline heavy and light chain sequences of the international ImMunoGeneTics information system® (http://imgt.org).

Reversion of Somatic Mutations Back into Germline Configuration

Double-stranded DNA encoding the variable regions of heavy and light chains obtained from ANA positive hybridomas as well as their germline and mutated versions were ordered as gBlock gene fragments from IDT (Integrated DNA Technologies). These fragments were cloned into heavy and light chain expression vectors driven by the human cytomegalovirus promoter and containing the human IgG_1 constant region for the heavy chain and the human kappa constant region for the light chain (28) (a kind gift from Dr. Hedda Wardemann, Deutsches Krebsforschungszentrum, Heidelberg, Germany). Subsequently, antibodies were produced in HEK 293 cells (ATCC, No. CRL-1573) and purified by affinity chromatography on Protein A Sepharose® (GE Health Care, Uppsala, Sweden) as described (28, 29).

RESULTS

IgG ANA Producing Hybridomas from Aging B6 Mice

Hybridomas were generated independently from spleen cells of seven individual aging B6 mice, which had high titers of serum ANA. The resulting IgG producing hybridomas were then tested for ANA reactivity by immunofluorescence, and positive cultures were sub-cloned. In total, 36 hybridomas producing IgG ANA were generated (**Table 1**). Of these, 16 were IgG_{2a}, 19 were IgG_{2b}, and only one was IgG_1. This heavy chain selection suggests that IgG ANA formation in aging B6 mice is mainly driven by a T_h1 response (30). All ANAs contained a kappa light chain. Sequence analysis of the corresponding Vh and Vk regions used by these hybridomas revealed a restricted repertoire (V, D, and

TABLE 1 | Characteristics of ANA-reactive mAbs derived from aging B6 mice.[a,b]

Mouse number	Hybridoma number	IgG isotype	Ig-heavy chain v-region			Ig-light chain v-region	
Aging B6 # 1	5.D3.D10	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	5.D3. E12	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	7.E8.B11	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	7.F9.F1	IgG2b	Vh1-75	D2-4	J2	Vk?	
	8G3.E6	IgG2a	Vh1-26	D1-1	J4	Vk4-74	Jk4
	8.H7.D3	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	15.G10.F10	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	15.H12.E4	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	16.B9.C9	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	16.D7.A9	IgG2a	Vh1-26	D1-1	J4	Vk4-74	Jk4
	17.A9.E6	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
	22.C9.G7	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk4
Aging B6 # 2	1.G5.B8	IgG1	Vh1-39	D4-1	J1	Vk4-57	Jk5
	8.G9.G4	IgG2b	Vh1-26	D1-1	J4	Vk6-32	Jk2
Aging B6 # 3	13.4.5.A	IgG2a	Vh1-26	D2-4	J3	Vk4-74	Jk2
	13.18.A	IgG2a	Vh1-26	D2-4	J3	Vk4-74	Jk2
	13.27.B	IgG2a	Vh1-26	D2-4	J3	Vk4-74	Jk2
	13.31A	IgG2a	Vh1-26	D2-4	J3	Vk4-74	Jk2
	13.69.B	IgG2a	Vh1-26	D2-4	J3	Vk4-74	Jk2
	13.85B	IgG2a	Vh1-26	D2-4	J3	Vk4-74	Jk2
Aging B6 # 4	1A2.1	IgG2a	Vh1-26	D2-2	J4	Vk4-74	Jk2
	2F8.1	IgG2b	Vh1-26	D1-1	J4	Vk4-74	Jk2
Aging B6 # 5	12.G3.G7	IgG2a	Vh1-26	D1-1	J4	Vk5-43	Jk2
	15.D10B3	IgG2a	Vh1-26	D1-1	J4	Vk5-43	Jk2
Aging B6 # 6	3.2.2A	IgG2b	Vh1-74	D2-4	J3	Vk6-23	Jk5
	3.10.1A	IgG2b	Vh1-26	D1-1	J2	Vk4-61	Jk1
	5.13.1A	IgG2b	Vh1-26	D1-1	J2	Vk4-74	Jk2
	6.15.1A	IgG2a	Vh8-12	D3-1	J1	Vk4-91	Jk2
	7.7.3A	IgG2b	Vh1-22	D2-2	J1	Vk10-96	Jk2
	20.15.1A	IgG2a	Vh1-50	D4-1	J2	Vk4-74	Jk2
	23.6.1A	IgG2a	Vh1-22	D2-2	J1	Vk10-96	Jk2
	24.18.1A	IgG2b	Vh1-22	D2-2	J1	Vk10-96	Jk2
	25.8.1A	IgG2b	Vh3-6	D3-3	J3	Vk4-74	Jk2
Aging B6 # 7	3.25A	IgG2b	Vh1-50	D2-4	J2	Vk?	
	3.36A	IgG2a	Vh1-80	D2-4	J3	Vk4-58	Jk2
	3.73A	IgG2a	Vh1-26	D1-1	J4	Vk4-74	Jk2

[a]*All the mAbs converted into germline sequences are boxed in orange.*
[b]*All the mAbs using Vh1-26 have been boxed in yellow and those using Vk4-74 in red.*

J annotations are according to the IMGT data base). Thus, 25 (69.4%) hybridomas used the Vh1-26 gene and this usage was found among hybridomas of all seven individual mice. Moreover, a very high frequency of Vk4-74 gene usage was also found. Thus, 23 (63.9%) hybridomas, derived from 5 of 7 individual mice, used this particular Vk light chain gene. Strikingly, 21 of the 23 Vk4-74 expressing hybridomas expressed the Vh1-26 heavy chain. Thus, the IgG ANA B cell repertoire of aging B6 mice is dominated by those expressing a Vh1-26 heavy chain gene in combination with a Vk4-74 light chain gene.

We also tested these ANAs for their capacity to bind to histones, Sm antigens, SS-B/La antigens, and DNA. By ELISA, 26 of the 36 showed strong and 5 showed weak histone binding, whereas binding to the other nuclear antigens was undetectable (**Figure 1A**).

In Individual Mice, Hybridomas Using Vh1-26/Vk4-74 Were Clonally Related

The fact that we obtained 11 hybridomas from mouse 1, and 6 hybridomas from mouse 3 using the Vh1-26/Vk4-74 combination with the same D, Jh, and Jk elements already strongly suggested a clonal relationship among the B cells that had fused to generate these hybridomas (**Table 1**). The finding that the amino acid sequences of the IgH and IgL CDR3 regions of these hybridomas were practically identical (Tables S1 and S2 in Supplementary Material) also supports this conclusion. Thus, at least in mouse 1 and 3, the ANA production seems to be dominated by a single B cell clone.

IgG ANA from B6 × bm12 Mice Undergoing a Chronic Graft-versus-Host Reaction

We then asked whether the restricted IgG ANA B cell repertoire in aging B6 mice, which do not show obvious signs of disease, was similarly restricted in B6 × bm12 mice undergoing a chronic graft-versus-host reaction and, thus, developing an SLE-like disease. Hybridomas were independently generated from spleen cells of five mice with the highest ANA titers. These fusions resulted in 34 hybridomas with IgG ANA activity (**Table 2**). A total of 23 ANAs (68%) reacted to histones but again, none reacted to the other nuclear antigens (**Figure 1B**). IgG constant region usage analysis revealed a distribution rather similar to that in aged mice with 18 IgG_{2a}, 13 IgG_{2b}, and only 3 IgG_1, the latter all from mouse 5. Thus, as in aging B6 mice, in B6 × bm12 mice undergoing a cGVHD, the IgG ANA formation appears to be T_h1 cell driven. Again, all ANAs contained a kappa light chain.

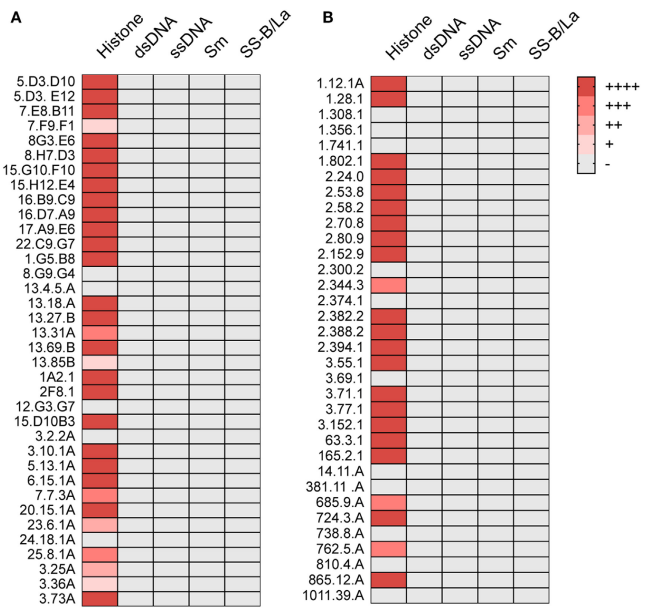


FIGURE 1 | ANA reactivity is directed against histones. Histone, dsDNA, ssDNA, Sm, or SS-B/La binding of ANA-reactive mAbs derived from **(A)** aging B6 (**Table 1**) or **(B)** young B6 × bm12 mice undergoing a cGVH reaction (**Table 2**). Significant binding was defined as an O.D. of five times over background in ELISA. (–), no significant binding at 5 μg/ml; (+), binding at 5 μg/ml; (++), binding at 1.7 μg/ml; (+++), binding at 0.55 μg/ml; and (++++), binding at 0.19 μg/ml or lower.

TABLE 2 | Characteristics of ANA-reactive mAbs derived from graft-versus-host disease (GVHD) mice.[a,b]

Mouse number	Hybridoma number	IgG isotype	Ig-heavy chain v-region			Ig-light chain v-region	
GVHD # 1	1.12.1A	IgG2b	Vh8-8	D2-14	J4	Vk4-74	Jk2
	1.28.1	IgG2a	Vh1-26	D1-1	J1	Vk4-74	Jk2
	1.308.1	IgG2a	Vh1-55	D2-4	J3	Vk3-4	Jk1
	1.356.1	IgG2a	Vh1-55	D2-4	J3	Vk3-4	Jk1
	1.741.1	IgG2a	Vh1-55	D2-4	J3	Vk3-4	Jk1
	1.802.1	IgG2b	Vh1-26	D1-1	J1	Vk4-63	Jk5
GVHD # 2	2.24.0	IgG2a	Vh1-52	D1-1	J1	Vk14-111	Jk5
	2.53.8	IgG2b	Vh14-2	D2-2	J2	Vk14-111	Jk1
	2.58.2	IgG2b	Vh14-2	D2-2	J2	Vk14-111	Jk1
	2.70.8	IgG2a	Vh14-4	D1-1	J3	Vk3-10	Jk1
	2.80.9	IgG2a	Vh14-4	D1-1	J3	Vk3-12	Jk1
	2.152.9	IgG2a	Vh1-52	D1-1	J1	Vk3-10	Jk1
	2.300.2	IgG2b	Vh1-55	D2-1	J2	Vk3-10	Jk5
	2.344.3	IgG2a	Vh1-52	D1-1	J1	Vk14-111	Jk5
	2.374.1	IgG2a	Vh1-26	D1-1	J1	Vk3-7	Jk1
	2.382.2	IgG2a	Vh14-4	D2-1	J3	Vk3-10	Jk1
	2.388.2	IgG2a	Vh1-31	D2-4	J4	Vk3-10	Jk2
	2.394.1	IgG2a	Vh14-4	D1-1	J3	Vk3-10	Jk1
GVHD # 3	3.55.1	IgG2a	Vh1-26	D1-1	J3	Vk4-74	Jk2
	3.69.1	IgG2b	Vh1-26	D1-1	J3	Vk4-74	Jk2
	3.71.1	IgG2a	Vh1-26	D2-5	J1	Vk4-74	Jk2
	3.77.1	IgG2a	Vh1-26	D1-1	J3	Vk4-74	Jk2
	3.152.1	IgG2b	Vh1-26	D1-1	J3	Vk4-74	Jk2
GVHD # 4	63.3.1	IgG2b	Vh1-55	D2-4	J2	Vk4-74	Jk2
	165.2.1	IgG2a	Vh8-12	D2-2	J1	Vk15-103	Jk5
GVHD # 5	14.11.A	IgG2b	Vh1-50	D1-1	J2	Vk1-117	Jk2
	381.11.A	IgG2b	Vh1-54		J4	Vk14-111	Jk2
	685.9.A	IgG2b	Vh1-59	D2-5	J3	Vk17-127	Jk5
	724.3.A	IgG1	Vh1-26	D1-1	J3	Vk4-91	Jk4
	738.8.A	IgG1	Vh1-26	D1-1	J3	Vk3-7	Jk1
	762.5.A	IgG2a	Vh5-17	D1-2	J4	Vk?	
	810.4.A	IgG1	Vh1-52	D2-12	J2	Vk3-10	Jk1
	865.12.A	IgG2b	Vh1-72	D3-3	J2	Vk4-74	Jk5
	1011.39.A	IgG2b	Vh1-53	D1-1	J2	Vk17-127	Jk5

[a]*All the mAbs converted into germline sequences are boxed in orange.*
[b]*All the mAbs using Vh1-26 are boxed in yellow and those using Vk4-74 in red.*

In aging B6 mice, a dominance of Vh1-26 and Vk4-74 usage by the IgG ANA producing hybridomas was observed. In B6 × bm12-derived hybridomas, the same genes were also found to be used, but at a much lower frequency. Thus, ten (29.4%) of these ANAs used Vh1-26 and nine (26.5%) used Vk4-74. Six of the hybridomas using Vk4-74 used the Vh1-26 heavy chain; however, five of these were derived from one mouse (GVHD mouse 3). These findings show that the IgG ANA B cell repertoires of aging B6 mice and B6 × bm12 mice undergoing a cGVHD are partially overlapping. However, the IgG ANA B cell repertoire seems to be more diverse in the B6 × bm12 mice than in the aging B6 mice. Therefore, the mechanisms underlying the generation of these autoreactive B cells might be different in the two model systems.

Somatic Mutations in the Light Chain Determine the ANA Reactivity of Vh1-26/Vk4-74 Using mAbs

Sequence analysis revealed that most mAbs with ANA reactivity carried somatic mutations in their Vh and Vk regions. These results are summarized in Tables S1–S4 in Supplementary Material.

Since in aging B6 mice the ANA reactivity was dominated by mAbs expressing a Vh1-26/Vk4-74 heavy and light chain combination, we tested if somatic mutations in the Vh and/or the Vk regions of these mAbs were required for their autoreactivity. Therefore, the Vh and Vk regions of six (derived from 4 individual mice) mAbs of aging B6 and two mAbs of GVHD mice using the Vh1-26/Vk4-74 combination were reverted to their germline configuration. After expression and purification, the ANA titers of these reverted mAbs were directly compared to their original, mutated forms. As shown in **Figures 2A,B**, the ANA titers of all mAbs, in which the Vh region had been reverted into germline configuration but the Vk regions were still somatically mutated, behaved like the original ANAs, continuing to recognize the histone antigens. However, all mAbs where the Vh was still somatically mutated but the Vk had been reverted to germline configuration lost ANA reactivity. Also, as expected, all mAbs in which both Vh and Vkappa regions had been reverted to germline configuration lost ANA reactivity. Thus, the ANA reactivity of the Vh1-26/Vk4-74 mAbs is due to somatic mutations in the Vk4-74 gene.

Due to the finding that somatic mutations within the Vk4-74 gene determined the ANA reactivity of Vh1-26/Vk4-74 mAbs and in order to identify a common motif that could account for this autoreactivity, we analyzed the sequence of these Vk4-74 genes in more detail. This analysis revealed that 20 of 23 Vh1-26/Vk4-74 mAbs derived from aging B6 mice had a mutation at position 30 (IMGT numbering) in their CDR1 region of the Vk4-74 gene. The germline-encoded serine in these mAbs was mutated into a positively charged arginine residue. Introduction of such a serine to arginine mutation in non-autoreactive germline versions of three different mAbs resulted in a complete gain of ANA reactivity for all of them (**Figure 2C**). Thus, antinuclear reactivity of these mAbs can be induced by a single base pair substitution changing the serine at position 30 in the CDR1 of the Vk4-74 light chain into an arginine.

B Cells Expressing a Germline-Encoded Immunoglobulin Vh Gene with a Negatively Charged CDR2 Region Contribute to the ANA-Reactive B Cell Repertoire

We also tested if non-Vh1-26/Vk4-74 using mAbs require somatic mutations in their Vh and/or Vk regions for ANA reactivity. Therefore, the Vh and Vk region of three mAbs of aging B6 mice and six mAbs of the GVHD mice were reverted to their germline configuration. The 1.G5.B8 mAb derived from an aging B6 mouse (No. 2) used a somatically mutated Vh region and a germline-encoded Vk region. Reversion of the Vh region of this mAb into germline sequences resulted in a complete abrogation of its ANA reactivity (**Figure 3A**). In contrast, upon reversion, the other two mAbs (7.7.3A and 6.15.1A, both from mouse 6) kept their ANA reactivity (**Figure 3A**). Thus, B cells expressing a germline-encoded immunoglobulin Vh can also contribute to the ANA-reactive B cell repertoire in aging B6 mice.

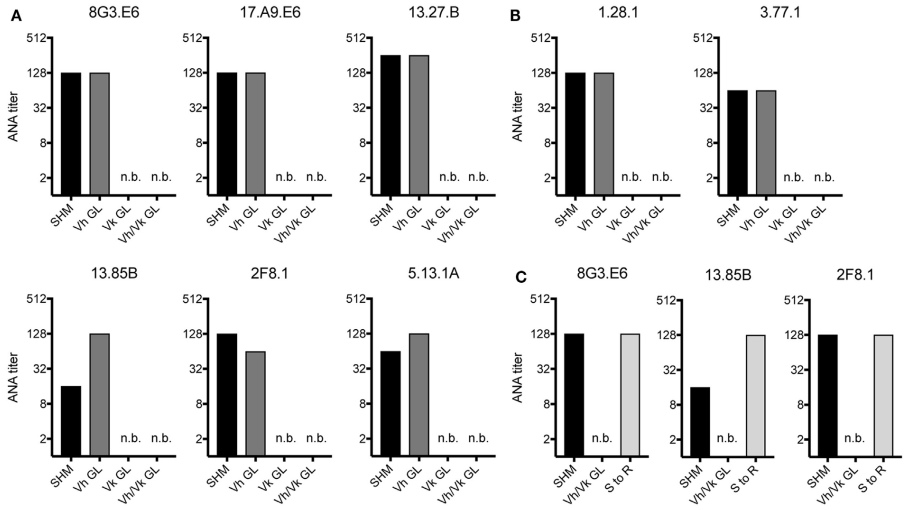


FIGURE 2 | ANA reactivity of Vh1-26/Vk4-74 using mAbs is caused by mutations in the light chain. ANA titers of different Vh1-26/Vk4-74 mAbs derived from **(A)** aging B6 (**Table 1**) or **(B)** young B6 × bm12 mice undergoing a cGvH reaction (**Table 2**). The histograms depict titers of individual mAbs, either with the original SHM, or Vh and/or Vk in germline configuration (GL), or **(C)** having a single mutation in their Vk CDR1 resulting in a serine to arginine conversion. A titer of one was defined as binding at a concentration of 5 μg/ml mAb, followed by 1:2 serial dilution steps and recording the last dilution where binding still was possible. SHM, somatic hypermutation; GL, germline; n.b., no binding detectable at 5 μg/ml of mAb.

From the six GVHD-derived mAbs, one (1.12.1A, mouse 1) lost ANA reactivity upon reversion of the Vk but not the Vh gene into germline configuration (**Figure 3B**). The fact that this mAb uses the Vk4-74 gene indicates that this Vk gene can also give rise to ANA reactivity when paired with a Vh gene other than Vh1-26. The other five mAbs kept their ANA reactivity upon reversion of their Vh and Vk genes to germline configuration (**Figure 3B**). Thus, B cells expressing a germline-encoded immunoglobulin can also contribute to the ANA-reactive repertoire in mice undergoing a cGVHD.

A remarkable observation was that 5 of 7 mAbs (one from aging B6 mice and four from GVHD mice), which kept ANA reactivity upon complete reversion of their Ig genes into germline configuration, had a negatively charged Vh-CDR2 region as defined by having at least two more negatively than positively charged amino acids in this region (see **Table 3**). This observation prompted us to test if these negatively charged amino acids are involved in ANA reactivity. Therefore, the negatively charged amino acids in the CDR2 regions of these five mAbs were mutated into neutral glycine. As shown in **Figure 3C**, one mAb derived from an aging B6 mouse (6.15.1A) and two from a GVHD mouse (2.58.2 and 2.394.1), completely lost ANA reactivity upon aspartic acid/glutamic acid conversion into glycine. The ANA titers of the other two GVHD-derived mAbs (2.344.3 and 165.2.1) diminished by a factor of 2 and 3, respectively (**Figure 3C**). Thus, B cells expressing a germline-encoded immunoglobulin with a negatively charged Vh-CDR2 region contribute to the ANA-reactive repertoire. At this point, it is noteworthy that 6 of 11 (54.6%) of the non-Vh1-26 using ANA mAbs from aging B6 mice and 14 of 24 (58.3%) from mice undergoing cGVHD indeed carry a negatively charged Vh-CDR2 (**Table 3**).

DISCUSSION

Two Pathways to ANA-Producing B Cells

In the present study, we compared antinuclear autoantibodies (ANAs) from aging B6 mice, which do not show signs of disease, with those derived from mice undergoing a chronic GVH reaction (GVHD mice) and which develop an SLE-like disease (23). In order to study the V-regions of ANAs, we generated B cell hybridomas from mice with high ANA titers in their serum. Almost all monoclonal ANAs bound to a mixture of histones. As expected from previous studies, ANA production was T cell and antigen dependent (1–3, 22), since virtually all V-regions of our monoclonal ANAs contained somatic mutations. To determine whether the ANA-producing B cells were derived from progenitors expressing B cell receptors (BCRs) for nuclear antigens, we reverted the mutated V-regions into the corresponding germline sequences and tested the resulting antibodies for ANA reactivity. Based on such analyses, we found that some ANA-producing B cells in both aging B6 mice and GVHD mice must be derived

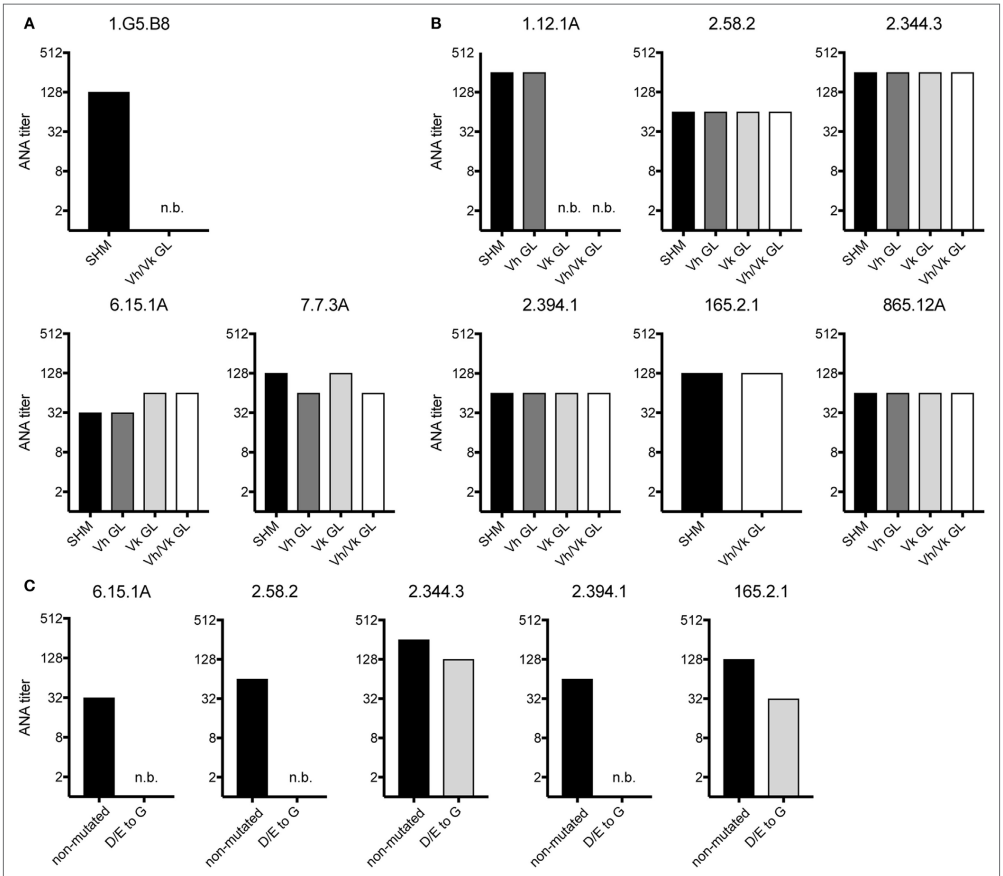


FIGURE 3 | ANA reactivity of non-Vh1-26/Vk4-74 using mAbs is mainly germline encoded. ANA titers of different non-Vh1-26/Vk4-74 mAbs derived from **(A)** aging B6 or **(B)** young B6 × bm12 mice undergoing a chronic graft-versus-host disease. The histograms depict titers of individual mAbs listed in **Table 3** containing V-regions, either with the original somatic hypermutations (SHM), or Vh and/or Vk in germline configuration (GL), or **(C)** containing a Vh CDR2 with conversions of aspartic and glutamic acids to glycine. Titers and legends as defined in **Figure 2**.

from progenitors expressing germline-encoded genes for BCRs specific for nuclear antigens, while several others were derived from progenitors expressing BCRs that showed no ANA reactivity. The vast majority of the latter were derived from progenitors using Vh1-26 in combination with Vk4-74. Antibodies with the germline sequences of these V-regions had no ANA reactivity. It has been shown previously that non-autoreactive B cells can become autoreactive upon acquiring somatic mutations in their Vh and/or Vl regions (6, 10, 13, 20). In contrast to the findings with Vh1-26/Vk4-74 expressing ANAs, the reversion of other Vh and Vk regions of several monoclonal ANAs to the corresponding germline sequences did not abolish ANA reactivity. The maintenance of ANA reactivity upon reversion into germline sequences was observed with 2 of 3 monoclonal ANAs from aging B6 mice, and with 5 of 6 monoclonal ANAs from GVHD mice. Thus, two pathways can be envisaged for the generation of ANA specific antibody forming B cells (**Figure 4**). In pathway one, they are derived from B cells undergoing somatic hypermutations in response to a foreign antigen (**Figure 4**, red line); in pathway two, they are derived from B cell progenitors expressing ANA-specific receptors that escape tolerance induction in the bone marrow, possibly by exposure to BCR crosslinking by nuclear antigens (**Figure 4**, blue line). Indeed, we showed previously that anti-dsDNA reactive B cells escaped tolerance

TABLE 3 | Vh-CDR2 regions of non-Vh1-26 ANA-reactive mAbs of graft-versus-host disease (GVHD) and aging B6 mice.[a,b]

Mouse number	Hybridoma number	Vh-region	Vh-CDR2 region	Mouse number	Hybridoma number	Vh-region	Vh-CDR2 region
GVHD # 1	1.12.1A	Vh8-8	I W W **D D D**	Aging B6 # 1	7.F9.F1	Vh1-75	I L P G S G S S
	1.308.1	Vh1-55	I Y P G S G S T				
	1.356.1	Vh1-55	I Y P G S G S T	Aging B6 # 2	1.G5.B8	Vh1-39	V N P N Y G T I
	1.741.1	Vh1-55	I Y P G S G S T				
				Aging B6 # 6	3.2.2A	Vh1-74	I H P S **D** S **D** T
GVHD # 2	2.24.0	Vh1-52	I **D** P S **D** G **E** T	Aging B6 # 6	6.15.1A	Vh8-12	I Y W **D D D K**
	2.53.8	Vh14-2	I **D** P **E D** G **E** T		7.7.3A	Vh1-22	I N P N N G G T
	2.58.2	Vh14-2	I **D** P **E D** G **E** S		20.15.1A	Vh1-50	I **D** P S **D** T F T
	2.70.8	Vh14-4	I **D** P **E** N G **D** T		23.6.1A	Vh1-22	I N P N N G **D** T
	2.80.9	Vh14-4	I **D** P **E** N G **D** T		24.18.1A	Vh1-22	I N P N N **D D** T
	2.152.9	Vh1-52	I **D** P S **D** G **E** T		25.8.1A	Vh3-6	I S C **D** G S S
	2.300.2	Vh1-55	I Y P G S V S T				
	2.344.3	Vh1-52	I **D** P S **D D E** T	Aging B6 # 7	3.25A	Vh1-50	I **D** P S **D** T Y T
	2.382.2	Vh14-4	I **D** P **E** N G **D** T	Aging B6 # 7	3.36A	Vh1-80	I Y P G **D** G **D** T
	2.388.2	Vh1-31	I F P Y N G V S				
	2.394.1	Vh14-4	I **D** P **E** N G **D** T				
GVHD # 4	63.3.1	Vh1-55	I Y P G S G S T				
	165.2.1	Vh8-12	I Y W **D D D K**				
GVHD # 5	14.11.A	Vh1-50	I **D** P S **D** S Y I				
	381.11.A	Vh1-54	I N P G S G G I				
	685.9.A	Vh1-59	I **D** P S **D** S S S				
	762.5.A	Vh5-17	I S **R** G S G I L				
	810.4.A	Vh1-52	I **D** P S **D** S **E** T				
	865.12.A	Vh1-72	I **D** P S S G G T				
	1011.39.A	Vh1-53	I N P S N **D** G T				
	1011.39.A	Vh1-53	I N P S N **D** G T				

Bold font highlights negatively and positively charged amino acids.
[a]All mAbs that were converted into germline sequences or in which the negatively charged amino acids in the CDR2 were converted into glycine are marked with orange boxes.
[b]All the negatively charged amino acids in Vh-CDR2 region are marked in yellow and the positively charged amino acids in green.

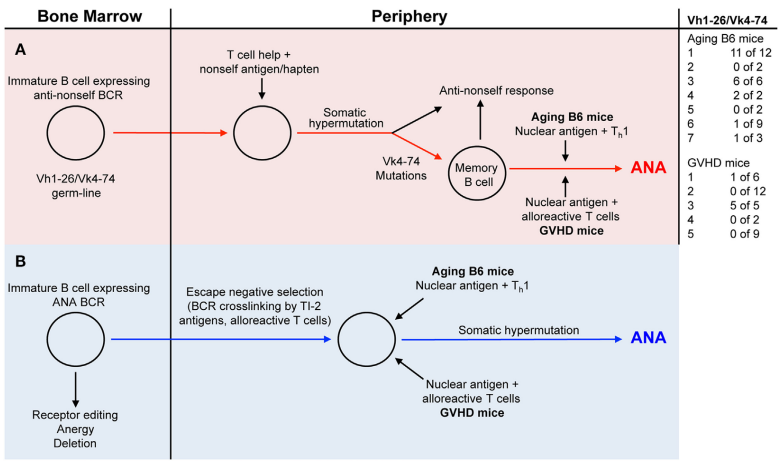


FIGURE 4 | Two distinct pathways generate ANA producing B cells. [**(A)** red background] B cells with a B-cell receptor (BCR) using a germline-encoded Vh1-26 in combination with a germline-encoded Vk4-74 gets stimulated by a non-self (NS) antigen/hapten. Due to somatic hypermutations in the Vk4-74 gene, ANA-reactive memory B cells are generated. In aging B6 mice, these memory cells are then activated by nuclear antigens and T_h1 cells to produce ANA. In the GVHD mice, nuclear antigens and alloreactive T cells activate these memory cells, resulting in ANA formation. [**(B)** blue background] Immature B cells in the bone marrow with germline-encoded BCRs specific for nuclear antigens escape tolerance induction mechanisms through BCR cross-linking by a T cell independent type 2-antigen (TI-2) antigens or a signal from alloreactive T cells. In the periphery of aging B6 mice, these cells get stimulated by nuclear antigens and T_h1 cells to produce ANAs. In the periphery of GVHD mice, nuclear antigens and alloreactive T cells activate these cells to ANA formation.

induction by the crosslinking of their BCRs by a T cell independent type 2-antigen (24).

Restricted Diversity of ANA Specific B Cells

Inspection of the V-region usage of ANA producing hybridomas shows that many ANAs derived from aging mice (21 of 36) (**Table 1**; **Figure 4**) and few from mice undergoing a cGVHD (6 of 34) (**Table 2**; **Figure 4**) express Vh1-26 in combination with Vk4-74. Interestingly this Vh/Vk combination is expressed in virtually all ANAs from aging mouse 1 (11 of 12), aging mouse 3 (6 of 6), and from the GVHD mouse 3 (5 of 5). Inspection of the V-regions strongly suggests that these ANAs are generated from single B cell clones with the exception of one clone of the GVHD mouse 3, which differs in the CDR3 compared to the other four. In other mice, ANAs are clearly generated from multiple B cell clones. In aging mouse 6, one ANA expresses the Vh1-26/Vk4-74 combination, one expresses the Vh1-26 with Vk4-61, two express the Vk4-74 with Vh1-50 and Vh3-6, respectively, and five express different Vh/Vk combinations. In GVHD mice, all except one mouse (No. 3) express ANAs derived from multiple B cell clones. Thus, in aging B6 mice, the ANA repertoire tends to be restricted, while in GVHD mice, the ANA repertoire tends to be less restricted. The more important question to be answered is why ANAs are oligoclonal in some mice and polyclonal in others. One reason for this restricted repertoire might be that Vh1-26 and Vk4-74 genes are overrepresented in the B cell repertoire of B6 mice in general. RNA sequence analysis of Vh1 usage by B cells from young and aging B6 mice showed that around 10% of these used Vh1-26 (our unpublished results). Since about 50% of all B cells use a Vh1 family member, Vh1-26 is expressed by 5% of them. We did not attempt to verify this number in the hybrids generated, by, for example, cloning and sequencing the Vh regions of random IgM or IgG antibodies not reacting to nuclear antigens. With respect to Vk4-74 usage, Aoki-Ota et al. (31) analyzed the kappa light chain repertoire in B6 mice. This analysis revealed that B6 B cells rarely use the Vk4-74 gene. We also have determined the Vk usage in developing B6 B cells and found that Vk4-74 was used less than 1% in single Vk-rearrangements of preBII cells and immature B cells of the bone marrow [unpublished observation, (32)]. Thus, the restricted ANA-reactive B cell repertoire does not simply appear to reflect a selective usage of the Vh1-26 and the Vk4-74 genes by the B6 B cells. Instead, and more interestingly, this restricted ANA repertoire may occur through selection by and as yet to be defined T cell dependent antigen.

Many years ago, it was shown that haptens such as NP (4-hydoxy-3-nitrophenyl)acetyl (33, 34), oxazolone (2-phenyl-5-oxazolone) (35, 36), and arsonate (p-azophenyl-arsonate) (37, 38) could elicit an oligoclonal humoral immune response, at least in certain inbred strains of mice. Based on this, one might envisage that a hapten-like structure is responsible for the restricted ANA-reactive B cell repertoire in aging B6 mice. Thus, one could imagine that the frequency of ANA-producing B cells that are derived from hapten-induced memory cells in aging mice (red pathway in **Figure 4**) is higher than the frequency of B cell progenitors with receptors for nuclear antigens that escape tolerance induction in the bone marrow (blue pathway in **Figure 4**).

Activation of ANA-Producing B Cells

In ANA-producing mice, B cells expressing nuclear antigen-specific BCRs must be activated by nuclear antigens and helper T cells. In GVHD mice, the alloreactive T cells act as helper T cells. In their initial report on the "allogeneic effect," Katz et al. reported a drastic enhancement of IgG responses as a result of a GVH reaction (39). Later, Osborne and Katz showed that a simple non-immunogenic hapten–polypeptide conjugate might elicit a vigorous primary IgG response as a consequence of the allogeneic effect (40). Likewise, Hamilton and Miller reported that otherwise tolerogenic hapten-conjugated syngeneic mouse erythrocytes would elicit a strong primary antibody response as a result of a GVH reaction (41). It is possible that this type of general B cell help by alloreactive T cells contributes to the tendency of the ANA response in cGVHD mice to be more diverse.

In aging mice, the specificity and origin of the T helper cells that are required for ANA production is not known. Based on the IgG class of the ANAs produced in these mice, it is conceivable that the helper cells involved in the ANA response are T_h1 cells (30).

Conclusions Regarding the Structure of Anti-Histone Antibodies

Almost 30 years ago, Weigert and coworkers showed that arginine residues in CDR regions play a crucial role for the specificity of anti-dsDNA autoantibodies (10, 11). In the present study, we find an important role of arginine in the Vk-region of certain ANAs. Site-directed mutagenesis revealed that somatic mutations in the Vk4-74 light chains of the Vh1-26/Vk4-74 mAbs determine ANA reactivity, since conversion into their germline sequence completely abolished ANA reactivity. Interestingly, and highly significant, 20 of 23 Vh1-26/Vk4-74-expressing ANAs derived from aging B6 mice had a serine to arginine mutation at position 30 in the CDR1 region of the Vk4-74 gene. In 1 of these 23 mAbs, this change to arginine was the only mutation present in the entire Vk4-74 gene. The finding that one of these (17A9E6) mAb loses ANA-binding activity upon conversion of this arginine into serine strongly indicates the importance of this mutation for ANA reactivity. Furthermore, the introduction of single serine to arginine mutations in the germline configuration of three different mAbs conferred their antinuclear reactivity. This proves that this single mutation can convert an ANA-negative mAb into a positive one and clearly shows how easily Vh1-26/Vk4-74 using mAbs may become autoantibodies. The ANA-reactive B cell repertoire has also been analyzed in B6 mice congenic for a SLE susceptibility locus. Also this analysis revealed a rather frequent usage of Vk4-74 light chains. Thus, Liang et al. (12) found that 9 of 30 ANA producing hybridomas used Vk4-74 light chain (herein called *ai4*). Moreover, it was shown that 3 of these 9 had a serine to arginine mutation at position 30 in the CDR1. In a report by Guo et al. (13), 9 of 33 ANA-producing hybridomas used a Vk4-74 light chain (herein also called *ai4*). At least 5 of these 9 had a serine to arginine mutation at position 30 in the CDR1, like we report herein.

However, since 5 of 6 Vh1-26/Vk4-74 using mAbs derived from the GVHD mice did not have this serine to arginine mutation, other mutations in the Vk4-74 gene can be involved in ANA reactivity.

Yet, another interesting finding reported here is that a high percentage of ANA-reactive mAbs not using the Vh1-26 gene, especially in the repertoire derived from GVHD mice, possess a germline-encoded negatively charged Vh CDR2 region. Thus, 14 of the total 34 monoclonal ANAs (41.2%) derived from the GVHD mice and 6 of 36 (16.7%) derived from the aging B6 mice have such a CDR2 region. Within the total Vh gene repertoire of B6 mice, 11.5% possess such a negatively charged CDR2 (42). Thus, our findings suggest that B cells with such a charged CDR2 might be positively selected into the ANA-reactive B cell repertoire, especially in GVHD mice. The finding that mutations of these negatively charged amino acids into glycine can result in a complete loss of ANA binding supports this hypothesis and, moreover, indicates the potential importance of this region in ANA reactivity. However, additional structural effects of the amino acid change to glycine cannot be excluded, especially since glycine is known to be a helix breaker.

It is noteworthy that despite high ANA serum titers, aging B6 mice do not show the typical lesions that are observed in multiple organs of SLE patients, whereas the GVHD mice do show this. Therefore, one could envisage that the ANA repertoire difference between aging B6 and GVHD mice is playing a role in the pathogenesis of the SLE-like disease observed in GVHD mice.

Overall, the findings described in the present study highlight various new characteristics of ANA-reactive B cell repertoires and argue for similar mechanisms of ANA generation in SLE patients. This might improve our understanding of the pathogenesis of this disease, thereby opening new concepts and therapies for its control.

ETHICS STATEMENT

The State veterinary authorities of Basel (Kantonales Veterinäramt, Basel-Stadt) had approved all animal experiments under permission numbers 1888 and 2434.

DATA AVAILABILITY

Sequencing data can be found in GenBank under accession numbers MG733774—MG733908.

AUTHOR CONTRIBUTIONS

AR conceived the study and designed experiments. All authors performed experiments and analyzed the data. AR, JA, and FK wrote the paper.

ACKNOWLEDGMENTS

We thank Drs. Rhodri Ceredig, Werner Haas, Fritz Melchers, Michael Parkhouse, and Panagiotis Tsapogas for helpful comments and critical reading of the manuscript. We thank Ricardo Koch, Hannie Rolink, and Mike Rolink for skillful technical assistance. This paper is dedicated to the memory of Antonius (Ton) Rolink, our principal investigator and professor, who unexpectedly died during the preparation of this manuscript.

FUNDING

AR is holder of the chair in Immunology endowed by F. Hoffmann-La Roche Ltd., Basel to the University of Basel. This study was supported by the Swiss National Science Foundation (310030B_160330/1). LA-S was supported by the People Program (Marie Curie Actions) of the European Union's Seventh Framework Program FP7/2007-2013 under Research Executive Agency Grant 315902.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fimmu.2018.00016/full#supplementary-material.

TABLES S1 AND S2 | Nucleotide sequences and their translation into amino acids of Vh- and Vk-regions of all hybridomas described herein, which were derived from aging B6 mice.

TABLES S3 AND S4 | Nucleotide sequences and their translation into amino acids of Vh- and Vk-regions of all hybridomas described herein, which were derived from young B6 × bm12 mice undergoing a GvH reaction.

TABLE S5 | Sequences of primers used for cDNA synthesis of total mRNA obtained from ANA-reactive hybridomas. The sequences of primers used for sequencing of Vh- and Vk-regions of the obtained cDNA are also shown.

REFERENCES

1. Kotzin BL. Systemic lupus erythematosus. *Cell* (1996) 85:303–6. doi:10.1016/S0092-8674(00)81108-3
2. Kaul A, Gordon C, Crow MK, Touma Z, Urowitz MB, van Vollenhoven R, et al. Systemic lupus erythematosus. *Nat Rev Dis Primers* (2016) 2:16039. doi:10.1038/nrdp.2016.39
3. Elkon K, Casali P. Nature and functions of autoantibodies. *Nat Clin Pract Rheumatol* (2008) 4:491–8. doi:10.1038/ncprheum0895
4. Tan EM. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv Immunol* (1989) 44:93–151. doi:10.1016/S0065-2776(08)60641-0
5. Hahn BH. Antibodies to DNA. *N Engl J Med* (1998) 338:1359–68. doi:10.1056/NEJM199805073381906
6. Schroeder K, Herrmann M, Winkler TH. The role of somatic hypermutation in the generation of pathogenic antibodies in SLE. *Autoimmunity* (2013) 46:121–7. doi:10.3109/08916934.2012.748751
7. Theofilopoulos AN, Dixon FJ. Murine models of systemic lupus erythematosus. *Adv Immunol* (1985) 37:269–390. doi:10.1016/S0065-2776(08)60342-9
8. Wakeland EK, Morel L, Mohan C, Yui M. Genetic dissection of lupus nephritis in murine models of SLE. *J Clin Immunol* (1997) 17:272–81. doi:10.1023/A:1027370514198
9. Morel L, Mohan C, Yu Y, Croker BP, Tian N, Deng A, et al. Functional dissection of systemic lupus erythematosus using congenic mouse strains. *J Immunol* (1997) 158:6019–28.
10. Shlomchik M, Mascelli M, Shan H, Radic MZ, Pisetsky D, Marshak-Rothstein A, et al. Anti-DNA antibodies from autoimmune mice arise by clonal expansion and somatic mutation. *J Exp Med* (1990) 171:265–92. doi:10.1084/jem.171.1.265
11. Radic MZ, Weigert M. Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu Rev Immunol* (1994) 12:487–520. doi:10.1146/annurev.iy.12.040194.002415
12. Liang Z, Chang S, Youn MS, Mohan C. Molecular hallmarks of anti-chromatin antibodies associated with the lupus susceptibility locus, Sle1. *Mol Immunol* (2009) 46:2671–81. doi:10.1016/j.molimm.2008.12.034

13. Guo W, Smith D, Aviszus K, Detanico T, Heiser RA, Wysocki LJ. Somatic hypermutation as a generator of antinuclear antibodies in a murine model of systemic autoimmunity. *J Exp Med* (2010) 207:2225–37. doi:10.1084/jem.20092712
14. Radic MZ, Erikson J, Litwin S, Weigert M. B lymphocytes may escape tolerance by revising their antigen receptors. *J Exp Med* (1993) 177:1165–73. doi:10.1084/jem.177.4.1165
15. Tiegs SL, Russell DM, Nemazee D. Receptor editing in self-reactive bone marrow B cells. *J Exp Med* (1993) 177:1009–20. doi:10.1084/jem.177.4.1009
16. Nemazee D. Receptor editing in lymphocyte development and central tolerance. *Nat Rev Immunol* (2006) 6:728–40. doi:10.1038/nri1939
17. Goodnow CC, Sprent J, Fazekas de St Groth B, Vinuesa CG. Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* (2005) 435:590–7. doi:10.1038/nature03724
18. Cambier JC, Gauld SB, Merrell KT, Vilen BJ. B-cell anergy: from transgenic models to naturally occurring anergic B cells? *Nat Rev Immunol* (2007) 7:633–43. doi:10.1038/nri2133
19. Wardemann H, Hammersen J, Nussenzweig MC. Human autoantibody silencing by immunoglobulin light chains. *J Exp Med* (2004) 200:191–9. doi:10.1084/jem.20040818
20. Diamond B, Scharff MD. Somatic mutation of the T15 heavy chain gives rise to an antibody with autoantibody specificity. *Proc Natl Acad Sci U S A* (1984) 81:5841–4. doi:10.1073/pnas.81.18.5841
21. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science* (2003) 301:1374–7. doi:10.1126/science.1086907
22. Nusser A, Nuber N, Wirz OF, Rolink H, Andersson J, Rolink A. The development of autoimmune features in aging mice is closely associated with alterations of the peripheral CD4(+) T-cell compartment. *Eur J Immunol* (2014) 44:2893–902. doi:10.1002/eji.201344408
23. Rolink AG, Pals ST, Gleichmann E. Allosuppressor and allohelper T cells in acute and chronic graft-vs.-host disease. II. F1 recipients carrying mutations at H-2K and/or I-A. *J Exp Med* (1983) 157:755–71. doi:10.1084/jem.157.2.755
24. Andersson J, Melchers F, Rolink A. Stimulation by T cell independent antigens can relieve the arrest of differentiation of immature auto-reactive B cells in the bone marrow. *Scand J Immunol* (1995) 42:21–33. doi:10.1111/j.1365-3083.1995.tb03621.x
25. Rolink AG, Radaszkiewicz T, Melchers F. The autoantigen-binding B cell repertoires of normal and of chronically graft-versus-host-diseased mice. *J Exp Med* (1987) 165:1675–87. doi:10.1084/jem.165.6.1675
26. Wellmann U, Werner A, Winkler TH. Altered selection processes of B lymphocytes in autoimmune NZB/W mice, despite intact central tolerance against DNA. *Eur J Immunol* (2001) 31:2800–10. doi:10.1002/1521-4141(200109)31:9<2800::AID-IMMU2800>3.0.CO;2-E
27. Benard A, Ceredig R, Rolink AG. Regulatory T cells control autoimmunity following syngeneic bone marrow transplantation. *Eur J Immunol* (2006) 36:2324–35. doi:10.1002/eji.200636434
28. Tiller T, Meffre E, Yurasov S, Tsuiji M, Nussenzweig MC, Wardemann H. Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* (2008) 329:112–24. doi:10.1016/j.jim.2007.09.017
29. Smith K, Garman L, Wrammert J, Zheng NY, Capra JD, Ahmed R, et al. Rapid generation of fully human monoclonal antibodies specific to a vaccinating antigen. *Nat Protoc* (2009) 4:372–84. doi:10.1038/nprot.2009.3
30. Coffman RL, Savelkoul HF, Lebman DA. Cytokine regulation of immunoglobulin isotype switching and expression. *Semin Immunol* (1989) 1:55–63.
31. Aoki-Ota M, Torkamani A, Ota T, Schork N, Nemazee D. Skewed primary Igkappa repertoire and V-J joining in C57BL/6 mice: implications for recombination accessibility and receptor editing. *J Immunol* (2012) 188:2305–15. doi:10.4049/jimmunol.1103484
32. Yamagami T, ten Boekel E, Schaniel C, Andersson J, Rolink A, Melchers F. Four of five RAG-expressing JCkappa-/- small pre-BII cells have no L chain gene rearrangements: detection by high-efficiency single cell PCR. *Immunity* (1999) 11:309–16. doi:10.1016/S1074-7613(00)80106-5
33. Reth M, Hammerling GJ, Rajewsky K. Analysis of the repertoire of anti-NP antibodies in C57BL/6 mice by cell fusion. I. Characterization of antibody families in the primary and hyperimmune response. *Eur J Immunol* (1978) 8:393–400. doi:10.1002/eji.1830080605
34. Bothwell AL, Paskind M, Reth M, Imanishi-Kari T, Rajewsky K, Baltimore D. Heavy chain variable region contribution to the NPb family of antibodies: somatic mutation evident in a gamma 2a variable region. *Cell* (1981) 24:625–37. doi:10.1016/0092-8674(81)90089-1
35. Kaartinen M, Griffiths GM, Hamlyn PH, Markham AF, Karjalainen K, Pelkonen JL, et al. Anti-oxazolone hybridomas and the structure of the oxazolone idiotype. *J Immunol* (1983) 130:937–45.
36. Kaartinen M, Griffiths GM, Markham AF, Milstein C. mRNA sequences define an unusually restricted IgG response to 2-phenyloxazolone and its early diversification. *Nature* (1983) 304:320–4. doi:10.1038/304320a0
37. Wysocki L, Manser T, Gefter ML. Somatic evolution of variable region structures during an immune response. *Proc Natl Acad Sci U S A* (1986) 83:1847–51. doi:10.1073/pnas.83.6.1847
38. Manser T. Evolution of antibody structure during the immune response. The differentiative potential of a single B lymphocyte. *J Exp Med* (1989) 170:1211–30. doi:10.1084/jem.170.4.1211
39. Katz DH, Paul WE, Benacerraf B. Carrier function in anti-hapten antibody responses. V. Analysis of cellular events in the enhancement of antibody responses by the "allogeneic effect" in DNP-OVA-primed guinea pigs challenged with a heterologous DNP-conjugate. *J Immunol* (1971) 107:1319–28.
40. Osborne DP Jr, Katz DH. The allogeneic effect in inbred mice. IV. Regulatory influences of graft-vs.-host reactions on host T lymphocyte functions. *J Exp Med* (1973) 138:825–38. doi:10.1084/jem.138.4.825
41. Hamilton JA, Miller JF. Induction of a primary antihapten response in vivo by a graft-vs.-host reaction. *J Exp Med* (1973) 138:1009–14. doi:10.1084/jem.138.4.1009
42. Johnston CM, Wood AL, Bolland DJ, Corcoran AE. Complete sequence assembly and characterization of the C57BL/6 mouse Ig heavy chain V region. *J Immunol* (2006) 176:4221–34. doi:10.4049/jimmunol.176.7.4221

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

VII. Acknowledgements

First of all I want to thank Ton for giving me the opportunity to do my PhD in his laboratory. I always admired his dedication to science and his inexhaustible motivation to further advance the field of immunology either in his office, where I was welcome at any time, or right at the bench. I want to thank him for his guidance during my PhD and for sharing his exceptional scientific knowledge and experience with me.

I want to thank Ed who agreed to be my new supervisor and provided all the support that I needed to finish my PhD.

I want to thank Daniela who agreed to be in my PhD committee and always supported me in any situation during my PhD and especially during the difficult time in the end.

I want to thank Jonas, Hanni, and Matthias for their scientific contribution to the project, the support during the years and the excellent and familiar teamwork.

I also want to thank Jan for constructive criticism and advice during all the years and the help he provided in revising my thesis drafts, which was probably very “mühsam”.

I want to thank all the people that were around me in the lab during all the years. I always appreciated and enjoyed the pleasant and mostly stimulating atmosphere that was only marginally disturbed by David Guetta from time to time. Thank you for making a sometimes very frustrating scientific life more enjoyable.

Most of all I want to thank my parents who always supported me and never gave up on me during all the years. Without their support all this would probably not have been possible.

Last but not least I want to thank my love Stefanie who suffered from and with me during stressful periods of my PhD.