Aus dem Deutsches Rheuma-Forschungszentrum
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Bone marrow maintains isotype switched memory B cells in stromal niches.

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1 Abstract

The adaptive immune system has the unique ability to remember and rapidly mount protective response against previously encountered pathogen. This feature of the immune system is termed immunological memory and the functional duty is carried out by differentiated T and B cells. The maintenance of memory cells is important to confer long-lasting protection for the organism. Also, strategic positioning of these memory cells throughout the organism in crucial to ensure timely response against recurrent antigenic stimulation. While the tissue distribution and maintenance of memory T and plasma cells has been described, the lifestyle of memory B cells (B_{mem}) has not been well studied so far. In my doctoral thesis I investigated the tissue organization and lifestyle of memory B cells in mice, which would serve as the starting point for further translational studies in humans. To determine the tissue distribution, isotype-switched B_{mem} of spleen, bone marrow (BM), peripheral blood, and lymph nodes were enumerated under different immunization and infection protocols. The majority of isotype-switched B_{mem} were localized in the spleen, but a significant population was also contained within the BM. Comparison of the repertoire of B cell receptor (BCR), a unique identifier of each individual B cell, the repertoires of isotype-switched B_{mem} of spleen and BM revealed limited overlap of B cells with same BCR (clonotypes) generated during a specific immune response. The majority of B_{mem} clonotypes are expressed exclusively in either organ, demonstrating that isotype-switched B_{mem} of the two organs represent distinct resident populations with minimal exchange between them via blood circulation. Phenotypically, isotype-switched B_{mem} of the two organs differ in surface protein expression of CD21 (complement receptor) and CD62L (L-selectin) with subsets of $CD21^{low}$ and $CD21^{high}$ populations in the BM but not in spleen, also, isotype-switched B_{mem} of BM express higher levels of CD62L compared to those in spleen. Isotype-switched B_{mem} of BM and spleen are resting in the G_0 of cell cycle as determined by the expression of the proliferative marker Ki67, and are refractory to *in vivo* treatment with cyclophosphamide (a DNA alkylating agent which kills proliferating cells). In the BM, isotype-switched B_{mem} are located in close proximity to reticular stromal cells expressing VCAM-1. To further understand the role of BM stromal cells in organization of survival niches for memory cells, the biology and functional properties of VCAM-1+ stromal cells were analyzed. Next generation sequencing single cell mRNA transcriptomes profiling of directly *ex vivo* isolated BM VCAM-1+ stromal cells revealed distinct subpopulation of stromal cells defined by the expression of cytokines and chemokines which have been described to be important for the maintenance and survival of subsets of hematopoietic and immune cells subsets. Altogether, the findings of this thesis demonstrate that murine B_{mem} are residing in the BM as distinct population of B cell memory and that

distinct subsets of BM stromal cells organize survival niches for different hematopoietic cells, including memory cells.

Abstract (German)

Das adaptive Immunsystem hat die einzigartige Fähigkeit sich an zuvor vorgefundene Krankheitserreger zu erinnern und schnell gegen diese schützend zu reagieren. Diese Fähigkeit des Immunsystems heißt immunologisches Gedächtnis und wird von differenzierten T- und B-Zellen ausgeübt. Die Aufrechterhaltung der Gedächtniszellen ist wichtig um dem Organismus einen dauerhaften Schutz zu verleihen. Weiterhin ist eine strategische Positionierung dieser Gedächtniszellen im gesamten Organismus entscheidend um eine zeitnahe Reaktion gegen wiederkehrende antigenische Stimulierungen sicherzustellen. Während die Gewebeverteilung und -erhaltung von Gedächtnis T-Zellen und Plasma Zellen bereits gut beschrieben wurde, wurde der Lebensstil von Gedächtnis-B-Zellen (B_{mem}) bislang nicht weiter untersucht. In meiner Doktorarbeit habe ich die Gewebeorganisation und den Lebensstil von Gedächtnis B-Zellen in Mäusen untersucht, welche als Ausgangspunkt für weitere translationale Studien am Menschen dient. Um die Gewebeverteilung zu untersuchen, wurden Isotyp-veränderte B_{mem} der Milz, des Knochenmarks, von peripherem Blut und von Lymphknoten unter verschiedenen Immunisierungs- und Infektionsprotokollen ausgezählt. Die Mehrheit der Isotyp-veränderten B_{mem} wurden in der Milz lokalisiert, es ist aber auch eine signifikante Population im Knochenmark enthalten. Der Vergleich des Repertoires des B-Zell-Rezeptors (BCR), einer einzigartigen Bezeichnung jeder individuellen B-Zelle, des Repertoires der Isotyp-veränderten B_{mem} der Milz und des Knochenmarks hat gezeigt, dass B-Zellen mit derselben BCR (Klonotypen), welche während einer spezifischen Immunreaktion generiert wurden, sich kaum überlappen. Die Mehrheit der B_{mem} Klonotypen wird ausschließlich in einem der beiden Organe exprimiert, was beweist, dass Isotyp-veränderte B_{mem} zweier Organe verschiedene Populationen mit nur minimalen Austausch über den Blutkreislauf repräsentieren. Phänotypisch, Isotyp-veränderte B_{mem} der beiden Organe unterscheiden sich in der Oberflächenproteinexpression von CD21 (Komplementrezeptor) und CD62L (L-Selektin) mit Untergruppen von $CD21^{low}$ und $CD21^{high}$ Populationen im Knochenmark, aber nicht in der Milz. Weiterhin beinhalten Isotyp-veränderte B_{mem} des Knochenmarks einen höheren Spiegel von CD62L im Vergleich zur Milz. Isotyp-veränderte B_{mem} des Knochenmarks und der Milz ruhen im G_0 des Zellzyklus, wie durch die Expression des proliferativen Markers Ki67 bestimmt wurde, und sind widerstandsfähig gegen *in vivo* Behandlungen mit Cyclophosphamid (einem DNA alkylierenden Wirkstoff, der

wuchernde Zellen tötet). Im Knochenmark befinden sich Isotyp-veränderte B_{mem} in unmittelbarer Nähe zu retikulären Stromazellen, die VCAM-1 exprimieren. Um die Rolle der Knochenmark Stromazellen bei der Organisation von Überlebensnischen für Gedächtniszellen genauer zu verstehen, wurden die Biologie und die funktionellen Eigenschaften von VCAM-1+ Stromazellen analysiert. Die Sequenzierung von Einzelzell-mRNA-Transkriptomen von direkt *ex vivo* isolierten Knochenmark VCAM-1+ Stromazellen ergab eindeutige Subpopulationen von Stromazellen, die durch die Expression von Cytokinen und Chemokinen definiert wurden, welche als wichtig für die Aufrechterhaltung und das Überleben von Subpopulationen von hämatopoetischen Zellen und Immunzellen beschrieben wurden.

Zusammenfassend beschreiben die Ergebnisse der Dissertation, dass murine B_{mem} im Knochenmark als ausgeprägte ansässige Population des B-Zell-Gedächtnisses vorkommen und das Subpopulationen von Knochenmark Stromazellen Überlebensnischen für verschiedene hämatopoetische Zellen, inklusive den Gedächtniszellen des Immunsystems, organisieren.

2 Introduction

2.1 Immunological memory

The adaptive immune system has the unique ability to remember and mount protective response against previously encountered pathogen in a more rapid and efficient manner. This ability of the immune system to confer protection against a previously encountered pathogen was already described in 430 BC by the ancient Greek historian Thucydides [1]. This feature of the immune system is termed immunological memory and the functional duty is carried out by well differentiated T and B cells [2]. The generation of protective immunological memory is also the underlying principle of immune protection acquired from immunization or vaccination.

2.2 Memory B cells

Antigen-experienced memory B cells (B_{mem}) are an essential component of immunological memory. They are functionally superior compared to naïve cells and have a lower activation threshold when re-encountering pathogens [3]. Isotype-switched B_{mem} (IgG or IgA) produce antibodies with higher affinity and specificity needed to clear foreign antigen or pathogen during immune response [3]. The organization of B cell memory remains however unclear in the scientific community. Both non-switched IgM^+ and isotype-switched B_{mem} have been described to be present in spleen, blood and bone marrow (BM) [4–6]. In humans, the spleen has been described as a major reservoir for *Vaccinia*-specific B_{mem} [7] and splenectomy leads to gradual loss of circulating B_{mem} [7,8]. This is however not the situation for tetanus toxoid (TT) infection as splenectomy does not lead to a loss of TT-specific B_{mem} [4], indicating that TT-specific memory B cells do not require the spleen for maintenance. These contrasting observations suggest that memory B cells which confer protective immunity against specific pathogens are also located in organs other than blood and spleen [9].

2.3 Tissue maintenance of memory lymphocytes

Subsets of long-lived memory lymphocytes (CD4+, CD8+ and plasma cells) have been shown to be maintained in different tissues including the BM. In the BM, dedicated niches provide the needed molecular signals to ensure the long term survival of these cells [10–14]. In contrast, knowledge about the role of BM in the maintenance of

memory B cells remains scarce. As with most hematopoietic and immune cells, the maintenance of B_{mem} is likely to be dependent on both intrinsic factors and external signals from their immediate microenvironment.

2.4 BM stromal niches in maintenance of memory lymphocytes

BM stromal cells are integral component of survival niches for different immune cells like memory CD4+, CD8+ and plasma cells [10–14]. *In vivo,* BM stromal cells express vascular cell-adhesion molecule 1 (VCAM1; CD106) [15], CXCL12 and IL7, collagen II and XI [10–14] among other factors necessary for the survival, maintenance and development of various cells of the hematopoietic and immune system [10–14]. Although eosinophils were initially reported as integral component of survival niches for plasma cells [12,16], recent research findings published in 2018 showed that eosinophils are redundant for maintenance of long-lived plasma cells [17,18]. Long-lived CD4+ and CD8+ memory cells contact IL-7 expressing BM stromal cells [10,19]. The functional organization of BM stromal cells is however not well addressed. For example, it is not known if distinct subpopulations of BM stromal cells are specialized for organization of niches for particular subsets of memory cells. Also, knowledge about other factors expressed by stromal cells which might play a role in the maintenance of memory cells is lacking.

3 Materials and Methods

3.1 Mice

All mice were housed under specific pathogen-free conditions at the Deutsches Rheuma-Forschungszentrum Berlin, a Leibniz Institute (DRFZ). C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany). Mice expressing GFP under the control of the *Prdm1* promoter (Blimp1-GFP)[20] were bred at the DRFZ animal facility. All animal experiments were performed according to institutional guidelines and licensed under German animal protection regulations.

3.2 Immunizations and infections

- 100µg NP-KLH (4-Hydroxy-3-nitrophenylacetyl (NP)) hapten conjugated to KLH (Keyhole Limpet Hemocyanin) with 10 µg LPS (*E. coli*, InvivoGen), subcutaneous (SC). For boost immunizations 10 µg NP-KLH without adjuvant was used.
- 100µg NP-CGG (NP hapten conjugated to Chicken Gamma Globulin (CGG)) in Incomplete Freund's adjuvant (IFA), three times (3X) at 21 days interval, intraperitoneal (IP) or subcutaneous.

- $2x10^5$ plaque-forming units of the Armstrong strain of lymphocytic choriomeningitis virus (LCMV), intraperitoneal.
- 10^6 colony-forming units of attenuated *Salmonella enterica* serovar typhimurium strain SL7207, intravenous.

3.3 Cyclophosphamide administration

C57BL/6J mice immunized three times (3x) IP with NP-CGG/IFA-immunized (at 21 days interval) to establish long-lived B_{mem} were treated twice (2X) with 50mg/kg cyclophosphamide (CyP), intravenous at 2 days interval and sacrificed on day 3 after the last treatment. Control mice were injected with Phosphate Buffered Saline (PBS) instead of cyclophosphamide.

3.4 Flow cytometric analysis and cell sorting (FACS)

Flow cytometric measurements and cell sorting were done according to standards defined in the guidelines to flow cytometry and cell sorting in immunological studies [21]. Antibodies were purchased from Miltenyi Biotec, Biolegend, eBioscience, or produced in DRFZ. All FACS data were acquired on MACSQuant (Miltenyi Biotech), BD FACSCanto II or BD FACSFortessa (BD Bioscience). BD Influx cell sorter (BD Bioscience) was used for cell sorting. Flow cytometric data were analyzed with FlowJo v10(Tree Star, Inc.). Total BM cell numbers were calculated based on cell numbers in a single femur of a mouse which is estimated to harbor 6.3% of total BM leading to a conversion factor of 7.9 for two femurs for total mouse BM [11].

3.4.1 Memory B cells

Antibodies directed against the following murine antigens were used for analysis of memory B cells: Ki-67 (B56, BD Biosciences), CD11c (N418), CD19 (1D3), CD38 (90), CD138 (281-2), GL7 (GL7), IgA (C10-3), IgD (11.26c), IgG1 (A85-1), IgG2a/b (R2-40), IgG2b (A95-1 and MRG2b-85), IgM (M41), CD93 (AA4.1), CD5 (19-3), B220 (RA3.6B2), CD21/35 (7G6), CD29 (HMß1-1), CD39 (Duha59), CD62L (MEL-14).

3.4.2 Bone marrow stromal cells

The following antibodies were used in analysis of BM stromal cells: anti-CD45 (30F11), anti-VCAM-1 (429), anti-CD31 (390) and anti-Ter119 (Ter119).

3.5 B cell receptor sequencing

B_{mem} from BM (tibiae, femurs, pelvics) and spleen of immunized mice (3x NP-CGG) were magnetically enriched using the Memory B cell Isolation Kit (130-095-838 Miltenyi). B_{mem} cells were FACSorted by gating on $CD19^+CD38^+CD138^-CD11c^-GL7^-IgM^-IgD^-$ small lymphocytes. Sorted cells per organ per mouse were split into equal halves to

give biological (cellular) replicates [22]. Biological replicates were processed independently from this point on. Total RNA was extracted from samples using the ZR RNA Miniprep Kit (Zymo Research) according to the manufacturer's protocol (Catalog nos. R1064 & R1065). Isolated RNA was splitted into equal halves and library preparation was performed to give technical duplicates. First-strand cDNA was synthesized with SMARTScribe Reverse Transcriptase (Clontech) using total RNA, a cDNA synthesis primer mix (mIgG12ab_r1(KKACAGTCACTGAGCTGCT), mIgG3_r (GTACAGTCACCAAGCTGCT), mIgA_r (CCAGGTCACATTCATCGTG) by metabion international AG) and a 5' – template-switch adaptor with unique molecular identifiers (UMI) (SmartNNNa (AAGCAGUGGTAUCAACGCAGAGUNNNNUNNNNUNNNNUCTT(rG)4)) according to the protocol "high-quality full length immunoglobulin profiling with unique molecular barcoding" by the Chudakov lab [23]. cDNA was purified with MinElute PCR purification Kit (Qiagen) and eluted in 10 µL 70°C nuclease-free H2O (Qiagen). The first and second PCR were performed according to the protocol by the Chudakov lab [23]. PCR 1 products were purified with MinElute PCR purification Kit (Qiagen) and eluted in 25 µL 70°C nuclease-free H2O (Qiagen). The products were also gel-purified from 2% agarose gels (extraction with MinElute gel extraction Kit (Qiagen); elution in 15 µL 70°C nuclease-free H2O (Qiagen). Adapter ligation was performed using the TruSeq® DNA PCR-Free Library Prep protocol (Illumina). The products were gel-purified from 2% agarose gels instead of bead purification as mentioned in the protocol (extraction with MinElute gel extraction Kit (Qiagen); elution in 10 µL 70°C nuclease-free H2O (Qiagen)). The quality of amplified libraries was verified by using an Agilent 2100 Bioanalyzer (2100 expert High Sensitivity DNA Assay). According to the fragment size, the libraries were quantified by qPCR using the KAPA Library Quantification Kit for Illumina platforms (KAPA Biosystems). Based on the result of the qPCR a final library pool with a concentration of 2 pM was used for sequencing with NextSeq 500 (Illumina) using the NextSeq 500/550 Mid Output Kit and 300 cycles (*2x150bp)*.

3.6 BCR repertoire analysis

B Cell Receptor (BCR) repertoire analysis was performed using MIGEC-1.2.4a [24] in default parameter settings while adding a demultiplexing step for identification of IgG1/2, IgG3 and IgA heavy chains. After the MIGEC pipeline's "checkout" step isotypes were classified according to presence of mIgG12_r2, mIgG3_r2 and mIgA_r2 primer sequences: AGTGGATAGACMGATG, AAGGGATAGACAGATG and TCAGTGGGTAGATGGTG, allowing for one mismatch against the primer sequence. Data were then processed independently for each isotype. The MIGEC segments file was adjusted to include only C57BL/6-specific V genes for mapping. MIGEC performs a UMI-guided correction to remove PCR as well as sequencing bias and errors. Each resulting consensus sequence was treated as one clone. Clones with identical V, D, and

J gene compositions and CDR3 nucleotide sequences were grouped together to define clonotypes. Solely clonotypes consistently found in both technical replicates of a given sample were considered in downstream analyses. Statistics on the overlap of repertoires between different samples were performed based on the presence of clonotypes. The degree of similarity between samples accounting for the abundance of clonotypes is represented by the cosine similarity [25].

3.7 Histology

3.7.1 Preparation of histological sections

Femoral bones were fixed in 4% PFA (Electron Microscopy Sciences) for 4 hours at 4°C, equilibrated in 30% sucrose/PBS, then frozen and stored at -80°C. 6µm cryosections of tissues were prepared. Tissue samples were first blocked with 1X PBS containing 10% FCS (Fetal Calf Serum) for 1h. Samples were then stained with antibodies in 0.1% Tween-20 (Sigma-Aldrich)/ 10% FCS/ 1XPBS for 1h. Antibodies towards following murine antigens were used: IgG2b (RMG2b-1), GFP (rabbit polyclonal), fibronectin (rabbit polyclonal), Ki67 (Sol-15), VCAM-1 (429), cadherin 17 (rabbit polyclonal), laminin (rabbit polyclonal, Sigma Aldrich), IgD (11.26c), Thy1 (T24, DRFZ), B220 (RA3.6B4), CD11c (N418), donkey anti-rabbit polyclonal IgG-AF488/647, strepatavidin-AF594/647, donkey anti-goat polyclonal AF488. For nuclear staining, sections were stained with 1 µg/ml DAPI in PBS. Sections were mounted in Fluorescent Mounting Medium (DAKO).

3.7.2 Confocal microscopy

For confocal microscopy, a Zeiss LSM710 with a 20×/0.8 numerical aperture objective lens was used. Images were generated by tile-scans and maximum intensity projection of 3-5 Z-stacks each with 1µm thickness. Image acquisition was performed using Zen 2010 Version 6.0 and images were analyzed by Zen 2012 Light Edition software (Carl Zeiss MicroImaging).

3.7.3 Manual image analysis

To determine the nearest neighbors of B_{mem}, cells in direct cell-cell contact or a position within a 10µm radius of cell boundaries of B_{mem} were enumerated manually using resolution images of immunofluorescence staining acquired by confocal microscopy.

3.7.4 Modelling random co-localization

To determine the probability of co-localization of BM B_{mem} to reticular stromal cells, cell-cell neighboring was modelled by random cell positioning [12]. Images of isotype-switched B_{mem} were positioned on histological images of VCAM-1 stained BM at random (1000 times), and the frequencies of co-localizing B_{mem} and stromal cells were then

determined. The modelled frequencies were then compared to the frequencies of the original histological images.

3.8 Single cell suspension of bone marrow

BM flush-out and the empty bones (tibia and femur) were digested using an optimized protocol with 0.5 mg/ml Collagenase IV (Sigma-Aldrich), 1mg/ml DNase I (Sigma-Aldrich), 0.25 mg/ml Dispase II (Roche), with or without 5µg/ml Latrunculin B (Sigma-Aldrich), for 30 min at 37°C.

3.9 Single cell RNA-sequencing

3.9.1 Single cell library preparation and RNA-sequencing

Ex vivo FACSorted VCAM-1+CD45-Ter119-CD31- BM stromal cells (IL-7-GFP knock-in mice) were applied to the 10x Genomics platform using the Single Cell 3' Reagent Kit V2 (10x Genomics) and following the manufacturer's instructions. Upon adapter ligation and index PCR the quality of the obtained cDNA library was assessed by Qubit quantification, Bioanalyzer fragment analysis (HS DNA Kit, Agilent) and KAPA library quantification qPCR (Roche). The sequencing was performed on a NextSeq500 device (Illumina) using a High Output v2 Kit (150 cycles) with the recommended sequencing conditions (read1: 26nt, read2: 98nt, index1: 8 nt, index2: n.a.).

3.9.2 BM stromal cells single cell RNA-*seq* analysis

Illumina output was demultiplex and mapped to the mm10 reference genome by cellranger-2.0.2 (10x Genomics Inc.) using refdata-cellranger-mm10-1.2.0 in default parameter setting and 3000 expected cells. Raw counts were further analyzed using R 3.5.1 with Seurat package (Seurat_2.3.4,) [26]. Potential lymphocyte and erythrocyte contamination cells expressing Ptprc (CD45) or hemoglobin subunits (Hba) respectively were detected and excluded prior the analysis, resulting in 1035 stromal cells. T-distributed Stochastic Neighbor Embedding (tSNE) and the underlying Principle Component Analysis was performed on 30 dimensions using variable genes as set by default (Fig. 4-15) or on a subset of 108 genes (Fig. 4-16), which are known for their role in mediating the communication between stromal cells and hematopoietic cells. Similarity of gene expression (co-expression) was estimated by the Jaccard similarity coefficient (Fig. 4-17). Heatmaps (Fig. 4-18) show the log2-transformed fold change of mean expression of positive and negative cells, displayed are the top 10 genes with the highest fold change. DiffExpTest-method was used for the statistical analysis of differential expressed genes [27] .

4 Results

4.1 Isotype-switched B_{mem} are abundant in spleen and BM

In order to investigate the tissue distribution of murine B_{mem}, single cell suspension of spleen, BM, peripheral lymph nodes (pLN), mesenteric lymph nodes (mLN), and Peyer's patches (PP) were analyzed by flow cytometry. Surface expression of immunoglobulin isotype (IgG_1, IgG_{2b}, or IgA) was used to identify isotype-switched B_{mem} by gating on $CD19^+CD38^+CD138^-GL7^-$small lymphocytes (Fig. 4-1). Mice were immunized with different experimental antigens (LCMV, NP-KLH, NP-CGG, *S.* typhimurium) via different routes of administration (sub-cutaneous, intravenous and intraperitoneal) and B_{mem} enumerated in the memory phase of immune response. Different routes of administration were used to investigate how the route of pathogen entry influences the distribution of B_{mem}.

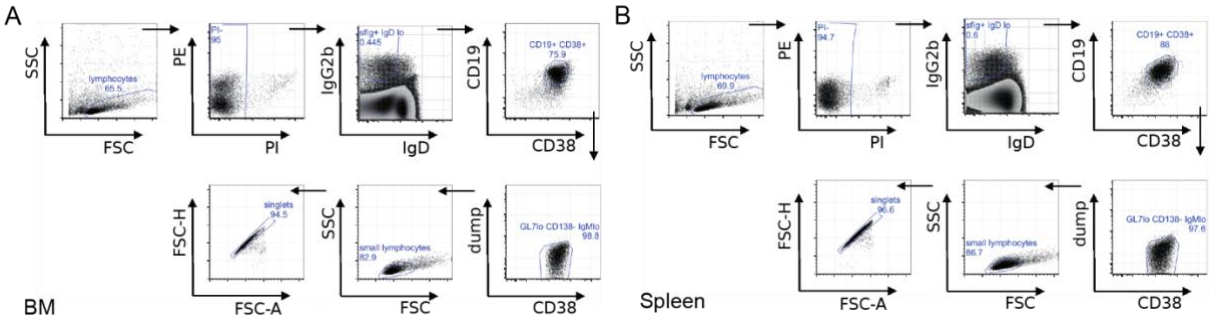

Figure 4-1 Gating for isotype-switched B_{mem} of BM and spleen.
Switched B_{mem} of BM (A) and spleen (B) were identified by expression of surface IgG_{2b}, IgG_1, or IgA and CD19, CD38 and lack of IgD, IgM, CD138, GL7 marker. Staining shown for IgG_{2b} exemplarily.

The quantification analysis revealed that besides the spleen, the BM hosts significant proportion of B_{mem}. Following the different immunization protocols, 32-60% of all isotype-switched B_{mem} were detected in the spleen, 18-41% of isotype-switched B_{mem} were located in the BM and 9-14% in peripheral lymph nodes (Fig. 4-2). The frequencies of memory B cells in peripheral lymph nodes (pLN) mesenteric lymph nodes (mLN), Peyers Patches (PP) and blood were consistently lower compared to BM and spleen (Fig. 4-2). The observed distribution of memory B cells was independent of either the antigen used or the route of administration of the antigen.

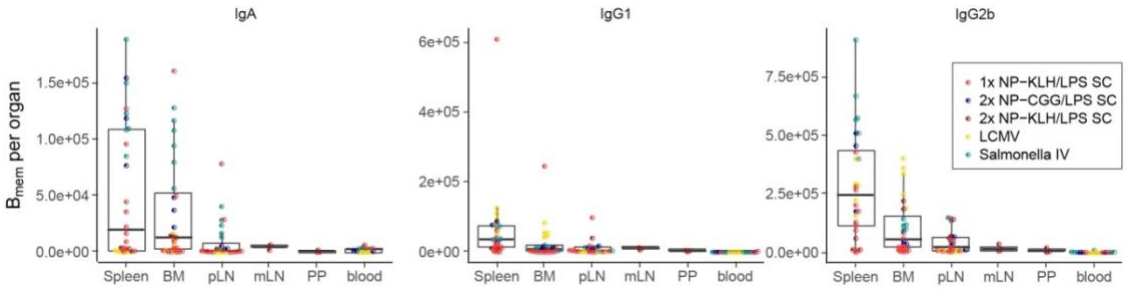

Figure 4-2 Spleen and BM harbor major populations of isotype-switched B_{mem}.
Cell numbers of isotype-switched B_{mem} per organ in C57BL/6 laboratory mice. Absolute cell numbers per organ calculated from flow cytometric counts (gated for IgG_1^+, IgG_{2b}^+, or IgA^+ $CD19^+CD38^+CD138^-GL7^-CD11c^-IgM^-IgD^-PI^-$ small lymphocytes); n=42, data pooled from 8 experiments with five different immunizations performed in mice aged 4-20 months and held under SPF conditions, colors indicate immunization.NP- 4-Hydroxy-3-nitrophenylacetyl; KHL-Keyhole Limpet Hemocyanin; CGG- Chicken Gamma Globulin; LPS-Lipopolysaccharide; LCMV-lymphocytic choriomeningitis virus

4.2 Exclusive antigen-receptor clonotypes identify distinct B_{mem} repertoire of BM and spleen

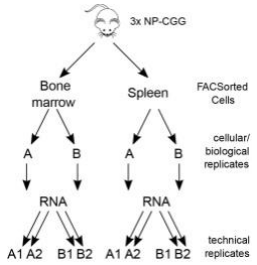

Figure 4-3 Experimental setup for the comparison of BCR repertoire of switched B_{mem} of BM and spleen:
After isolation, cells of the same organ were divided into equal proportions and processed as biological replicates. After RNA isolation, samples were split and processed as technical replicates.

Every B cell carries a unique membrane bound immunoglobulin (Ig) which is also known as the B Cell Receptor (BCR) [28]. BCRs are assembled during the B-cell development that involves random somatic recombination of V, D, J gene segments of the heavy chain locus and V, J gene segments of the light chain locus resulting in a huge diversity of BCRs [29]. Each single BCR (B cell clone) can be identified by its unique complementarity-determining region 3 (CDR3), part of the variable chain in BCR that bind to a specific antigen. The unique V, D, J gene segments rearrangement process makes it highly unlikely that any two naïve B cells express the same BCR although all progeny of a particular B cell keep the BCR with some additional mutations to the parent's BCR [28]. In this way B cell clones (progeny) of a particular initial B cell generated during an immune response can be traced and identified by their BCR. This unique property of BCR is valuable in the analysis of repertoire of B_{mem} generated in an immune response. BCR repertoire comparison of B_{mem} of different organs helps to

determine tissue exclusive (resident) populations and also to address the extent of exchange (via circulation) of B_{mem} populations between the different tissues.

High-throughput BCR RNA sequencing of IgG_1^+ and IgG_2^+ ($IgG_{1/2}^+$), IgG_3^+, and IgA^+ isotype-switched B_{mem} was performed using cells isolated from spleen and BM of three individual C57BL/6J mice, which had been immunized three times with NP-CGG minimum 73 days prior to analysis (Fig. 4-3). For all repertoire analyses, unique B cell clones were defined by 100% amino acid sequence identity of CDR3 regions. All clones with single counts were excluded to minimize confounding effects of sequencing errors and sequencing depth. Clonotypes (clonally related B cells generated from same single same naive B cell) were defined as clones with a common unique CDR3 sequence and, shared V, D, and J gene segment usage.

Determination of V_H gene family usage showed that $IgG_{1/2}^+$, IgG_3^+, and IgA^+ isotype-switched B_{mem} of BM and spleen per mouse are highly divergent in the distribution of V_H gene family usage (Fig. 4-4).

To determine repertoire overlap, the distribution of clonotypes between B_{mem} of BM and spleen was also analyzed. To minimize confounding effects of sequencing errors and sequencing depth, only those clonotypes consistently found in technical replicates were considered for repertoire comparison [30,31]. Isotype-switched B_{mem} of spleen and BM expressing $IgG_{1/2}$, IgG_3, and IgA, respectively, showed a considerable fraction of exclusive organ clonotypes: $IgG_{1/2}$=46.1%-89.8%, IgG_3=43.7%-93.7%, IgA=49.9%-90.8% (Fig. 4-5). The proportion of clonotypes shared between $IgG_{1/2}^+$, IgG_3^+, and IgA^+ B_{mem} of spleen and BM of individual mice was consistently significantly lower= $IgG_{1/2}^+$=9.8%-29.4%, IgG_3^+=6.5%-29.8%, IgA^+=6.3%-34.5% (Fig. 4-5).

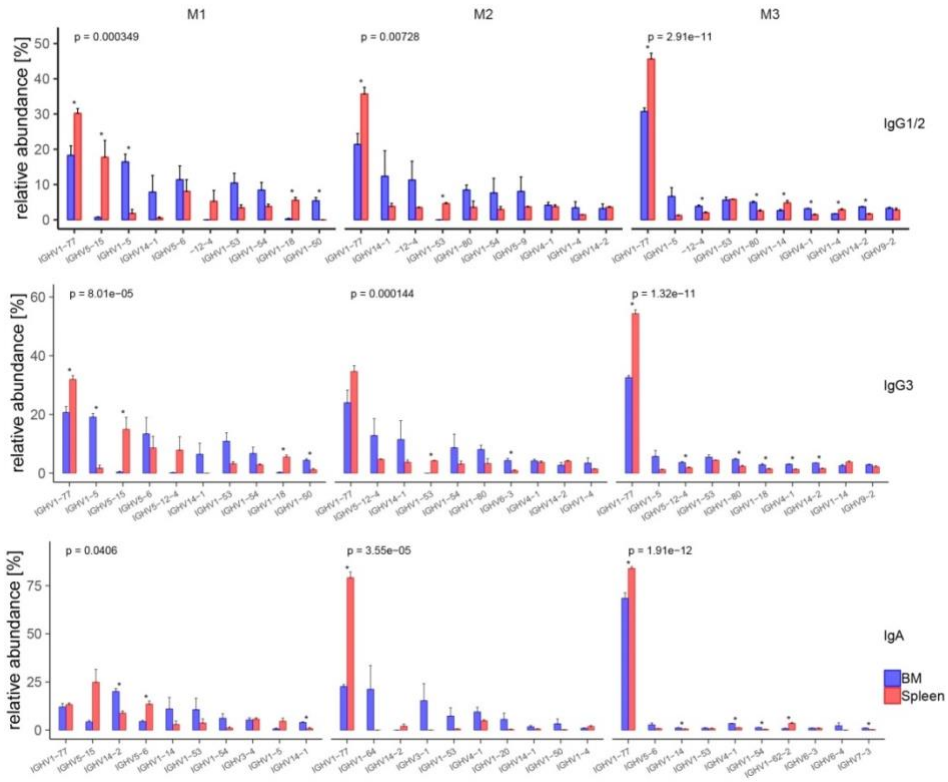

Figure 4-4 V_H gene recruitment to spleen and BM switched B_{mem}, represented as frequency of a particular V_H gene among total CDR3s per isotype per organ sample.
Bars show relative abundance of the 10 most frequent V_H genes, error bars indicate SEM. Significance of difference in V_H gene distribution to Spleen and BM assessed by multivariate ANOVA, p values corrected for multiple testing (Benjamini-Hochberg), * indicates significant difference in means for a particular V_H gene (Welch's test). M1-M3: 3 replicates

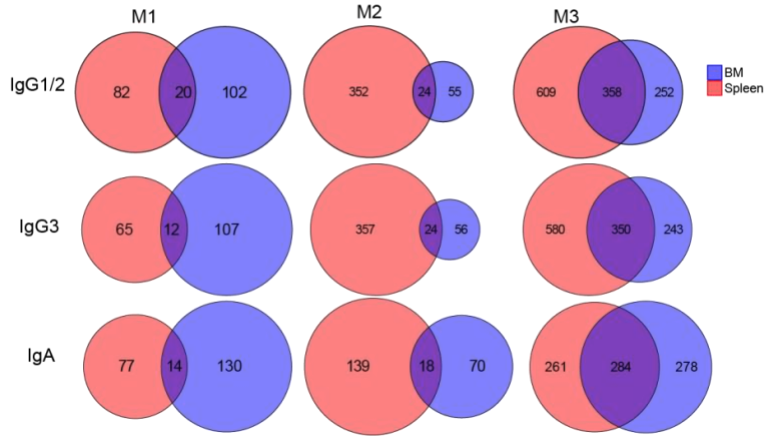

Figure 4-5 Overlap of the BCR (heavy chain CDR3) repertoire of switched B_{mem} from spleen and BM
Venn diagrams represent clonotype presence for $IgG_{1/2}$, IgG_3 and IgA B_{mem} in a given sample: numbers indicate clonotypes present in one organ exclusively or in both (overlap). M1-M3: 3 replicates

Due to the huge diversity of the BCR repertoire, sample size is a crucial factor in the comparison of the repertoire any two organs. To confirm that the sample sizes analyzed were sufficient to represent the repertoire of the different organs, clonotype repertoires of biological (cellular) replicates of isotype-switched B_{mem} isolated from spleen of each mouse were compared (Fig. 4-6). In those biological replicates (each containing half of

spleen B_{mem}), the cosine similarity which measures the correlation of frequency of clonotypes in the samples was consistently higher: mean cosine similarity of 0.83-0.92 for $IgG_{1/2}$, 0.83-0.96 for IgG_3, 0.78-0.99 for IgA. This means that sampling half of spleen B_{mem} is enough to identify true clonotype and confirms that the size of the spleen and BM B_{mem} samples was enough to make meaningful conclusion of the BCR repertoire comparison. This high correlation of biological replicates is comparable to that of the RNA-technical replicates. On the contrary, the cosine similarity analysis of shared clonotypes between BM and spleen ($IgG_{1/2}$= 0.5-0.54, IgG_3= 0.54-0.55, IgA= 0.26-0.55) was significantly lower compared to that of organ replicates (biological and technical) (Fig. 4-6). The cosine similarity between BM and spleen B_{mem} is significantly minimal and suggests distinct subpopulations.

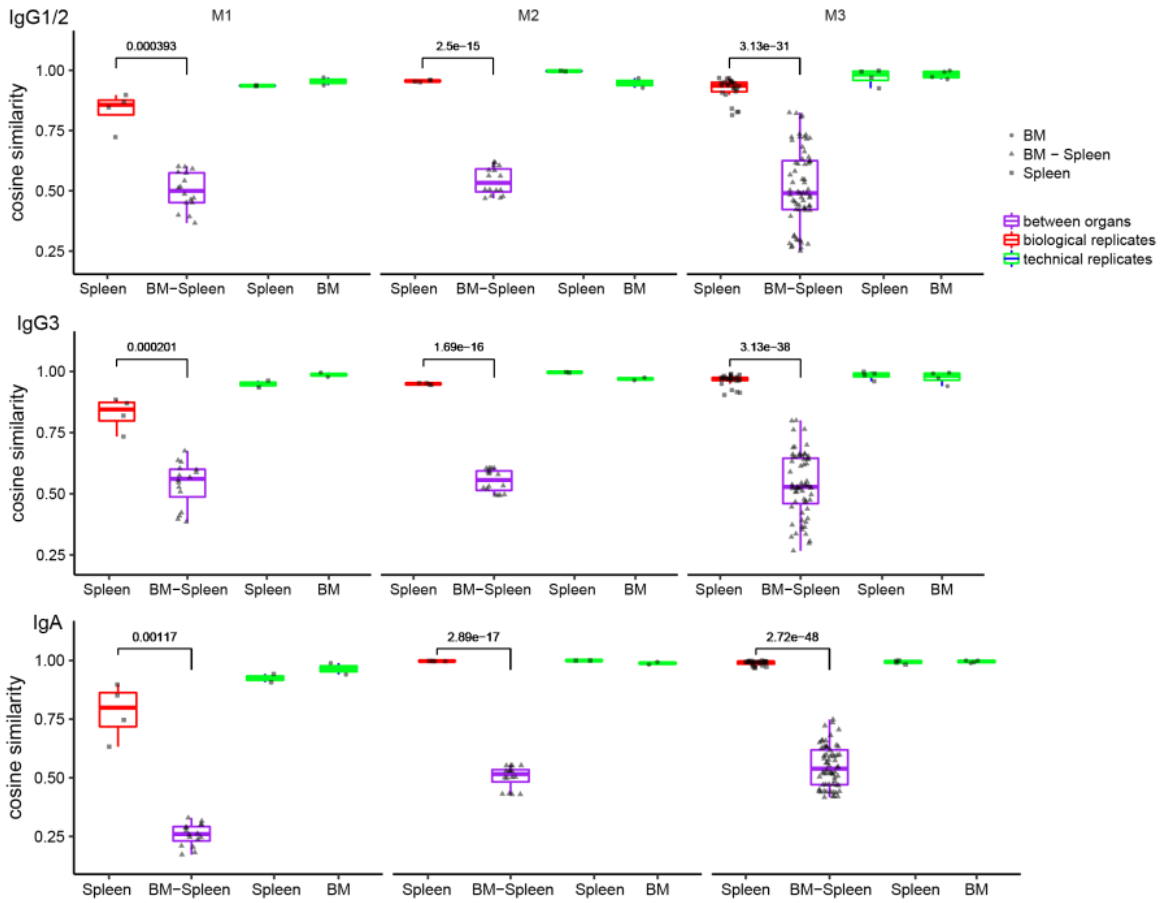

Figure 4-6 Cosine similarity comparison of BCR (heavy chain CDR3) repertoires of switched B_{mem}

Cosine similarity comparison (accounting for clonotype frequencies) within technical replicates of $IgG_{1/2}$, IgG_3 and IgA B_{mem} of spleen and BM (blue), within cellular replicates from spleens (red), and between spleen and BM (BM-Spleen, purple) of three individual mice. p values (Welch's test for difference of means of cosine similarity within shared IgH repertoire (spleen cellular replicates) and between spleen and BM replicates are indicated.

4.3 B_{mem} of BM and spleen differ in their expression of CD21 and CD62L

To determine whether B_{mem} of BM and spleen differed in their surface protein expression, the expression of more than 200 different surface markers comprising CDs and other cell surface markers (LegendScreen Mouse Cell Screen (PE) (# 700005 Biolegend) on $CD19^+CD138^-CD38^+CD11c^-GL7^-IgM^-IgD^-IgG_{2b}^+$ B_{mem} of BM and spleen was analyzed using flow cytometry. Gating was performed analogously to the strategy displayed in Fig. 4-1.

The surface markers screen analysis showed that IgG_{2b}^+ B_{mem} of BM and spleen differ in their expression pattern for CD21/35 and CD62L (Fig. 4-7). Expression levels of several surface molecules, such as CD20, TACI (CD267), and MHC class II was similar for IgG_{2b}^+ B_{mem} of BM and spleen. The expression of CD62L (L-selectin) was higher on IgG_{2b}^+ B_{mem} of BM compared to spleen. Expression of CD21/35 (CR2/1), the complement receptor [32,33] was reduced in BM IgG_{2b}^+ B_{mem} with about half of the cells expressing lower levels compared to those of spleen. The differences in expression of CD21/CD35 and CD62L identified from the high throughput screen were confirmed in independent experiments. The functional implication of these differences in protein expression is currently not clear and remains to be investigated.

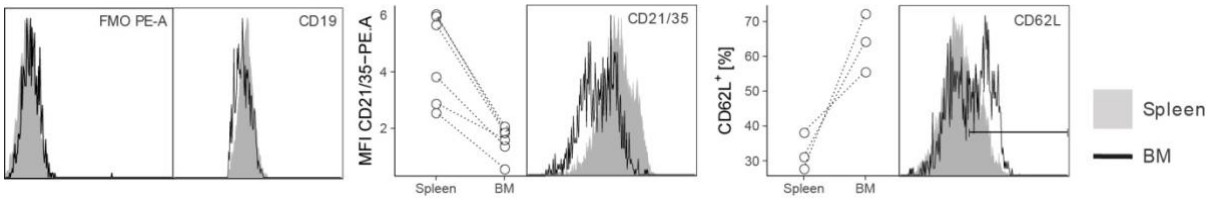

Figure 4-7 Differential marker expression between spleen and BM IgG_{2b}^+ B_{mem}
Mean fluorescence intensity (MFI) or frequency of cells positive for a marker for paired bone marrow and spleen IgG_{2b}^+ B_{mem} is shown next to corresponding representative histogram. Gated for $IgG_{2b}^+CD19^+CD38^+CD138^-GL7^-CD11c^-IgM^-IgD^-PI^-$ small lymphocytes, histogram plots are representative for five or more biological replicates from 3 independent experiments FMO PE: PE-channel fluorescence minus one control, FSC-A: forward-scatter area. (15 aged and immunized C57BL/6J mice)

4.4 Memory B cells are quiescent and resting in G_0 of cell cycle

Long-lived memory lymphocytes (CD4+, CD8+ and plasma cells) have been described to be maintained in tissues in a state of quiescence (in terms of activation and proliferation) [10,34,35]. To investigate whether this is also the case for B_{mem}, the proliferative state of isotype-switched B_{mem} of BM and spleen was determined by intranuclear expression of the proliferative marker Ki67 using flow cytometry. Ki67 is expressed in all phases of the cell cycle, except G_0 [36]. Ki-67 was expressed by as many as 9.3% (median: 8.0%) of switched IgG_{2b}^+ B_{mem} cells in spleen, and by no more than 4% (median: 2.6%) of isotype-switched B_{mem} of BM, showing that more than 90% of cells in the spleen and more than 95% in the BM were in the G_0 phase of cell cycle, i.e. resting in terms of proliferation (Fig. 4-8).

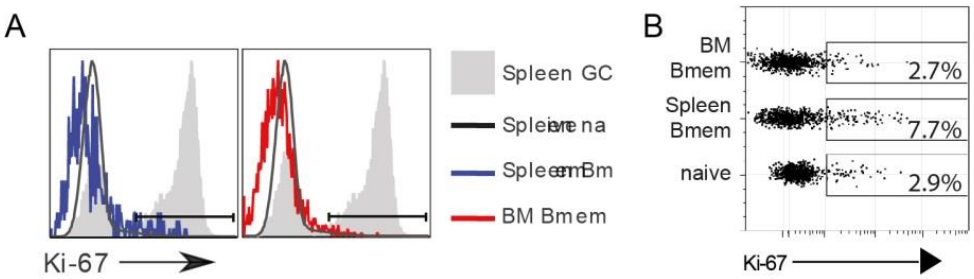

Figure 4-8 IgG_{2b}^+ B_{mem} of BM and spleen rest in G_0 of cell cycle
(A) Flow-cytometric quantification of Ki-67expression in IgG_{2b}^+ B_{mem},($IgG_{2b}^+CD19^+CD38^+CD138^-GL7^-CD11c^-IgM^-IgD^-PI^-$ small) splenic naïve ($IgM^+IgD^+IgG_{2b}^-CD19^+CD38^+CD138^-GL7^-CD11c^-$ PI^- small lymphocytes) and germinal center (GC) ($CD19^+CD38^{lo}GL7^+CD11c^{--}PI^-$ lymphocytes) B cells. (B) frequencies of $Ki\text{-}67^+$ cells within the population indicated. Data is representative of 2 independent experiments.

To confirm *in vivo* quiescence (non-proliferative) of B_{mem} as observed with the Ki67 staining, we treated immunized mice (in the memory phase) with cyclophosphamide. Cyclophosphamide is a DNA alkylating agent which kills proliferating cells [37]. B_{mem} of both spleen and BM were refractory to treatment with cyclophosphamide. The number of isotype-switched B_{mem} (IgG_{2b}, IgG_1, IgA) was not affected by the cyclophosphamide compared to the control treatment with PBS, whereas, overall CD19+ B cell populations of both organs were significantly reduced (Fig. 4-9).

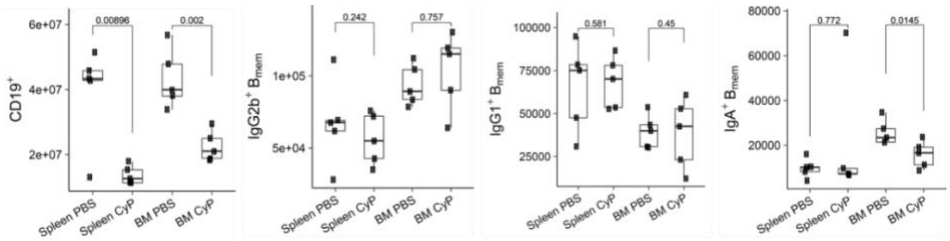

Figure 4-9 Switched B_{mem} are refractory to cyclophosphamide treatment
Flow-cytometric quantification of $CD19^+$ B cells and IgG_{2b}^+ B_{mem} in mice treated with Cyclophosphamide (CyP) or untreated controls (PBS) after immunization with 3x NP-CGG/IFA. Analysis was performed after 7 days of CyP. p value (Welch's test indicated). Representative data shown for one out of two independent experiments.

4.5 B_{mem} co-localize with $VCAM\text{-}1^+$ cells in the bone marrow

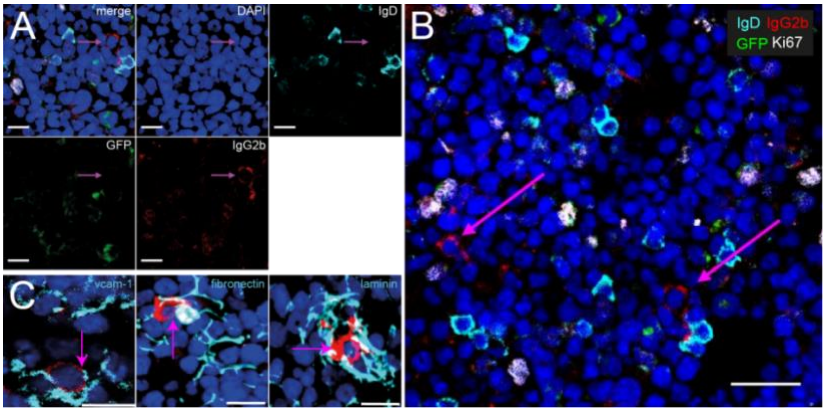

Figure 4-10 IgG_{2b}^+ B_{mem} of BM localize next to cells expressing VCAM-1, laminin and fibronectin
A) Identification of BM IgG_{2b}^+ $B_{mem.}$ naive B cells and plasma cells were excluded by IgD and Blimp1-GFP, respectively. Cell nucleus was identified with DAPI (blue). Scale bar: 10µm. B) IgG_{2b}^+ B_{mem} (Ki-67$^-$ IgD$^-$ Blimp1-GFP$^-$) are dispersed as single cells throughout the BM. Arrows indicate IgG_{2b}^+-staining DAPI$^+$ cells. Scale bar: 20µm. C) Co-localization of IgG_{2b}^+GFP$^-$IgD$^-$ IgG_{2b}^+ cells (arrows) with mesenchymal stromal cells (VCAM-1, fibronectin, laminin). Arrows indicate IgG_{2b}^+ staining DAPI$^+$ cells. Representative micrograph. Scale bars: 10µm.

The BM is an important organ in the tissue maintenance of long-lived memory lymphocytes (CD4+, CD8+, plasma cells). In the BM, plasma cells, memory CD4+ and CD8+ are localized in niches organized by reticular stromal cells [10,34,35]. BM niches for memory B cells have not been described. To investigate how the BM survival niches of B_{mem} is organized; histology sections of BM (Blimp1-GFP mice) were analyzed by immunofluorescence. Switched IgG2b$^+$ B_{mem} were identified as IgG_{2b}^+IgD$^-$Ki-67$^-$ nucleated cells. GFP+IgG2b$^+$ plasma cells [20] were excluded from analysis (Fig. 4-10A). Switched IgG_{2b}^+ memory B cells were dispersed as single cells throughout the BM (Fig. 4-10B). 75% of IgG_{2b}^+ memory B cells were in direct contact with reticular cells expressing VCAM-1 and fibronectin, and another 15 to 20% within 10µm vicinity of such cells (Fig. 4-10C, Fig. 4-11A). 53% of IgG_{2b}^+ B_{mem} were directly contacting laminin-expressing stromal cells, and another 26% were in the 10µm vicinity of such cells (Fig. 4-10C, Fig. 4-11A).

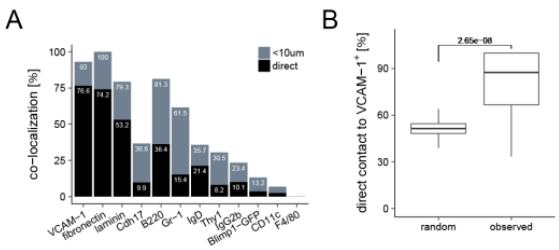

Figure 4-11 Colocalization of B_{mem} with stromal cells is deterministic
(A) Co-localization of with mesenchymal stromal cells. Graph shows frequency of IgG_{2b}^+ cells in direct contact (black) or within 10µm (grey) of a cell stained for the molecule indicated. (C) Graphs represent direct co-localization of than 12000 simulated (random) cells (images from 7 BM slides) versus co-localization observed per slide for 28 slides from 4 mice with two or more analyzed B_{mem} per slide (mean=5.66 cells per slide), p-value (Welch's test) indicated on graph.

4.6 B_{mem} - stromal co-localization is deterministic

The analysis of BM sections showed that the majority of B_{mem} co-localize with VCAM-1+ reticular stromal cells. To determine whether the contact of B_{mem} to VCAM-1 expressing stromal cells is not a mere random association between the two cell types, co-localization of these cells types was simulated by randomization modelling [12]. The randomization modelling confirmed that the co-localization between B_{mem} and reticular stromal cells was deterministic and cannot be attributed to mere randomness. The observed frequencies of co-localization (recorded at microscope) were significantly higher than in the randomly simulated co-localization (Fig. 4-11B). The co-localization of BM B_{mem} and stromal cells is in line with expression of VLA4 (CD49d/CD29), a receptor for fibronectin and VCAM-1, and VLA6 (CD49f/CD29), a receptor for laminin [38], by IgG_{2b}^{+} B_{mem} (Fig. 4-12).

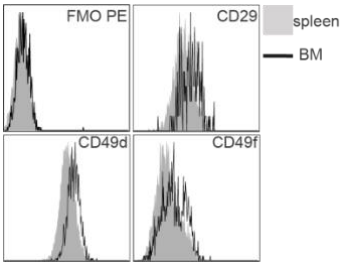

Figure 4-12 B_{mem} express receptors to VCAM-1, laminin and fibronectin
surface expression of VLA-4 and VLA-6 components CD29, CD49d, CD49f in spleen and bone marrow IgG_{2b}^{+} B_{mem}. Gated for $IgG_{2b}^{+}CD19^{+}CD38^{+}CD138^{-}GL7^{-}CD11c^{-}IgM^{-}IgD^{-}PI^{-}$ small lymphocytes; histogram plots are representative for three biological replicates.

4.7 BM stromal cells exhibit enormous heterogeneity

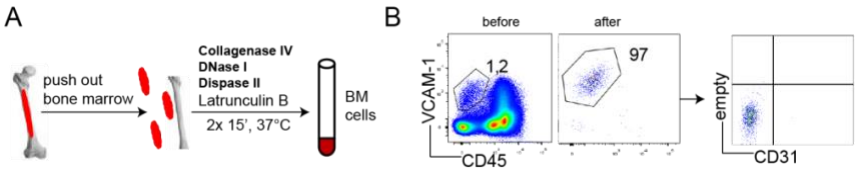

Figure 4-13 Schematic overview of isolation of BM stromal cells.
(A) Enzymatic digestion of BM.
(B) Cytometric isolation of VCAM-1+CD45-Ter119-CD31- BM stromal cells.

BM VCAM-1+ stromal cells express cytokines and chemokines which attract and help in the maintenance of different subsets of memory T and long-lived plasma cells [39]. Although much is known about the organization of memory lymphocytes, corresponding research on the organization of BM stromal cells is scarce. Taking advantage of the latest single cell RNA sequencing high throughput technology, the phenotype and functional organization of BM stromal cells was addressed at the single cell resolution. BM cells (tibia and femur) were isolated by enzymatic digestion to generate single cell suspension (Fig. 4-13A). *Ex vivo* VCAM-1+CD45-Ter119-CD31- BM cells were then

sorted by FACS to 97% purity (Fig. 4-13B) and transcriptomes of individual cells were determined using 10X genomics-based droplet sequencing (Fig. 4-14). Transcriptomes of 1,167 individual stromal cells were analyzed with a mean of 398,739 reads per cell (Fig. 4-14A) resulting in a saturation rate of 95.6%, i.e. more than 95% of the total transcriptome was captured (Fig. 4-14B). 16,142 genes were detected in total, with a median of 1,538 genes per cell (Fig. 4-14A, C). Transcriptomes of individual cells were projected on a t-distributed stochastic neighbor embedding (t-SNE) analysis [40] to visualize the basic heterogeneity of the stromal cells (Fig. 4-15).

A

Parameter	Output
number of cells	1,167
total number of reads	465,328,482
mean reads per cell	398,739
median UMI counts per cell	3,441
total genes detected	16,142
median genes per cell	1,538

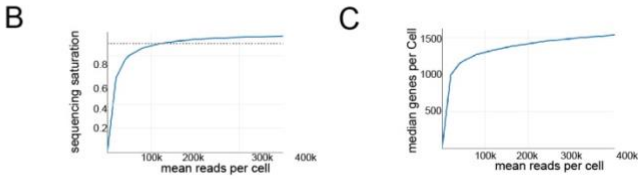

Figure 4-14 Single cell RNA sequencing (sc RNA-seq) of BM stromal cells
(A) Quality control (QC) summary of single cells sequencing output. (B) 10X genomics-based plot showing the mean read per cells, against the sequencing saturation. (C) Plot of the median number of genes detected per cell in relation to total reads per cell

Factors like *Vcam1*, *Cxcl12, and Kitl* important in the maintenance and development of different hematopoietic cells were expressed by more than 90% of the BM stromal cells. *Pdgfrb, Cadherin 11*(*Cdh11*), genes encoding for mesenchymal surface proteins were expressed by majority of cells, qualifying these genes as genuine stroma cells markers [41] (Fig. 4-15). On the level of single cell transcriptomes, cells expressing the various CD genes are dispersed within the t-SNE plots. Genes encoding cell surface molecules like *Lamp1* (CD107a), *Ox2* (CD200), *Cd1d1*, *Eng* (CD105), and *Cd44* were expressed individually by the stromal cells without defining unique subpopulations of stromal bone marrow (Fig. 4-15).

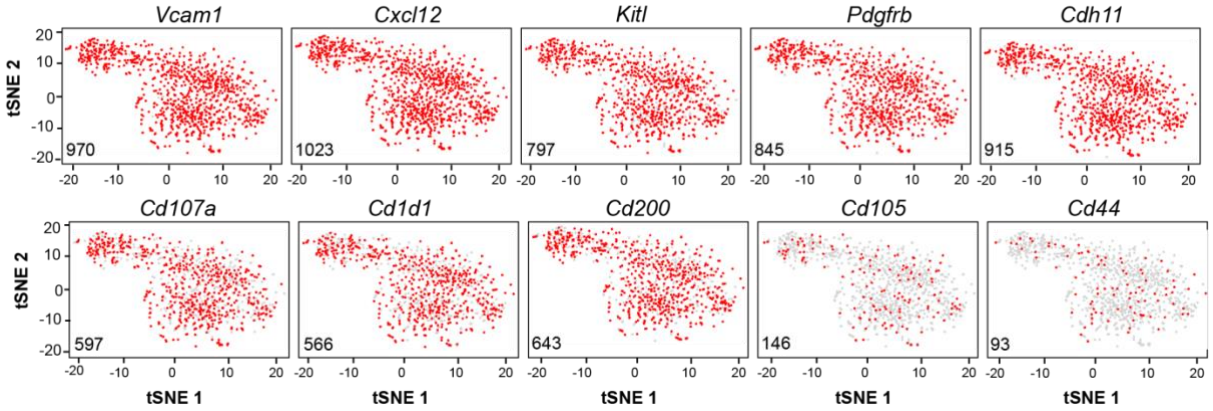

Figure 4-15 t-SNE plots highlighting the expression (red) of individual genes

4.8 Distinct subpopulations of BM stromal cells for the maintenance of immune and hematopoietic cell subsets

In the interaction between stromal cells and hematopoietic cells, the expression of chemokines and cytokines by stromal cells is essential for them to attract and control hematopoietic cells. The transcriptomes of individual BM stromal cells were analyzed for the expression of genes which encode for secreted proteins (cytokines and chemokines). A total of 108 genes were selected for further analysis, based on their established role in the communication of stromal cells with cells of the hematopoietic system.

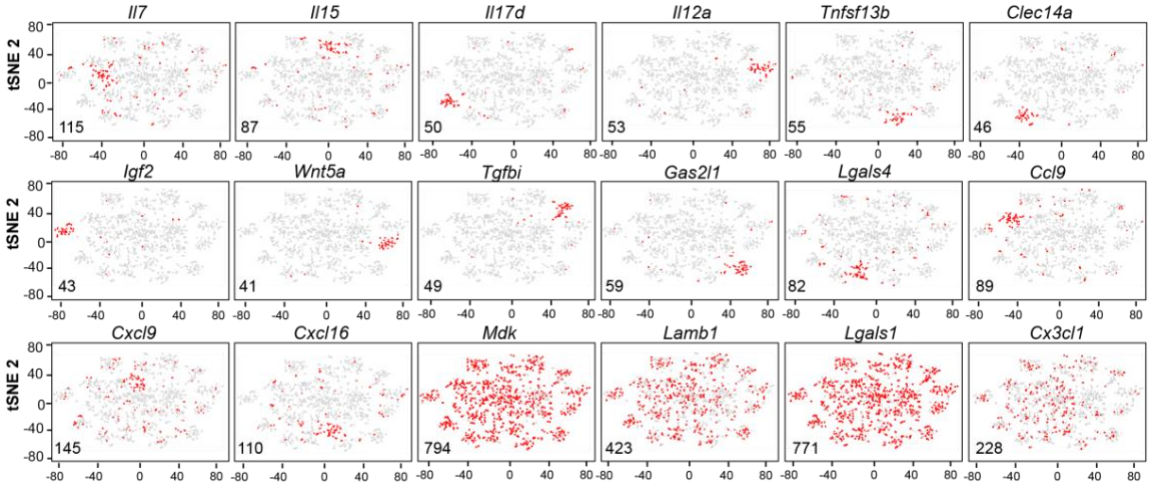

Figure 4-16 t-SNE plots highlighting the expression (red) of individual genes

Supervised clustering analysis of expression of these genes identified 14 non-overlapping subsets of stromal cells (Fig. 4-16). Genes like *Cxcl12*, *Kitl*, Colony Stimulating Factor 1 (*Csf1*) and Laminin B1 (*Lamb1*), were expressed by most stromal cells, hence they do not define distinct subpopulations of stromal cells (Fig. 4-16). Cells expressing the cytokines *Il7, Il15, Il12a, Il17d, Clec14a, Igf2, Lgals4, Tnfsf13b, Il4, Wnt5a and Tgfbi* formed unique subsets (Fig. 4-16). IL17D is a novel cytokine which inhibits the development of myeloid progenitor cells [42]. CLEC14A is a type I transmembrane involved in cell-to-cell adhesion, and thus shaping immune response [43]. IL12A has multiple effects on T and natural killer cells [44]. Expression of the chemokines *Ccl9, Cxcl9 and Cxcl16* was restricted to distinct subsets, too. CXCL16 attracts memory T cells which express CXCR6 [45]. CCL9 and CCL7 attract subsets of dendritic [46] and T cells [47] respectively.

Expression of genes encoding for any of these chemokine/cytokines was exclusive to distinct subset of stromal cells, with less than 10% of cells co-expressing any two of these genes (Fig. 4-17). Stromal cells expressing these cytokines and chemokines expressed defined gene signatures, based on their entire transcriptomes, qualifying them as distinct subpopulations of stromal cells (Fig. 4-18).

The non-overlap expression of key cytokines and chemokines demonstrates that different subsets of BM stromal cells potentially attract and organize survival niches for the different memory lymphocytes. This demonstrates for the first time, existence of potential specialized stromal niches for the support and long-term maintenance of immune cells and other cells of the hematopoietic system

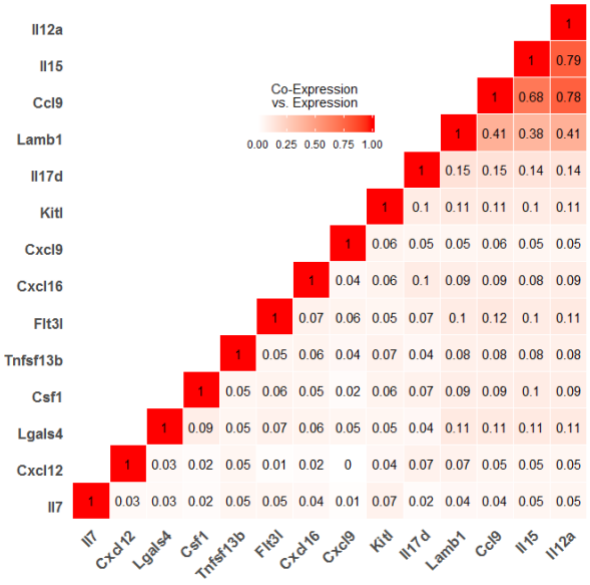

Figure 4-17 Co-expression matrix showing the correlation of two genes.
The similarity of gene expression (co-expression) of gene A and gene B was computed by Jaccard similarity coefficient ((A!=0 && B!=0) / (A!=0 || B!=0) where A and B refers to expression of genes A and B respectively)

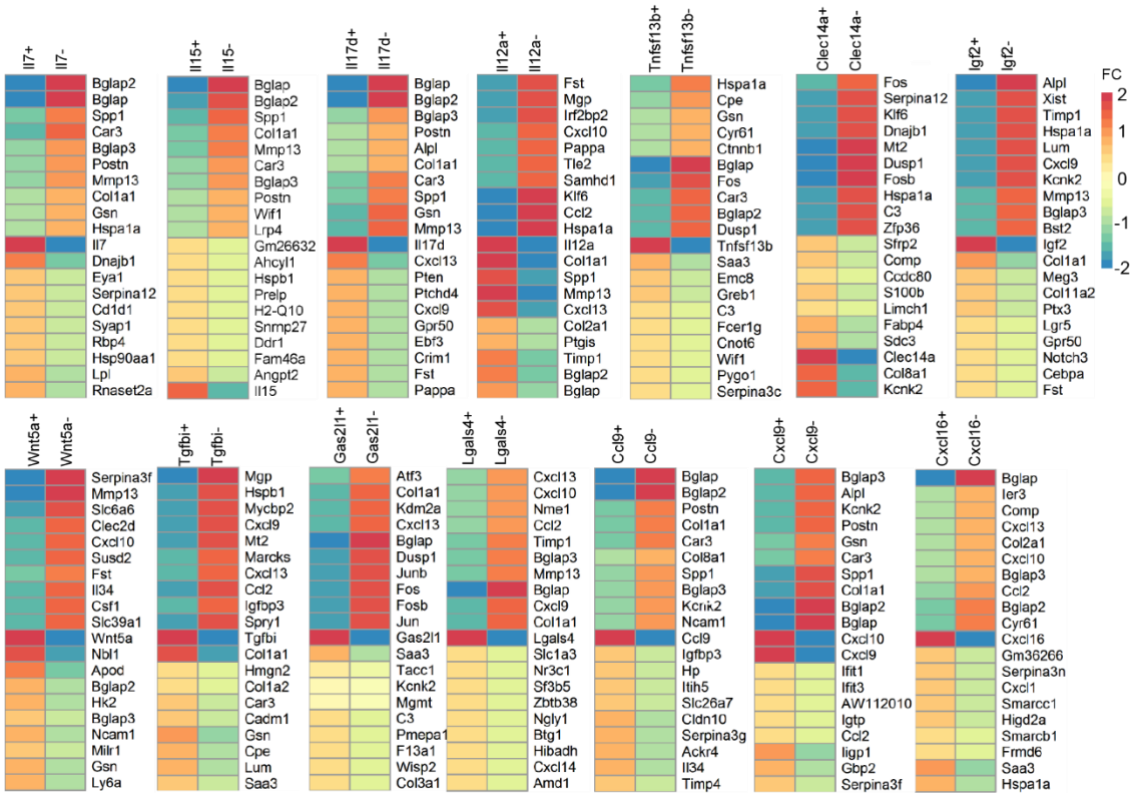

Figure 4-18 Heatmap comparison of the gene expression profile of cells which express or do not express a single gene.
Fold change (FC) shows the log2(Average Expression positive cells) - log2(Average Expression negative cells), displayed are the top 10 genes with the highest fold change. DiffExpTest-method was used for the statistical analysis of differential expressed genes

5 Discussion

The findings of this doctoral thesis demonstrate that B cell memory is compartmentalized similarly to T and plasma cells with significant populations of isotype-switched B_{mem} in BM and spleen. Isotype-switched B_{mem} of BM and spleen differ in BCR repertoires, suggesting that they might constitute separate compartments of B cell memory. It remains unclear whether (sub-) populations of these tissue resident isotype-switched B_{mem} contribute to the pool of circulating B cell memory. Also, memory B cells of the two organs show differences in their phenotype. The majority of B_{mem} of BM and spleen are quiescent and refractory to therapeutic targets which kill proliferative cells. In the BM, B_{mem} co-localize to reticular stromal cells in a deterministic manner guided by receptor-ligand interactions. BM stromal cells exhibit structural and functional organization with distinct sub-populations of BM stromal expressing factors important in the maintenance of subsets of memory lymphocytes. Subset of BM stromal cells (approximately 10%) express BAFF (B-cell Activating Factor) which is crucial for B and

plasma cells survival is also evident for the single cell RNA-sequencing transcriptome analysis.

5.1 Tissue distribution of memory B cells

These findings provide evidence for the existence of a resting population of B_{mem} in the murine BM. Overall, in mice the BM contains a significant number of isotype-switched B_{mem}. Isotype-switched B_{mem} in spleen and BM differ in presence and frequency of BCR repertoire. A significant proportion of 40% to 80% of the BCR clonotypes are expressed exclusively in either spleen or BM. Exclusive clonotypes indicate tissue residency and minimal exchange of the repertoire between the two organs. For those shared B_{mem} clonotypes (10-35%) between BM and spleen, the situation is less clear, they could be resident or constantly exchanged between the two organs. Although this doctoral thesis addressed the tissue distribution of isotype-switched memory B cells, it is likely that non-switched B_{mem} exhibit similar distribution pattern. Studies on tissue distribution of B cell memory in human has mostly being restricted to peripheral blood partly due to the difficult of access to tissues like BM and spleen. Human studies in this direction are needed to translate and better understand the tissue distribution of B_{mem} in humans. Strategic positioning and functional specialization of subsets of B_{mem} would ensure rapid, effective local and systemic protection during immune challenge.

5.2 Lifestyle of memory B cells

Switched memory B cells of BM and spleen are resting in terms of proliferation (G_0 phase of the cell cycle) as shown by expression of the cell cycle marker Ki-67. *In vivo*, B_{mem} of BM and spleen are refractory to treatment with cyclophosphamide, a therapeutic agent which kills proliferating cells. This finding points to the longterm survival and resistance of memory B cells to therapeutic agents which eliminate other subsets of hematopoietic and immune cells. The finding on CD21 expression adds an additional level of complexity and heterogeneity of B cell memory. The functional implication of these phenotypic differences remains a matter of investigation. $CD21_{low}$ B cells have been described as atypical, characterized by functional exhaustion and with a potential role in infection or autoimmunity [48,49].

5.3 Bone marrow niches for memory cells

In the BM, switched B_{mem} localize individually to VCAM-1+ reticular stromal cells, similar to those maintaining memory T and plasma cells. The multiple receptor-ligand (survival factors) interactions between memory cells and stromal cells construe the importance of BM stromal cells in maintenance and organization of the immunological memory. The single cells transcriptome analysis shows for the first time that distinct subsets of BM stromal cells express the factors IL7, IL15, and Tnfsf13b (BAFF) which are important in the maintenance of memory CD4+, CD8+, B and plasma cells respectively via signaling through corresponding receptors expressed on the memory cells. It has long been shown that T cell support and antigenic stimulation of BCR are dispensable for the long-term maintenance of B_{mem}. Stromal cells express several factors important for the survival of B_{mem} in a receptor-ligand manner. Thus, stromal cells of the BM are also potentially autonomous in providing niches for the long-term maintenance of immune memory cells without the need for accessory cells in the niche. The findings of this thesis suggest the existence of specialized stromal niches for different subsets of immune memory cells. The exact survival signaling pathways induced in B_{mem} by stromal cells should be investigated to help devise ways to boost protective immunological memory or deplete pathological memory cells in autoimmunity, chronic inflammation and cancer.

In conclusion, my doctoral thesis described the bone marrow as a major organ in the maintenance of long-lived quiescent memory B cells and that memory B cells of bone marrow differ from those in spleen in terms of BCR repertoire and surface protein expression. In the bone marrow, a subpopulation of reticular mesenchymal stromal cells organizes survival niches for the maintenance of memory B cells through the expression of important cytokines and chemokines.

6 Literature

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7 Statutory Declaration

“I, **Richard Kwasi Addo**, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic: **BONE MARROW MAINTAINS ISOTYPE SWITCHED MEMORY B CELLS IN STROMAL NICHES**, independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I am aware of the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice and that I commit to comply with these regulations.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

8 Declaration of your own contribution to any publications

Publication 1:

Single-Cell transcriptomes of murine Bone Marrow Stromal Cells Reveal Niche-Associated Heterogeneity

Richard K. Addo, Frederik Heinrich, Gitta Anne Heinz, Daniel Schulz, Özen Sercan-Alp, Katrin Lehmann, Cam Loan Tran, Markus Bardua, Mareen Matz3, Max Löhning, Anja E. Hauser, Andrey Kruglov, Hyun-Dong Chang, Pawel Durek, Andreas Radbruch, and Mir-Farzin Mashreghi.

European Journal of Immunology 2019

Contribution:

- First authorship
- Planning and preparation of the project together with Prof. Andreas Radbruch, Dr Hyun-Dong Chang and Dr. Mir-Farzin Mashreghi
- Conducting the animal experiment
- Enzymatic digestion and single cell isolation of bone marrow cells
- FACS sorting and analysis of data
- Independent graphical representation of the results in all Figures
- Essential contribution to the critical appraisal of the results with identification of the relevant findings of the study including their limitations
- Significant contribution to the processing and evaluation single cells RNA sequencing data
- Development of the manuscript leading to publication together with Prof. Dr. Andreas Radbruch, Dr. Hyun-Dong Chang and Dr. Mir-Farzin Mashreghi
- Collaboration with Prof. Dr. Andreas Radbruch, Dr. Hyun-Dong Chang and Dr. Mir-Farzin Mashreghi on the revision and implementation of the reviewers' comments

Publication 2:

MicroRNA-31 reduces the motility of proinflammatory T helper 1 lymphocytes

Bardua M, Haftmann C, Durek P, Westendorf K, Buttgereit A, Tran CL, McGrath M, Weber M1, Lehmann K, **Addo RK**, Heinz GA, Stittrich AB, Maschmeyer P, Radbruch H, Lohoff M, Chang HD, Radbruch A, Mashreghi MF.

Frontiers in Immunology, 2018

Contribution:

- Co-authorship
- Essential contribution to the critical appraisal of the results with identification of the relevant findings of the study including their limitations.

- Significant contribution to the processing and evaluation of qRT-PCR data measuring miR-31, Foxo1, Sell, Klf2 and Cd69 expression in Figure 5
- FACS data analysis of Tbet (Figure 5D, 5F), Foxp3 (Figure 5G)
- collaboration on the revision and implementation of the reviewers' comments

Publication 3:

The Regulation of IFN Type I Pathway Related Genes RSAD2 and ETV7 Specifically Indicate Antibody-Mediated Rejection After Kidney Transplantation

Matz M, Heinrich F, Zhang Q, Lorkowski C, Seelow E, Wu K, Lachmann N, **Addo RK**, Durek P, Mashreghi MF, Budde K.

Clinical Transplantation, 2018

Contribution:

- Co-authorship
- Essential contribution to the critical appraisal of the results with identification of the relevant findings of the study including their limitations Figure 2 and Figure 3.
- Significant contribution to the processing and evaluation high throughput RNA sequencing data including selection of candidate genes Figure 2 and Figure 3

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Signature, date and stamp of supervising university professor / lecturer

_____

Signature of doctoral candidate