Analysis of the early B cell development in the domestic chicken by reverse genetics

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To my family

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ABBREVIATIONS

7AAD	7 Amino actinomycin D
Ab	Antibody
AGM	Aorta-gonad-mesonephros region
Aqua bidest.	Double distilled water
Aqua dest.	Distilled water
APC	Allophycocyanin
BSA	Bovine serum albumin
Bu1	Type I glycoprotein
CALT	Conjunctiva associated lymphoid tissue
Cas9	CRISPR associated protein 9
CCL	CC-Chemokine ligand
CCR	CC-Chemokine receptor
CD40L	CD40 ligand
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CEFs	Chicken embryonic fibroblasts
ch	Chicken
CLP	Common lymphoid progenitor
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCL	CXC ligand
CXCR	CXC receptor
DAPI	4,6 Diamidino-2-phenylindole
D_H	Heavy chain diversity segment
DNA	Deoxyribonucleic acid
E2A	Transcription factor E2 alpha
ED	Embryonic day
FAE	Follicle associated epithelium
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
FLT3	Fms related tyrosine kinase 3
FSC	Forward scatter
G0	Generation 0
G1	Generation 1
GALT	Gut associated lymphoid tissue
G-CSF	Granulocyte colony stimulating factor
GFP	Green fluorescence protein
HEV	High endothelial venules
HGF	Hepatocyte growth factor
HIF-1a	Hypoxia inducible factor 1a
HIV-1	Human immunodeficiency virus 1
HSC	Hematopoietic stem cell
IFE	Interfollicular epithelium
IFN	Interferon
Ig	Immunoglobulin
IgD	Immunoglobulin D
IgM	Immunoglobulin M
IgY	Immunoglobulin Y
Igα	Immunoglobulin α
Igβ	Immunoglobulin β

IL4	Interleukin 4
IL6	Interleukin 6
IL7	Interleukin 7
IL15	Interleukin 15
ITAM	Immunoreceptor tyrosine-based activation motif
i.v.	Intravenously
J_H	Heavy chain joining segment
JH-KO	Heavy chain joining segment knockout
J_L	Light chain joining segment
L	Ligand
LESTR	Leukocyte derived seven transmembrane domain receptor
LSL	Lohmann's Selected Leghorn
mAb	Monoclonal antibody
MIF	Macrophage migration inhibitory factor
MPP	Multipotent progenitor cell
NALT	Nasal associated lymphoid tissue
NK	Natural killer cell
Pax5	Paired box 5
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PBSF	Pre-B cell growth stimulating factor
PCR	Polymerase chain reaction
PNAd	Peripheral lymph node addressin
qRT-PCR	Quantitative real time polymerase chain reaction
R	Receptor
RAG	Recombination activating gene
RCAS	Replication-competent avian leucosis virus
SALT	Skin associated lymphoid tissue
SCF	Stem cell factor
SDF-1	Stromal cell derived factor-1
sIg	Surface immunoglobulin
s.c.	Subcutaneous
TGFβ	Transforming growth factor β
T_H	Helper T cell
UNLB	Unlabeled
UV	Ultraviolet
VCAM	Vascular cell adhesion molecule
V_H	Heavy chain variable segment
V_L	Light chain variable segment
WT	Wild type
α	Alpha
β	Beta
γ	Gamma
δ	Delta
κ	Kappa
λ	Lambda
μ	Micro

1. INTRODUCTION

In the past few decades the chicken has become a very important animal model in several research fields. Beside its contribution to immunology research it is one of the most used animal models in developmental biology. Furthermore, the chicken is one of the most important sources for animal protein in the human diet. Because of intensive housing conditions, however, the health of the species has often been at risk. Thus, a better understanding of the immune system is essential to maintain or improve animal health and welfare.

A solid immune system relies on functional B lymphocytes which can differentiate into antigen specific antibody producing plasma cells. During their development B lymphocytes undergo several migration steps between lymphoid organs.

Chicken hematopoietic cells need to migrate from the aorta to hematopoietic tissues (mainly the spleen) where the first B cell precursors are localized. From there they migrate into the bursa of Fabricius. The most critical part of B cell development and differentiation takes place in the bursa. B cells emigrate from there and back to the periphery as mature B cells around hatch, where they are maintained till activation in germinal centers in response to antigen contact.

The pathways and the mechanisms of the chicken B cell development are relatively well studied. However, important knowledge is missing on the regulatory signals framing the developmental processes and especially the factors driving the migration of the cells throughout development.

In mammalian B cell development one significant factor regulating migration of the cells during their early development is the interaction of the chemokine CXCL12 and its chemokine receptor CXCR4. Bone marrow stromal cells produce the chemokine CXCL12, while B cell progenitors express the chemokine receptor CXCR4 on their surface. Chemokine ligand and receptor tend to bind and thereby the interaction of CXCR4 and CXCL12 regulates the trafficking of the cells.

Due to parallels in mammalian and avian B cell development but also based on results of *in vitro* studies, the CXCR4-CXCL12 axis seems to be a much promising candidate being involved in bursal colonization.

To analyse the role of the CXCR4-CXCL12 interaction for the early B cell development *in vivo,* a method which enables the intravenous manipulation of the chicken embryo for both

intravenous drug and cell administration and blood sampling had to be established. The method had to be standardized to a level that multiple manipulations of the same embryo are possible. Furthermore, adoptive transfers of B cell precursors into chicken embryos using immunoglobulin knockout chickens had to be established. By adoptive transfer of B cell precursor into immunoglobulin knockout chickens it was possible to analyse the role of CXCR4-CXCL12 in B cell migration *in vivo*. Two different approaches have been used to block the interaction of CXCR4 and CXCL12 either by intravenous injection of a CXCR4 inhibitor or by blocking the CXCR4 receptor on the cells prior to adoptive transfer.

2. LITERATURE

The health of any organism depends on a functional immune system and its efficient immune responses. The immune system can be separated in two important and to each other complementary parts, the innate and the adaptive immune system [1]. The innate immune system constitutes the first line of defense of the organism. Reactions within the innate immune response are non-specific but very fast and act as a first barrier to infectious agents. By means of chemokines and cytokines, cells are recruited to the site of infection. At the same time while trying to defend the organism, the innate immune responses promote the activation of the adaptive immune system. The antigen is presented to specialized cells of the adaptive immune system. The specific adaptive immune responses need up to seven days to develop, but after the primary exposure and response to an antigen, any further exposure leads to immediate response [1, 2].

2.1 THE ROLE OF B LYMPHOCYTES IN THE IMMUNE SYSTEM

B lymphocytes play an essential role in the adaptive immune response. Originating from hematopoietic stem cells (HSCs), they have common origin with T lymphocytes and natural killer cells (NK) [3, 4] but at the same time significant differences. As antigen presenting cells [5] and precursors of plasma cells, B cells contribute significantly to the adaptive immunity. With their vast antigen receptor repertoire (a human can produce more than 10^{12} different antibody molecules) they are able to recognize and bind antigens of enormous variety [6, 7]. After antigen recognition, B cells proliferate and produce a clone of antigen specific B cells [8]. Only then do they divert into effector plasma cells.

Plasma cells secret antigen specific antibodies [9] which can act in three different ways for the host defense. They bind to the antigen in order to inactivate it, a procedure called neutralization. They lead to opsonisation of the antigen by building a coating surrounding the antigen and making it recognizable for other cells to ingest and destroy it. Finally, antibodies activate the complement system by forming the first receptor of the complement cascade. The complement system then promotes phagocytosis by phagocytes [1].

2.2 B CELL DEVELOPMENT IN MAMMALS

As mentioned before, B cells originate from the hematopoietic stem cells and have to commit at first either to the myeloid or the lymphoid lineage [3, 4]. From the stage of multipotent progenitor cells (MPPs), they differentiate into common lymphoid progenitors (CLPs). CLPs differentiate then into antigen specific lymphocytes, the B and T cells, as well as into not antigen-specific NK cells. Cells committed to the lymphoid lineage will later on have to commit either to the B or the T cell lineage and will give rise to B and T cells respectively.

2.2.1 The role of the B cell receptor complex in B cell development

The main role of the B cells is to produce immunoglobulins in order to fulfill their function in the host defense. Each clone of B cells produces a unique immunoglobulin molecule [10].

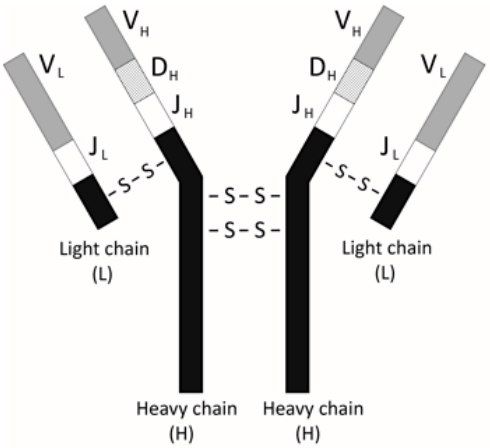


Fig. 1 Antibody molecule structure

The immunoglobulin molecules consist of two heavy and two light chains, symmetrically bound. Heavy chains consist of a variable, a diversity, a joining and a constant region. The light chain lacks diversity segments.

Immunoglobulins consist of two light and two heavy chains, which are bilaterally symmetric, organized in a Y-shape [11]. The chains have a variable region responsible for recognizing the antigens and a constant region which is isotype-specific. The light chain consists only of variable (V) and joining (J) segments, while the heavy chain consists of variable, joining and diversity (D) segments (Fig. 1). There is a pool of gene segments for the different regions which rearrange in order to build a wide immunoglobulin diversity repertoire [1].

The first developmental stage of B cells is the early pro-B cell [1, 12]. During the stages of pro-B cells (early and late) the cells undergo several stages of gene rearrangement through which pro-B cells turn into pre-B cells which express the B cell receptor (BCR) mainly intracellularly but also on their surface.

The first gene rearrangement takes place at the heavy chain (H) locus and affects the diversity (D_H) and joining (J_H) gene segments. Following the D_H and J_H rearrangement, a V_H to DJ_H rearrangement takes place [13] (see Fig. 2). Many of the recombinations can lead to unsuccessful rearrangements. In order to check if the recombination was productive, the heavy chain needs to signal [14]. However, therefore pairing with light chains is essential and since they have not rearranged at this point, pro-B cells produce two proteins which are very similar to the light chain and known as 'surrogate light chains' [1]. The surrogate light chains can bind with the μ chain and build together the pre-B cell receptor [15], allowing its surface expression. The surrogate light chains which have associated with the heavy chains possess amino-terminal tails which bind to amino-terminal tails of neighbouring pre-B cell receptors [1]. Additionally, pro-B cells begin to express the transmembrane proteins Igα and Igβ. Both of them are being expressed from the stage of the pro-B cells on and are necessary not only for the pre-B cell receptor but also for the expression of a functional BCR. Igα and Igβ, associated with the B cell receptor, build the B cell receptor complex [16]. The association of the corresponding amino-terminal tails of the pre-B cell receptor leads to dimerization of the pre-B cell receptors and mediates signalling through the immunoreceptor tyrosine-based activation motifs (ITAMs) of the Igα and Igβ signalling chains [17]. Signalling through the Igα and Igβ ITAMs results in inhibition of the expression of recombination activating gene 1 and 2 (RAG-1 and RAG-2) and proliferation of the cells [1, 18].

Furthermore, signalling by the pre-B cell receptor leads to allelic exclusion [19]. Allelic exclusion ensures that only one of the two alleles is being expressed. Each B cell expresses that way only one type of B cell receptor. If gene rearrangements in both alleles are non-productive, the cells are driven to apoptosis. A successful process leads to termination of the heavy chain gene rearrangements [20].

After production of the pre-B cell receptor and passing this checkpoint between the stage of pro-B cell and pre-B cell, the cells proceed to the next step of rearranging the light chains (Fig. 2). However, before they rearrange their light chains, they first proliferate, producing clones of cells expressing the same productive heavy chain (up to 100 fold) [21]. This way cells rearranging their light chain lead to an even higher diversity of the receptor.

The pre-B cells express the RAG proteins again, whose expression was reduced before by the signalling through the ITAMs of the Igα and Igβ signalling chains (see Tab. 1). RAGs expression is as necessary for the rearrangement of the light chains as for the heavy chains [22]. There are two types of light chain genes, the κ and the λ light chain gene [23]. Murine

and human κ light genes are usually rearranged prior to the λ light chain locus [24]. Additionally, beside allelic exclusion, which also applies to the light chain rearrangement procedure, a further mechanism for exclusion applies to the light chain, that of the isotypic exclusion. Isotypic exclusion ensures that only one of the κ or λ light chain type is expressed by a single B cell [13].

Once again, only pre-B cells that have successfully rearranged their light chain can further develop since not all unsuccessful joins undergo apoptosis. Productive rearrangements and pairing of the light chain with a μ chain leads to a functional surface immunoglobulin (sIg) on the cell surface and the cell turns into an immature B cell [25]. In order for immature B cells to further develop they are not allowed to be reactive to self-antigens [26]. Self-reacting cells are either undergoing apoptosis (i.e. clonal deletion) [27] or are driven to anergy. Therefore, the whole B cell receptor complex interacts with the bone marrow environment. The signals that occur from that interaction define the next step. This procedure is called central tolerance. Cells that are not self-reactive are positively selected and can develop further. Finally, beside the μ chain, non self-reactive cells produce a δ chain. The production of the δ chain leads to the expression of the IgD molecule on the surface of the cell (Fig. 2). When cells express both IgM and IgD on their surface, the rearrangements cease.

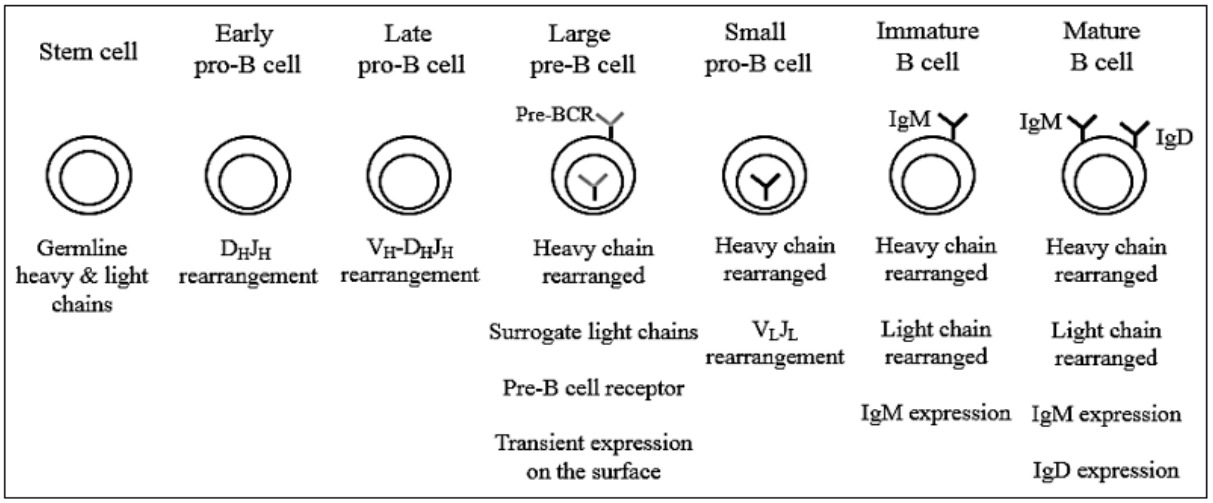


Fig. 2 Developmental stages of B cell lineage in mammals, modified from *Janeway's Immunology [1]*

The H-chain locus is the first to rearrange. Early pro-B cells rearrange their D to J segments. Then a V_H to DJ_H rearrangement takes place at the stage of late pro-B cells. If successful, an immunoglobulin heavy chain is expressed, also known as the pre-B cell receptor, which is first found in the cytoplasm and then partially on the surface of the cells. At the stage of small pre-B cells, the cells express only the μ heavy chain in their cytoplasm while the light chain genes rearrange. After successful rearrangement cells turn into immature B cells, expressing the IgM on their surface. Finally, the cells produce a δ heavy chain, leading the IgD expression on the cell surface of the mature B cells [1].

2.2.2 B cell migration during development in mammals

The first HSCs are localized in the aorta-gonad-mesonephros region (AGM) and specifically first detected in the dorsal aorta area [28, 29]. In mice, these cells migrate to the embryonic liver where they massively proliferate [30]. The following migration target of the cells is the bone marrow (see Fig. 3). Throughout life, HSCs are retained in the bone marrow for a very long period and divide themselves almost every fifth month [31]. When divided, one of the cells starts differentiating and the other cell stays in the bone marrow as an HSC [32, 33]. In the bone marrow the cells are distributed in different areals, according to their developmental stage. Early stages are mostly localized in the low oxygen region [34], while the later stages move closer to the vessel-rich areas of the bone marrow from where, after a short intravascular maturation phase, they can then migrate through the blood stream to the peripheral lymphoid tissues [35, 36]. Migration of B cells from the bone marrow through the blood stream and into the spleen is driven by sphingosine-1-phosphate-mediated (S1P-mediated) chemoattraction [37, 38], which overcomes the retention mediated by the CXCR4 and CXCL12 interaction, dominating during the prior stages [21]. Mature B cells undergo migration processes in order to encounter antigens and interact with helper T cells (T_H), to be activated and to differentiate in germinal centers into either antibody secreting plasma cells or memory B cells [1]. Finally, plasma cells exit the germinal centers to either move to the medullary cord of the lymphoid tissue or to migrate back to the bone marrow.

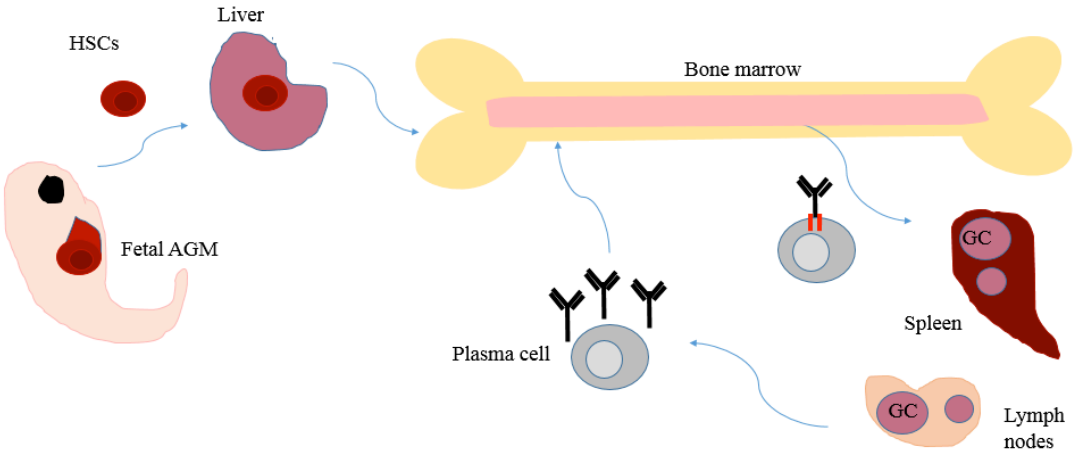


Fig. 3 B cell development in mammals

Hematopoietic stem cells (HSCs) from the fetal aorta-gonad-mesonephros region (AGM) migrate to the liver and from the liver to the bone marrow where they differentiate into B cells and produce surface immunoglobulins. In order to mature they migrate back to the periphery in the spleen and lymphoid organs where they are activated and proliferate in germinal centers to differentiate into antigen specific antibody producing plasma cells and memory cells that migrate back to the bone marrow.

The migration processes (shown in Fig. 3) as well as the differentiation processes of the cells

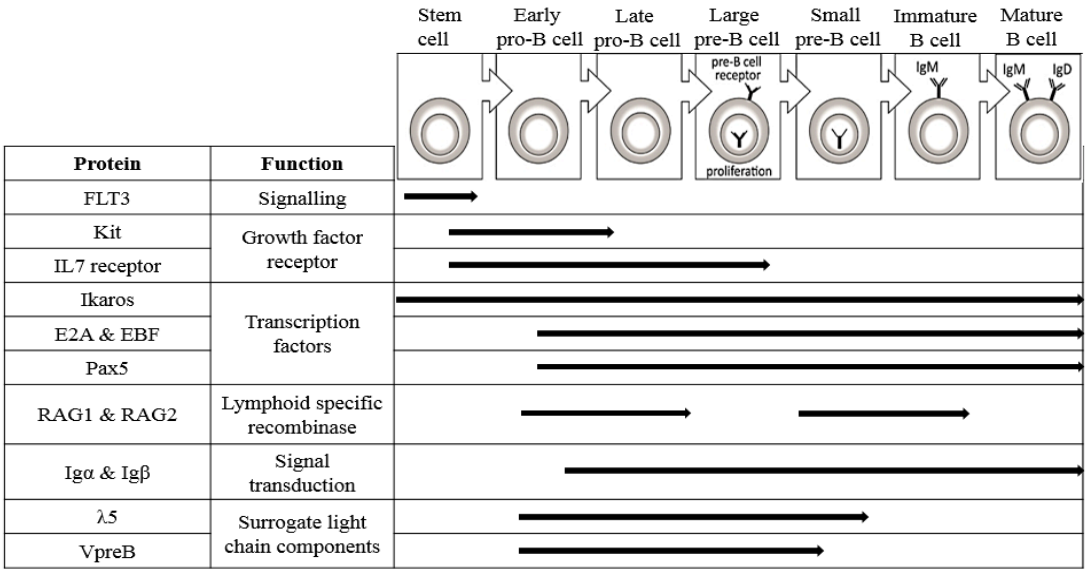
are driven and regulated by diverse factors produced from the cells themselves or from the cells of their environment (i.e. stromal cells of the bone marrow).

2.2.3 Regulation of the B cell development in mammals

There are several factors that regulate the developmental procedure of B cells. These factors can be originating in the cells themselves or in their environment (i.e. bone marrow stromal cells). The regulation is provided by signalling or by acting as essential growth and transcription factors or by transduction of signals [1].

2.2.3.1 Factors expressed by B cells, regulating cells' own development

HSCs and MPPs express the receptor tyrosine kinase FLT3 (see Tab. 1). In order to differentiate to the next stage of the CLPs, MPPs require signalling through the FLT3 [39]. FLT3 expressing cells bind to the stromal cells of the bone marrow expressing the membrane bound FLT3L ligand. Furthermore, the interleukin 7 (IL7) receptor is expressed at this stage (see Tab. 1). The expression of the IL7 receptor is driven by the FLT3 signalling in combination with the transcription factor PU.1 [40, 41]. Pro-B cells express on their surface the receptor tyrosine kinase Kit (CD117) which binds to the stem-cell factor (SCF). This activates the kinase which then induces the B cell progenitors' proliferation. Moreover, the transcription factors PU.1 and Ikaros are expressed very early in the B cell developmental process and are essential for the induction of the transcription factors E2A and EBF expression [42, 43]. The significance of the transcription factor Ikaros is demonstrated by Ikaros-deficient mice which completely lack B cells but also show decreased number of fetal T cells and NK cells [44, 45]. Both E2A and EBF transcription factors promote together the expression of further proteins, essential for the development of the B lineage cells [1]. One of them is the transcription factor Pax5, a protein coding gene whose target is, among others, the Igα gene. Under Pax5 deficiency, pro-B cells might turn into T cells but also to myeloid cells and do not develop to further stages [46]. Beside the Pax5 expression, E2A and EBF are involved in the regulation of the RAG1 and RAG2 expression [47], which are necessary for both heavy and light chain rearrangement (see Tab. 1).



Tab. 1 Expression of proteins during B cell development, modified from *Janeway's Immunology [1]*

HSCs and CLPs express the FLT3 receptor. Pro-B cells also express the Kit and the IL7, while the stage of pre-B cell can be distinguished by the absence of the Kit and the IL7 receptor. The transcription factors Ikaros, E2A, EBF and Pax5 are first expressed by the early pro-B cells and throughout their development. RAG1 and RAG2 are present for the heavy and light chains rearrangements and after each successful rearrangement they are downregulated. λ5 and VpreB are components of the surrogate light chains and are expressed prior to the light chain rearrangement.

2.2.3.2 Factors expressed by the bone marrow driving B cell differentiation and development

The bone marrow constitutes an essential environment for the lymphocytes' development. Its stromal cells provide factors which promote the differentiation and development of the lymphocytes and at the same time regulate the motility or maintenance of the cells in and out of the bone marrow. The most important signallings provided by the bone marrow are induced by the IL7 and the stem cell factor (SCF), the FMS-like tyrosine kinase (FLT3L) and last but not least the CXCL12 chemokine [48].

IL7 is secreted by the bone marrow stromal cells, a cytokine essential at the pro-B cell stage. IL7 binds to its receptor which is expressed on the surface of the B lineage cells. IL7-deficient mice have strongly reduced pro-B cells and pre-B cells while the B cell progenitors, CLPs, are present in normal levels [49, 50]. Furthermore, the cytokine is inducing proliferation of the pro-B cells [13], but also recombination of the D_H to distal V_H segments in pro-B cells [51]. Based on further studies it is likely that IL7 already shows activity at the stage of CLPs [52].

Both IL7 and SCF are essential for the growth and survival of the lymphocytes [49]. SCF is a membrane-bound cytokine, which is also produced by the bone marrow stromal cells. The cytokine binds to the receptor tyrosine kinase Kit, expressed by the B cell progenitors during

their early development. The role of the SCF-Kit interaction is to stimulate the growth of the early B lineage cells. However, various studies support that SCF might be iterating for the embryo and the young mice, but crucial for the adult mice [53]. FLT3L is a membrane-bound ligand for FLT3 expressed by the bone marrow stromal cells and plays a crucial role in the regulation of the CLPs [39, 54]. The signalling by FLT3 starts very early in the development and prior to the CLP stage. In combination with the transcription factor PU.1, it drives the expression of the IL7 receptor which is required already in the CLP stage.

CXCL12, whose significance is undeniable, has also been known as stromal derived factor-1 (SDF-1). This chemokine is produced by the bone marrow stromal cells [55] and was primarily recognized as growth-stimulating factor and was, therefore, initially named pre-B cell growth stimulating factor (PBSF). Although the chemokine, in combination with IL7, induces proliferation [56], its main role is the regulation of the migration processes of diverse types of cells. Its high expression by the bone marrow stromal cells drives the homing and then the retention but also proliferation of the differentiating cells [57, 58]. That is succeeded in cooperation with the expression of its ligand CXCR4 by the target cells. The primary physiological receptor of the CXCL12, the CXCR4 chemokine receptor [56, 59], is expressed by the lymphocytes in different stages, not only during their generation and development in the bone marrow but also later on, depending on trafficking requirements (i.e. homing of the end stage plasma cells back to the bone marrow).

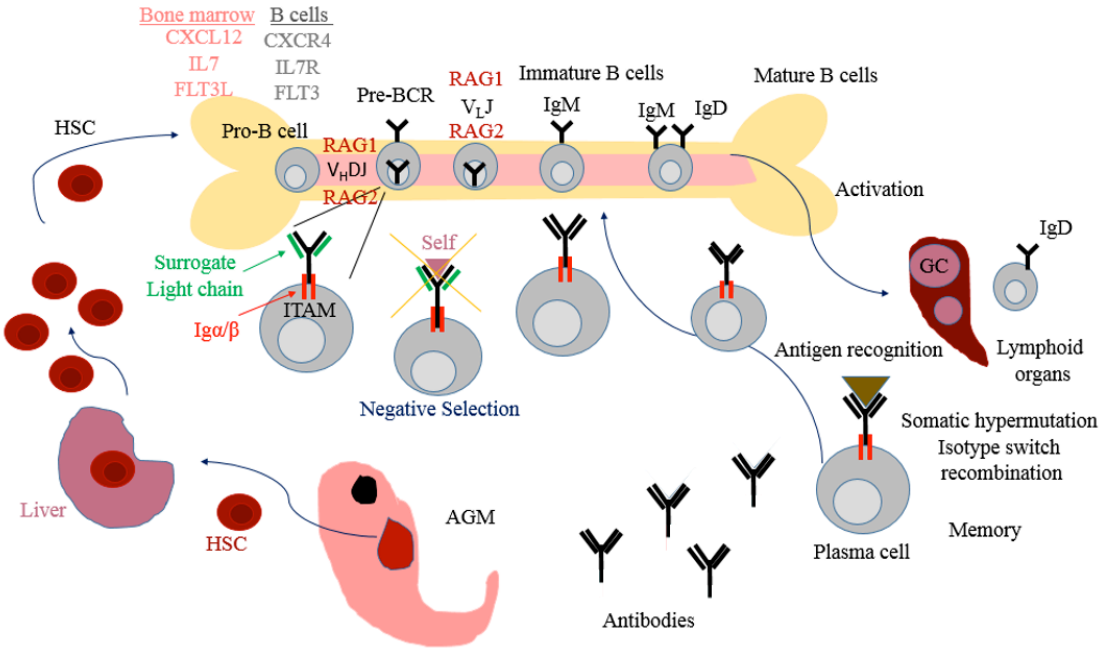


Fig. 4 B cell development and migration pathways in mammals

Hematopoietic stem cells (HSCs) migrate from the embryonic aorta-gonad-mesonephros (AGM) region into the liver and then to the bone marrow. In the bone marrow under the influence and through signalling of proteins

expressed either from the cells themselves or the bone marrow microenvironment, further differentiation and development is supported into pro-B cells, pre-B cells and immature B cells. During these stages heavy and light chain loci rearrange to produce a diverse B cell receptor repertoire. Mature B cells emigrate from the bone marrow to the spleen and other lymphoid organs where upon antigen mediated activation, they proliferate in germinal centers (GCs). Antigen specific mature B cells differentiate into antibody secreting plasma cells, while part of the differentiated cells migrate back to the bone and form a memory population.

2.2.4 The role of CXCR4 and CXCL12 in mammals

The main receptor for the CXCL12 chemokine is the CXCR4 chemokine receptor [60]. As all chemokine receptors, CXCR4 is a G protein coupled receptor [61]. The receptor's amino acid sequence is highly conserved and shows great similarity to other receptors of the same group. Intracellular signalling is mediated through the heterotrimeric G-proteins (with Gα, Gβ and Gγ subunits). Activation of the receptor leads to dissociation of the G-protein. The Gα monomer inhibits the adenylyl cyclase activity and initiates the MAPK and PI3K pathways, while the Gβγ dimer leads to intracellular calcium mobilization through the activation of phospholipase C [62]. The CXCR4 receptor is also known as leukocyte derived seven transmembrane domain receptor (LESTR) or fusin [63] and acts as a co-receptor for the HIV virus which allows its entry when co-expressed with the CD4 molecule on T cell's surface [60, 64, 65].

The interaction of CXCL12 and CXCR4 is connected with many and very diverse physiological processes. Signalling through this axis has a crucial cell migration regulatory role not just for lymphocytes [66] and HSCs [67] but also for the angiogenesis [68] and neurogenesis [59, 69]. In B lymphopoiesis, CXCL12 secreted by the bone marrow stromal cells selectively attracts CXCR4 expressing early B cell precursors into the bone marrow and retains them there [70]. CXCR4 knockout mice die perinatally, mostly on ED18.5. However, analyses of fetal organs have been performed prior to the embryos death and one important finding is the hypocellularity of the fetal bone marrow. Stromal cells and osteoblasts dominate the bone marrow, while cells of hematopoietic origin are severely reduced [69]. In the fetal liver, the pro B population was also severely reduced [70]. Mice lacking the CXCL12, beside the occurring perinatal deaths, presented severely low B cell progenitors' levels both in liver and bone marrow. While the myeloid lineage was affected in the bone marrow, the fetal liver was not affected [66]. Further deficits occurred in the mutants such as abnormalities in the vascularization of the gastrointestinal tract and in cardiogenesis [66, 71]. Moreover, it has been shown that the axis is important for the migration of primordial germ cells to the gonads [72]. The CXCL12 chemokine is also expressed in the thymus, from where, with other

chemokines, it controls the migration processes of differentiating thymocytes [73].

During immunoresponses, germinal centers are formed from the B cell follicles located in the lymphoid organs. In the germinal centers (GC) B cells undergo rapid proliferation [74]. At this stage the cells are localized at the dark zone of the GC and are called centroblasts. The centroblasts express two chemokine receptors, the CXCR4 and CXCR5. At the same time, the stromal cells of the dark zone produce the chemokine CXCL12, retaining the CXCR4 expressing cells in this region. When the CXCR4 expression declines, the cells emigrate from the dark zone to the light zone as centrocytes [75]. In this area, follicular dendritic cells (FDCs) produce CXCL13, the ligand for the CXCR5 receptor which is expressed by the centrocytes [76, 77].

To conclude, during ontogenesis the interaction of CXCR4 and CXCL12 is essential for the myelopoiesis and lymphopoiesis but also for vascularization and cardiogenesis [66, 68, 69]. Deficiency in one of two components leads to perinatal deaths in mice [68]. In B cell development, blocking of the interaction between the two components leads to a vast B cell deficit and a clear deficit in the colonization of the bone marrow by hematopoietic cells and developing B lymphocytes [69].

2.2.4.1 CXCR4 and CXCL12 signalling pathways and alternative bindings

Although CXCR4 is the unique physiological receptor for the CXCL12, the latter can alternatively bind to another receptor, the CXCR7 [78]. CXCR7 regulates the CXCL12 concentrations through its function as a decoy receptor, reducing the CXCL12-CXCR4 signalling [79]. CXCR7-CXCL12 binding leads partially to CXCR4 endocytosis [80] mediated through β-arrestin but also to β-arrestin-mediated MAPK activation (see Fig. 5). Moreover, lysosomal degradation of the ligand CXCL12 is mediated through β-arrestin [81, 82]. Upon overexpression, CXCR7 can furthermore dimerize with the CXCR4 and then bind to the CXCL12 (see Fig. 5). The binding of the CXCL12 with the dimer leads to β-arrestin mediated MAPK signalling and receptor internalization [83].

CXCR4 can bind to an alternative ligand, the chemokine MIF (see Fig. 5), which plays an important role in cell trafficking by binding to the chemokines receptors CXCR4 and CXCR2. MIF has a clear association with pro-inflammatory processes and its production is stimulated by inflammatory stimuli [84].

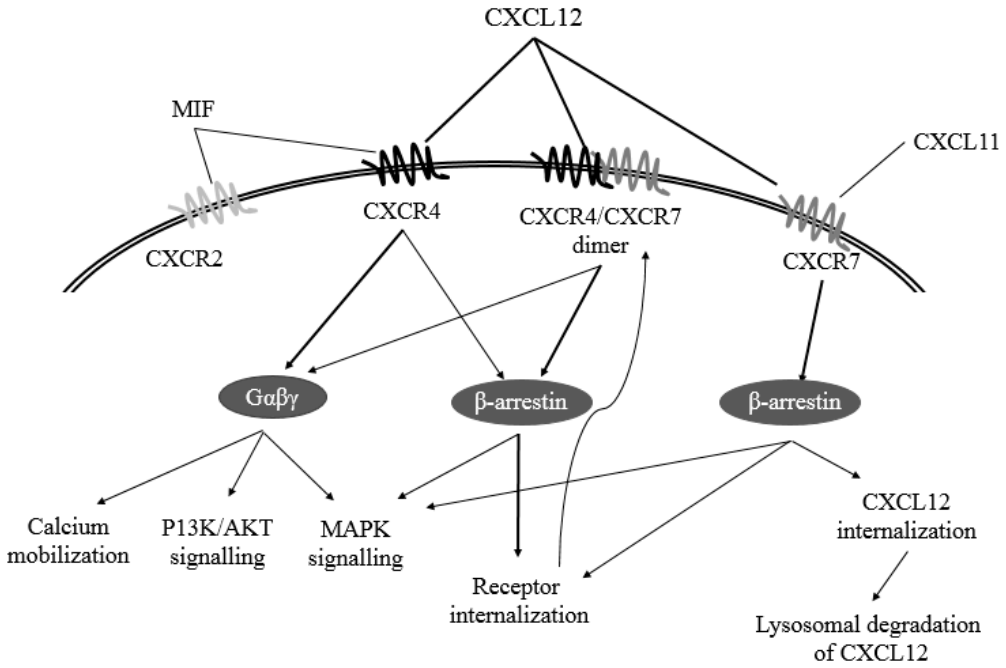


Fig. 5 Signalling pathways of CXCR4 and CXCL12 in mammals, modified from *Doring et al. [85]*

CXCR4 is the physiological receptor of the chemokine CXCL12 and mediates signalling through the G-proteins. Activation of the receptor leads initiation of the MAPK and PI3K pathways, but also to intracellular calcium mobilization. An alternative signalling pathway is mediated by β-arrestin and leads either to initiation of the MAPK pathway or to the internalization of the receptor. The same effect mediated by β-arrestin is demonstrated when CXCR4 dimerizes with the CXCR7, an alternative receptor for the CXCL12. Binding of CXCR7 with the CXCL12 leads again to β-arrestin mediated MAPK signalling, receptor internalization or CXCL12 internalization and degradation. Finally, CXCR4 can also bind with the macrophage migration inhibition factor (MIF). CXCR2 is the alternative receptor for the chemokine MIF.

CXCR2 is the other available receptor for the MIF chemokine (see Fig. 5). CXCR2 interacts antagonistically with the CXCR4, regulating the mobilization of the neutrophils from the bone marrow [86].

2.2.5 CXCR5 and CXCL13 in the post bone marrow development

The CXCR5-CXCL13 axis has been primarily studied in mice and has been formerly known as BLR1-BCA1 axis [87], standing for Burkitt lymphoma receptor 1 and B cell attracting chemokine 1. The receptor CXCR5 is mainly expressed by B cells [88], however other cell populations, such as the T follicular helper cells [89], also express it. Naive B cells express the CXCR5, while the B cell area in the lymphatic tissues are producing the ligand chemokine CXCL13 [90]. It has been shown that the axis plays an important role in the development of lymphatic tissue, specifically for the physiological formation of the B cell areas. Mice with deficiency in the CXCR5-CXCL13 axis show presence of B cells in the lymphatic tissues, however there is no B cell zone organization detected [91]. Germinal centers can only be

formed under physiological function of the CXCR5-CXCL13 axis [92], while some lymph nodes (i.e. inguinal) also require physiological function of the axis [93].

In the germinal center's dark zone the chemokine CXCL12 is highly expressed while the centroblasts express the receptor CXCR4. When the cells differentiate into centrocytes CXCR4 expression decreases while CXCR5 expression increases. Since the ligand chemokine CXCL13 is expressed in the light zone of the germinal center, the CXCR5 expressing centrocytes start migrating toward the light zone [76].

2.3 B CELL DEVELOPMENT IN THE CHICKEN

Chickens are one of the most important animal models in biomedical research. Significant discoveries like graft versus host disease were made in chicken embryos. Beside their importance in research, chicken protein is one of the major sources for animal protein in human diet. But chicken health is threatened by a plethora of pathogens causing disease outbreaks. In order to improve the protection mechanisms of the chicken flocks it is necessary to better understand the development and function of the chicken immune system and especially the adaptive immune response. B lymphocytes with their ability to produce antibodies play an important role in the protection of the chickens against pathogens.

During B cell development, a wide variety of B cell receptors is generated in order to provide the organism with a vast antibody repertoire for the recognition of all kinds of antigens [6, 7]. Even though there are a lot of similarities between B cell development in mammals and birds, there are also significant differences. While the mechanism of antibody diversification in rodents and primates is based on random combination of genetic elements encoding the heavy and light chain loci [6], this mechanism is not as efficient in the avian species due to the limited number of segments available for rearrangement (see Fig. 8) [94-96]. Therefore, another mechanism has to compensate this inefficiency to provide the organism with the required antibody diversity. The process is called gene conversion [97] .

While the most important organ in the development of B cells in mammals is the bone marrow, birds have a unique organ, the bursa of Fabricius where the B cell development occurs [98]. The bursa involutes around sexual maturity of the animals [99].

2.3.1 The role of the Bursa of Fabricius

One main difference in the early development of chicken B lymphocytes is the minor role the bone marrow has in avian species and the presence of a lymphoid organ which overtakes the role of the bone marrow in B cell development: the bursa of Fabricius.

The bursa is a gut-associated lymphoid tissue which is located between the cloaca and the sacrum [100]. It is a hollow organ and through a short duct it communicates with the proctodeal region of the cloaca [101]. Due to its localization, the bursa was first recognized by Hieronymus Fabricius in 1621 as semen-storing organ but this theory was proven wrong. It was eventually recognized as an important organ for the development of the B lymphocytes by Bruce Glick in 1956 [102].

The bursal lumen is present by ED7. By ED10 longitudinal folds start to appear and finally about 15 to 20 folds protrude in the lumen of the bursa [103]. Throughout the folds, which are in contact to each other, there are follicles, organized in two layers and separated by connective tissue [104]. In the connective tissue between the follicles, blood and lymphatic vesicles are localized. The folds are surrounded by epithelium which can be separated in interfollicular (IFE) and follicle-associated (FAE) epithelium [105] (see Fig. 6). The IFE covers most of the folds' surface (around 90%) while the rest (10%) consists of the FAE [106]. The FAE constitutes the contact and incentive point of the follicles' medulla with the lumen of the bursa and consequently with the antigens coming from the proctodeum [107].

The follicles, when completely developed, have a size of 0.2 to 0.4mm each and are organized in medulla and cortex [108]. These two compartments are fully separated by the basal lamina. Each follicle has an independent blood supply through capillaries that build up a fine network at the border between cortex and medulla. The medulla does not include vessels. The medullary anlage is seen between ED11 and ED12 while the formation of the follicle associated epithelium takes place on ED14 to ED15. The organization of the follicles in cortex and medulla starts around hatch [103].

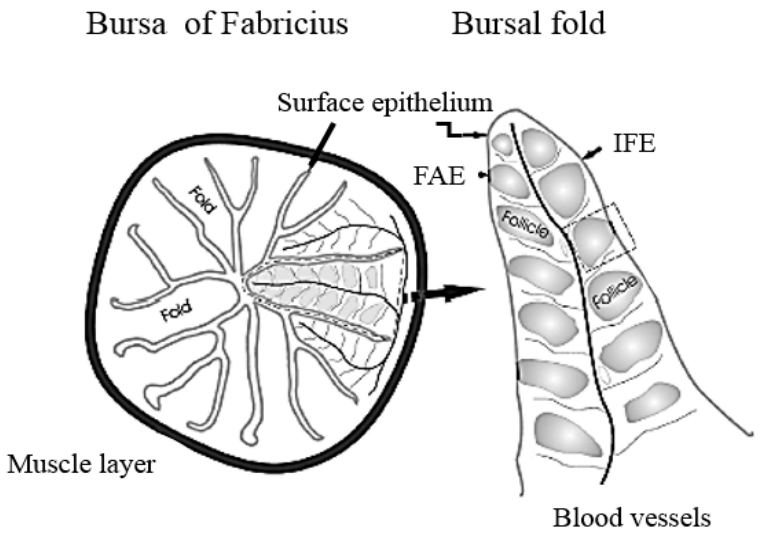


Fig. 6 Organization of the bursa of Fabricius and its bursal follicles, modified from *Avian Immunology [100]*

The bursa of Fabricius is organized in longitudinal, follicle containing, folds. The folds are covered completely by the interfollicular epithelium (IFE) and at the points where follicles are present by the follicle associated epithelium (FAE).

In the bursa around ten to twelve thousand follicles [108, 109] are built until the age of four weeks after hatch. These not only contain lymphocytes, but also stromal cells such as the bursal secretory dendritic cells. Other cells that are found are macrophages, epithelial reticular cells, corticomedullary archforming cells (which separate medulla from cortex) and mesenchymal reticular cells in the cortex [110] .

2.3.2 Stages of B lymphocyte development in chicken

The B cell development in avian species is separated in three phases: the pre-bursal, bursal and post-bursal development [97]. Each phase plays an important but also different role in the process of development.

2.3.2.1 Pre-bursal B cell development

Hematopoietic cells from the dorsal aorta give rise to CLPs. CLPs develop in pre-bursal and pre-thymic lymphocytes in hematopoietic tissues such as the spleen [100]. B cell progenitors are mainly found in the spleen at this stage (see Fig. 7). In the spleen, but also in other sites of hematopoiesis, pre-bursal B cells undergo rearrangement of the immunoglobulin loci [111] and commit to the B cell lineage prior to their migration into the bursa [112].

Gene rearrangement events occur in the chicken only during embryonic development [113], in contrast to mammalian B cell development where Ig gene rearrangements are detected in B cell progenitors throughout life [114]. The mechanism is similar to that in mammals but the

events can occur at the heavy and light chain locus at the same time. Light chain rearrangement can even occur prior to completed heavy chain rearrangement [115]. As mentioned in 2.3, gene rearrangement events in the chicken are not as efficient in creating diversity, since the available gene segments are very limited. The heavy chain locus contains unique V_H1 and J_H segments and only 15 highly-conserved D_H segments [94, 96]. Similarly, the light chain locus contains only unique V_L and J_L segments [96]. Consequently, the rearrangement of these segments produces a very limited diversity (around 150 combinations of the heavy chain and about ten of the light chain, which after combining them lead to a combinatorial diversity of 1500 combinations VDJ_H/VJ_L [100]).

B lymphocyte precursors, which have undergone rearrangement, migrate into the bursal mesenchyme through the blood in a single wave between ED10 and ED15 [116-118]. They are characterized by the expression of the chicken B6 or else known as Bu1 antigen [119]. These cells are $CD45^+$, $CD1^+$ and express the sialyl LewisX carbohydrate epitope [118, 120, 121].

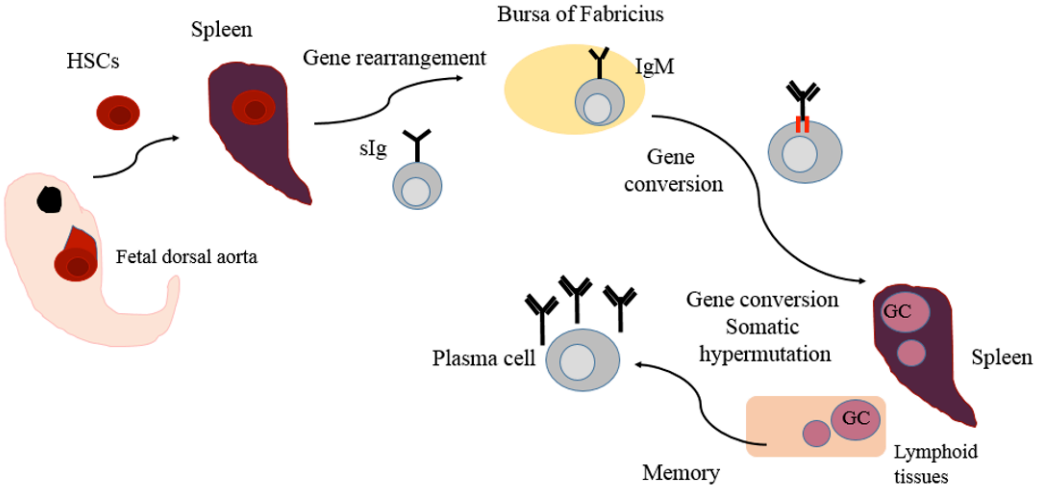


Fig. 7 B cell developmental pathway in the chicken

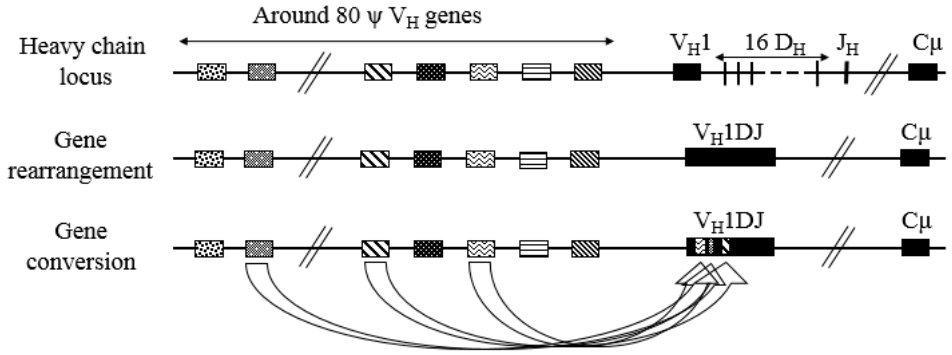
The hematopoietic stem cells from the dorsal aorta of the embryo migrate mainly into the spleen. During ED10 and 14 they migrate to the bursa of Fabricius. Heavy and light chain rearrangement as sIg (surface immunoglobulin) expression is independent of the bursal microenvironment and detected prior to the colonization of the bursa. For the development of the cells into mature B cells, a productive IgM expression in the bursa is required. Further diversification of the antibody repertoire relies on several rounds of gene conversion. Besides in the bursa, gene conversion also takes place in the lymphoid tissues. Somatic hypermutation plays a role in the diversification of the repertoire, too. Mature and activated B cells produce antibody secreting plasma cells. A part of them will be maintained as 'memory' for a faster immune response after contact with the same antigen.

2.3.2.2 Bursal B cell development

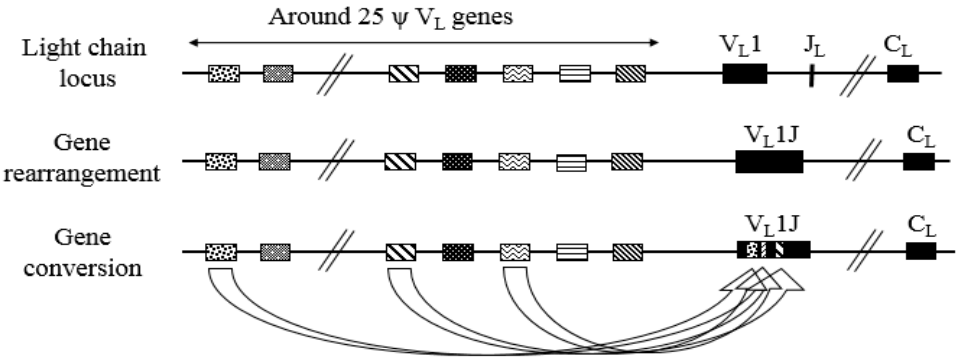
The bursa of Fabricius has a significant role for the B cell development, although gene rearrangements occur independent of the bursal microenvironment and neither the induction

nor the completion of the immunoglobulin gene rearrangements require the presence of the B cells in the bursa [122-124]. However, in order for the cells to further develop into mature and fully functional B cells, they require the bursal microenvironment. Lymphocytes cross the basal membrane, form follicles and proliferate. It has been shown that B cell precursors do not need to express the surface immunoglobulin in order to be able to migrate across the basement membrane [125].

Each follicle is colonized by two to five cells which rapidly proliferate [109]. Finally, a follicle consists of about $2x10^5$ to $4x10^5$ cells, mainly B lymphocytes, dendritic cells, macrophages and epithelial cells [109]. Cells in the follicles undergo somatic gene conversion during which, pseudogenes located upstream of the functional V gene segments, lacking promoter, exons and VDJ recombination sequences, are used as donor sequences to diversify both heavy and light chain loci as shown in Fig. 8. The procedure of gene conversion repeats for several rounds [126, 127]. The chicken antibody repertoire is generated during the late embryonic stage and for a short period after hatching. As the chick ages, its B cells undergo additional rounds of somatic gene conversion and the antibody repertoire expands [128] until a wide antibody repertoire is achieved (10^{11} different antigen recognizing receptors [95, 129]) at the age of five to seven weeks when the bursa is fully developed [100].



A. Organization of the heavy chain locus, VDJ rearrangement and gene conversion



B. Organization of the light chain locus, VJ rearrangement and gene conversion

Fig. 8 Organization of the chicken heavy and light chain loci, gene rearrangement and gene conversion

Heavy chain locus rearranges the variable (VH), diversity (DH) and joining (JH), while light chain locus rearranges variable (V_L) and joining (J_L) segments. Due to the limited available segments created repertoire is poor. However, with several rounds of gene conversion a repertoire of 10^{11} unique BCRs is achieved. Gene conversion is based on the donation of sequences from pseudogenes which are located upstream of the functional V gene segments. There are around 80 pseudogenes available in the heavy chain locus (ψV_H) and around 25 in the light chain locus (ψV_L).

The surface immunoglobulin (sIg) complex consists of the immunoglobulin heavy and light chains. The chains are in association with the chicken homologs of the mammalian Igα/β proteins, CD79α and CD79β. CD79α and CD79β are transmembrane proteins which consist of an extracellular domain, a transmembrane region and a cytoplasmic tail [100]. The role of Igα/β proteins in mammals is the transduction of signalling through ITAM motifs [130, 131], which is essential for the B cell development [132, 133]. Studies by Ratcliffe *et al.* support that in the absence of ligation, basal signalling through Igα is sufficient to drive early B cell development, in contrast to Igβ [134]. However, the role of these proteins needs to be further investigated.

Around hatch, B cells start to reorganize in the bursal follicles. B cells emigrate from the bud, across the basement membrane and start forming the cortex, while the initial follicle bud forms the medulla of the follicle. The cortex contains high proliferative B cells while the proliferation of the cells in the medullary region stagnates [135, 136]. The cortex develops fully in the first two weeks after hatch [103, 137, 138].

2.3.2.3 Post-bursal development

Around hatch B cells emigrate from the bursa [139] (see Fig. 7). Most of the B cells in the embryo bursa undergo apoptosis rather than emigrating to the periphery [140]. Although surface immunoglobulin expression is not essential for the migration of the cells into the bursa, the expression of productive sIg is essential for the survival, for further development of the cells after hatch and for the emigration of the cells from the bursa to the periphery [125, 141]. Most of the emigrating cells are short-lived cells, while a population of long-lived B cells emigrates from the bursa to peripheral lymphoid tissues [128]. The lymphoid tissues colonized by the post bursal B cells include the spleen, the bone marrow, but also gut-, bronchus-, nasal-, conjunctiva- and skin- associated lymphoid tissues (GALT, BALT, NALT, CALT and SALT respectively).

In response to antigens, B cells proliferate in germinal centers in secondary lymphoid organs and in the other lymphoid tissues mentioned above (see Fig. 7). It takes about seven days until the germinal centers are formed after the initial antigen contact [127, 142, 143]. The

immunoglobulin genes of the antigen-activated B cells are diversified by gene conversion and somatic hypermutation in the early stages of the germinal center reaction. Later, most of the changes take place by somatic hypermutation and less by gene conversion [144, 145].

2.4 REGULATION OF B CELL DEVELOPMENT IN THE CHICKEN

Though the regulatory mechanisms of the B cell development in the chicken are not as well studied as in other species, there are already some aspects known.

2.4.1 Gene rearrangement and gene conversion in chicken B cell development

In contrast to mammals, gene rearrangement of heavy and light chains in the chicken is independent of the microenvironment [122-124]. Colonization of the bursa with a single wave of pre-bursal B cells happens during embryonic life in a germ-free environment independently of sIg expression [128, 146]. B cell precursors that have not completed the VDJ recombination can further rearrange in the bursa [128].

Splenocytes that were transferred from wild type embryos to immunocompromized embryos demonstrated expression of the B cell surface antigen chB6 (a mostly used B cell marker [147]) and potential to colonize the bursa [148]. Furthermore, B cells with light and heavy chain rearrangements have been isolated from several hematopoietic tissues [111, 115, 122]. Finally, B cells with immunoglobulin rearrangements and with the capacity to migrate into the bursa were detected in the bone marrow [123]. Consequently, the bursal microenvironment is not essential for the immunoglobulin rearrangement processes in the chicken. Heavy and light chains can undergo rearrangement in any order between embryonic day ED10 and ED12, prior to the migration of the cells to the bursa [115].

In contrast, in mammals the heavy chain needs to rearrange first in order for the light chain to undergo rearrangement, too. Since completion of the rearrangement of the heavy chain in the chicken does not require the light chain expression, there is no expression of a pre-B cell receptor complex as seen in mammals. However, as in mammals, so do the rearrangement of the heavy and light chains chicken immunoglobulin, require RAG1 and RAG2 expression. The expression of RAG1 and RAG2 is detected in chicken B cell precursors undergoing immunoglobulin gene rearrangement [123, 149, 150].

Around hatch about 90% of the bursal B cells express sIgM. Allelic exclusion described in mammals also applies to chicken B cells. Consequently, follicles may maintain a mixture of sIgM1a and sIgM1b expressing cells. But each of the cells can express only one of the allotypes [151] which confirms that bursal follicles are colonized at first by more than one cell [122].

Nonetheless, gene conversion, which actually creates the antibody repertoire in the chicken, takes place in the bursal microenvironment independent of antigen ligation. In the absence of the bursa, in the very early developmental stages (i.e. ED2 to ED3), there is production of non-specific IgM on the surface of the B cells, without any specific antibody response [100]. Isotype switching for IgY and IgA production is consequently not possible. However, in the absence of the bursa in the later stages of development but still before ED18 (prior to the B cell emigration to the periphery) the hatched chicks lack circulating Ig and are also incapable of specific antibody response [100]. Furthermore, gene conversion requires the activation-induced cytidine deaminase (AID) [152]. In DT40 cells, a B cell line, the disruption of the AID gene leads to inhibition of gene conversion [127].

2.4.2 Regulation of the B cell migration in the chicken embryo

Since the migration of B cell precursors into the bursa is essential for the development into functional mature B cells, it is important to better understand this process. As mentioned before, Ig expression is not necessary for the migration of the cells and therefore does not seem to have a regulatory role in the process. Based on what is known in mammals, it is most likely that chemokines regulate the B cell migration processes in the chicken.

Chemokines have been studied thoroughly in mice and humans and several of them are taking part in the regulation of the B lymphocyte development [61]. As mentioned in chapter 2.2.3, the most important pair is CXCR4 and CXCL12. They regulate the trafficking of B cells from the periphery to the bone marrow and in the germinal center's reaction, they retain the cells in the dark zone.

The interaction of CXCR4 with CXCL12 equally regulates the migration of plasma cells from the periphery to the bone marrow at the latest developmental pathway of B cells. The role of the axis in primates and rodents leads to the assumption that the axis might also be very important in the migration regulation during B lymphocyte development in the chicken.

2.4.3 Role of CXCR4 and CXCL12 in the chicken B cell development

The chicken CXCR4 was cloned in 2001 [153] while the chicken CXCL12 was characterized in 2005 [154]. Studies have shown that CXCL12 is highly expressed in the bursa [155, 156], while the CXCR4 receptor is expressed by the chicken B cells on their surface. The highest expression level of CXCL12 was detected on ED14 in the bursa, while on ED18 the expression decreased. Post hatch the expression level decreased further to one seventh of the expression on ED14. On the other hand, CXCR4 expression is also present and detected in the bursa already on ED14. In contrast to CXCL12, its expression increases towards hatching. This is not the result of up-regulation by the cells, but of the much higher numbers of B cells localized in the bursa at this stage. Furthermore, *in vitro* studies have shown that CXCR4 expressing DT40s (chicken B cell line) migrate towards the ligand CXCL12 in migration assays [155].

Other cells of the immune system also express the CXCR4 receptor. $CD4^+$ and $CD8^+$ T lymphocytes express the receptor. Furthermore, almost all $Kul01^+$ cells (marker for myeloid lineage cells such as blood monocytes and tissue-resident macrophages) express the CXCR4 on their surface [155].

2.4.4 Role of CXCR5 and CXCL13 in the chicken B cell development

In the chicken, three CXCL13 isoforms have been detected [157]: CXCL13 L1, CXCL13 L2, and CXCL13 L3 [156]. The expression of the CXCL13 isoforms has been detected mainly in the bursa and in the spleen. Isoforms L1 and L2 are expressed prior to hatch, while post-hatch the expression slightly decreases in the bursa and highly increases in the spleen. The L3 isoform is the only isoform whose expression does not decrease post hatch in the bursa and its expression is higher in the bursa than in the spleen post-hatch [156]. The CXCR5 receptor is expressed by B cells in the spleen and the bursa, both prior and post-hatch. Macrophages and T cells also express the CXCR5 receptor [155].

2.5 INHIBITION OF THE CXCR4/CXCL12 INTERACTION

Several studies have been performed on the effect of inhibiting the interaction of CXCR4 and CXCL12 not only in the B cell development but also in different types of cell populations. First of all, mice deficient in CXCR4 [69] or CXCL12 [66] have been generated. CXCR4

deficient animals were generated by homologous recombination. Heterozygous CXCR4 deficient mice have a phenotype similar to the WT. Homozygous animals of both types of deficiency die perinatally. Their bone marrow is hypocellular and B lymphopoiesis is severely reduced. In both $CXCR4^{-/-}$ and $CXCL12^{-/-}$ deficient mice B lymphopoiesis is severely reduced, the bone marrow is hypocellular and myelopoiesis in the bone marrow is absent [69]. Another way of inhibition is the application of CXCR4 antagonists. One of the antagonists is the inhibitor AMD3100, or else known as plerixafor [158]. AMD3100 is a bicyclam derivative and a specific CXCR4 antagonist [159] which by blocking the binding pocket of the CXCR4 interferes with the interaction of CXCR4 with its ligand CXCL12 [160]. AMD3100 has been used in the HIV infection research field to block the CXCR4 receptor [161]. CXCR4 is the co-receptor for HIV virus and when co-expressed with the CD4 on T cell's surface, the entry of the virus into the cell and thereby the infection of the cell is allowed. The purpose of the blocking of the CXCR4 receptor with the AMD3100 was to inhibit the binding of the co-receptor CXCR4 and prevent the infection of the cells by the HIV virus [162]. Meanwhile, AMD3100 is used for bone marrow stem cell mobilization in humans [163]. In the first studies in humans examining the AMD3100 safety and pharmacokinetics, 10μg to 80μg/kg were applied *i.v.* but also *s.c.* [164]. In another study for $CD34^{+}$ cell mobilization, patients were treated with a single application of 1000μg/kg AMD3100 *s.c.* [165].

AMD3100 has also been used in *in vitro* studies with chicken cells, blocking the CXCR4 receptor -and thereby the CXCR4-CXCL12 interaction- to examine the effect of the blocking on B cell migration. DT40 cells which migrate toward the ligand CXCL12 were inhibited from migrating when AMD3100 was added [155]. It has been shown that AMD3100 inhibits the interaction of the CXCR4-CXCL12 axis by occupying the binding pocket of the CXCR4 [166] and thereby not allowing the CXCL12 to bind with CXCR4.

2.6 TRANSGENIC CHICKEN MODELS IN THE ANALYSIS OF B CELL DEVELOPMENT

The heavy chain knockout chicken line (*JH*-KO) is an immunoglobulin knockout chicken line, which was generated by targeting the J_H gene segment by homologous recombination [125]. Adult homozygous birds carrying the knockout in both alleles, are free of postbursal B cells. Immunization of the birds does not lead to an antibody response, since the heavy chain

has been knocked out and the immunoglobulin molecule cannot be expressed on the surface of the cells. However, immature B cells are present in the pre-bursal phase and show the capacity to colonize the bursa. It was already shown that B cells in which only a truncated μ chain is express have the capacity to colonize the bursa. The truncated μ chain was sufficient to support early B cell development [167]. The heavy chain knockout chicken model revealed that despite total loss of heavy chain expression, these B cells can migrate into the bursa, cross over the base membrane, form follicles and even proliferate in the follicles. These B cells do not develop further into mature B cells and consequently there are no B cells detected in the periphery. The B cells detected in the embryonic bursa are $Bu1^+$ but IgM^- [125]. Though the B cells of the *JH*-KO embryo colonize the bursa and form follicles, the bursal follicle development is compromised. While bursae of WT birds show a clear organization of the follicles in cortex and medulla, the bursae of the *JH*-KO embryos do not show any similar organization. The T cell development of the *JH*-KO line stays unaffected and shows a normal development.

The line offers the possibility to further study the B cell development in the chicken in the absence of endogenous BCR and B cells.

A second transgenic chicken line has been used in this project, the GFP 165-2 chicken line. The chickens of this line are expressing GFP (green fluorescence protein) under the control of the beta-actin promoter. The line was produced at Crystal Bioscience, Inc., Emeryville, California, USA, by deriving primordial germ cells and transfecting them with GFP and injection of the transfected primordial germ cells into embryos for the creation of G0 chimeras. The G0 chimeras with GFP germline transmission were mated to wild type animals. From the generation produced by this mating (G1), the line of birds expressing GFP was established [168].

2.7 *IN OVO* MANIPULATION OF CHICKEN EMBRYOS

Several methods have been used in order to manipulate chicken embryos for various studies. While it is easy to draw blood from hatched birds, it requires a lot of experience to draw sufficient amounts of blood from embryos. In the past white blood cells counts have been performed mainly by blood smears and histological analysis [169, 170]. More recently, a method for an automated whole white blood cell count based on flow cytometry became available [171]. However, few studies have investigated the composition of chick embryo

blood samples at various points in times during development [172, 173]. One interesting method that has been performed for blood gas analysis between ED15 and 18 has been published by Tazawa *et al*. [174]. Sampling was succeeded by means of catheterization of a vessel and fixation of the catheter with clay for sequential samplings. 75% of the catheterization trials were successful, although it is not clear how success was determined (maintenance of the catheter in its right position until the end of the experiment or survival of the embryos till termination of the experiment) [174]. In an experimental metastatic assay in the chick, a method for intravenous injection has been described in detail and offers solid information and base for intravenous manipulation of the chicken embryo [175]. Furthermore, intravenous injections have been performed *in ovo* in order to inject an antigen in the embryonic blood circulation (100µl *i.v.* injected in ED11 embryos) and investigate the responses on later stages [176].

In order to gain insight into the early B cell development adoptive cell transfer experiments have also been used. Adoptive transfer experiments have been performed to treat cells of an individual *ex vivo* and provide cells with new properties, or to transfer cells of a foreign organism to another. Most commonly, adoptive transfer is used to transfer cells (mostly lymphocytes) from immunized donors to a non-immune host. In the chicken adoptive cell transfer experiments have been performed, among others, to better analyse both B and T lymphocyte development. Adoptive cell transfer experiments in which $2.5x10^5$ to $2x10^6$ spleen or bone marrow cells were transferred *i.v.* with 150µl medium into ED14 embryos, have shown the early separation of the B cell and T cell lineage [148]. Pink *et al.* adoptively transferred 1 to 20 million post bursal B cells *i.v.* in age-matched six-weeks-old chickens that were neonatally treated with cyclophosphamide (CP-leads to suppress host's responsiveness) in order to study the capacity of the CP treated chickens to form antibodies after reconstitution of the B cell population [177].

A method of similar principle, which has significantly contributed to the knowledge we have on neural development [178] but also in the understanding of early lymphocyte development [179], is the generation of quail-chick chimeras. Quail grafts are transplanted on chicken embryos, allowing later investigation of the origin of the cells. For example, quail grafts were placed on chicken yolk sac in the earliest possible stage (before heart beating) to examine if the hematopoietic cells source is the yolk sac. A marker allows the differentiation between quail and chicken cells [180]. From the chimeras experiments it was concluded that hematopoietic cells originate from the embryo and not the yolk sac [179, 181, 182].

Moreover, it was possible to determine that bursal lymphocytes originate from precursor cells than migrate into the bursa through the blood stream and not from the epithelial cells or the mesenchyme [146].

Another way to study the function of different components of the B cell receptor *in vivo* was the transduction of embryos with retroviral vectors coding for various elements of the B cell receptor [176]. Ratcliffe *et al.* examining self-tolerance during B cell development, induced the expression of antigen specific truncated μ chains in immature B cells by means of *in vivo* transduction. Therefore, chicken embryonic fibroblasts (CEFs) were transfected with a construct of the replication-competent avian leucosis virus (RCAS) in which the genes of interest were cloned. In order to infect the chicken embryos, one million transfected CEFs were inoculated in the embryos. The study concluded that B cells expressing a foreign antigen specific BCR proceeded to further development after ligation with the antigen, while B cell expressing self-specific antigen BCR underwent deletion. Furthermore, self-tolerance is independent of the bursa and dependent on signalling downstream of the B cell receptor [176].

Before the generation of immunoglobulin knockout chicken B cell deficient birds were either generated by bursectomy or by cyclophosphamide treatment. Bursectomy is a method which has been used for many decades in order to examine its role [101]. In the absence of the organ, the effects could be examined and conclusions on the role of the bursa were made. 'Deactivation' of the bursa per bursectomy or CP treatment has been performed in various developmental stages, from very early stages (i.e. 50-70 hours of incubation) [183] but also in neotanal stages, directly after hatch [177].

One of the most important advantages of the chicken embryo as an animal model in developmental biology is the possibility to easily access the embryo independently of the maternal animal. A further advantage is the completed organogenesis already on ED9 (35^{th} to 36^{th} stage according to Hamburger and Hamilton stages) [184].

3. PURPOSE OF THE PROJECT

The purpose of the current study was to analyse early B cell development in the chicken embryo. The goal was the establishment of a method for intravenous manipulation and adoptive cell transfer in the chicken embryos. By using the established techniques and immunoglobulin knockout chickens the factors involved in immigration of B cells in the bursa of Fabricius were analysed *in vivo*.

4. MATERIAL AND METHODS

4.1 ANIMALS AND ANIMAL BREEDING

The chicken lines GFP and *JH*-KO were kept at the Institute for Animal Physiology, LMU Munich in a conventional breeding system. The animals were fed with commercial diet for egg layer chickens and for young chicks. Feed and water were provided *ad libitum*. Euthanasia of the animals was performed, after sedation, per decapitation.

The chicken lines used in this project are listed in the following table:

Line	Breeding / Origin
Lohmann's Selected Leghorn Classic (LSL Classic)	Gut Heinrichsruh LSL Rhein-Main, Berglern
GFP 165-2 (GFP)	Institute for Animal Physiology, LMU Munich/Crystal Bioscience, Emeryville, California, USA
Heavy Chain Knockout (*JH*-KO)	Institute for Animal Physiology, LMU Munich

Tab. 2 Chicken lines used in this project

4.2 INCUBATION OF THE EGGS

Material

Incubator, Heka Favorit-Olymp [34] and Grumbach BSS [35]

Aqua dest.

The eggs were incubated in the incubators HEKA Favorit Olymp and Grumbach BSS. Eggs were candled on embryonic day seven and eighteen. Infertile eggs or eggs in which the embryo had died, were discarded. The eggs were set for further incubation up to the day of further experiments.

The parameters of the incubations are listed in the following table (Tab. 3).

Parameters	Embryonic day (ED) 0-17	ED18	ED20-Hatch
Temperature (°C)	37.8°C	37.2°C	37.0°C
Humidity (%)	55%	55%	80%
Rocking	Yes (3 times per day for 120 minutes)	No	No

Tab. 3 Egg incubation parameters

4.3 INTRAVENOUS APPROACH TO THE EMBRYO

4.3.1 Intravenous approach to the chicken embryo after transfer in a surrogate egg shell

Material

Multifunction tool (Dermal tool) [33]

Weight boat [29]

Plastic wrap [38]

Q-tips [29]

20% Penicillin/Streptomycin [20]

Insulin one-way syringes 0.3x12mm, 40 I.U. [22]

Turkey eggs [23]

Scalpel [24]

Phosphate-buffered saline (PBS), pH 7.2

8.00gr. Sodium chloride (NaCl) [1]

1.45gr. Disodium hydrogen phosphate dihydrate ($Na_2HPO_4x2H_2O$) [1]

0.2gr. Potassium chloride (KCl) [1]

0.2gr. Dipotassium hydrogenphosphate (KH_2PO_4) [1]

Ad 1000ml aqua dest.

pH was adjusted at 7.2 by using HCl [1] and NaOH [1]

The chicken eggs were weighed and numbered. Turkey eggs were weighed and sorted. A 30 to 40gr bigger turkey egg was chosen for each chicken egg. The chicken eggs were set for incubation (for incubation parameters see chapter 4.2) for three days. Three days later the turkey eggs were scored at their pointy side. About 1/3 of the egg shell was cut off to discard

the egg content. After the preparation of the surrogate turkey egg shells, the chicken eggs were also scored. The scoring line was drilled in the middle of the chicken eggs cautiously in order to not damage the underlying membrane, known as outer and inner shell membrane. The chicken eggs were carefully opened with a scalpel (Fig. 9) without coming in contact with the embryo or the yolk. After opening the eggs, the embryos were first transferred to a weight boat and then into the surrogate turkey egg shell (Fig. 9). In case of damage of the embryo or of the egg yolk, the embryos were discarded.

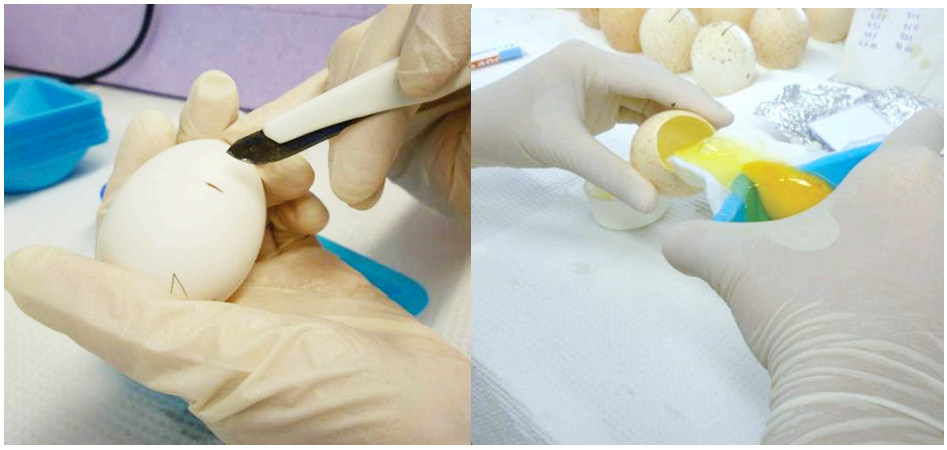


Fig. 9 Opening of the chicken egg and transfer from the weight boat into a surrogate turkey egg shell

Before sealing the surrogates, 1ml of 20% Penicillin/Streptomycin was added to prevent infections. The eggs were then sealed by means of a Q-Tip, chicken egg white with 5% Penicillin/Streptomycin and a piece of plastic wrap (4x4cm pieces) (Fig. 10).

Fig. 10 Sealing of the surrogate egg by means of egg white with P/S, Q-tip and plastic wrap

The embryos were incubated in their surrogates up to the day of further experiments (Fig. 11). On the day of the intravenous injections the eggs were taken from the incubator one after another to avoid their undercooling. To approach the vessels of the embryo, the eggs were held so that the surface vessels would come closer to the edge of the surrogate egg shell (that is usually succeeded with an angle of about 60° degree). The plastic wrap was opened and 50 or

100µl of substance were injected intravenously (*i.v.*) with an one-way 30G insulin syringe. The eggs were sealed again as described before and set back for further incubation.

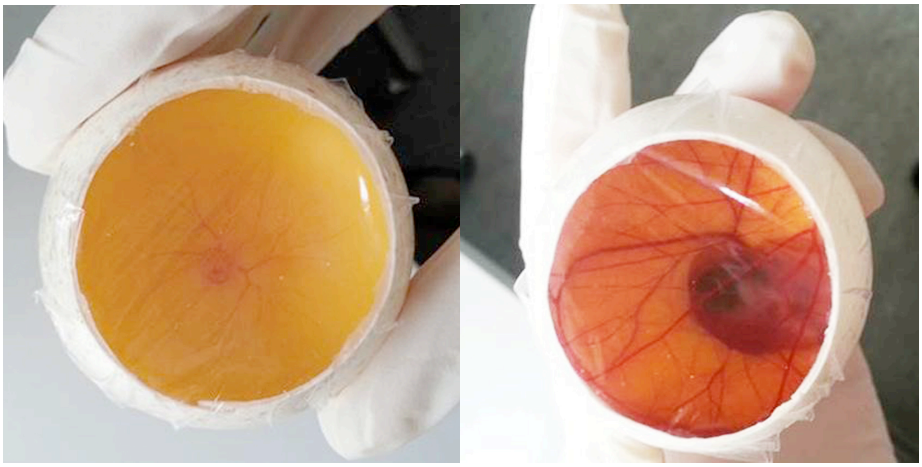


Fig. 11 Chicken embryos on ED3 and ED10 after transfer into surrogate turkey egg shells

4.3.2 Intravenous approach to the chicken embryo in its own shell (*in ovo*)

Material

Candling light with fixation system [42]

Multifunction tool (dermal tool) [33]

Insulin one-way syringes 0.3x12mm, 40I.U. [22]

Needle 22G [24]

Paraffin oil [1]

Labelling tape [28]

Substance to be injected

On the day of injections, the eggs were candled to define the position of vessels that were available for injection. A triangle (1x1x0.5cm) was drown with a pencil over a vessel that was stable and would not move when rotating the egg. The position for the injection was chosen close to a bifurcation of the vessel. The eggs were carefully scored with the dermal tool in order to avoid damaging the underlying membrane (outer and inner shell membrane) (Fig. 12, A.). The drilled triangle, was carefully removed from the egg shell (Fig. 12, B.). The egg was then fixated at the candling light with the fixating system and a drop of paraffin oil was set on the membrane of the egg, where the triangle was removed (Fig. 12, C.). That way the membrane becomes transparent and the vessel is clearer to see. The injection was performed with an one-way 30G insulin syringe (Fig. 12, D.)

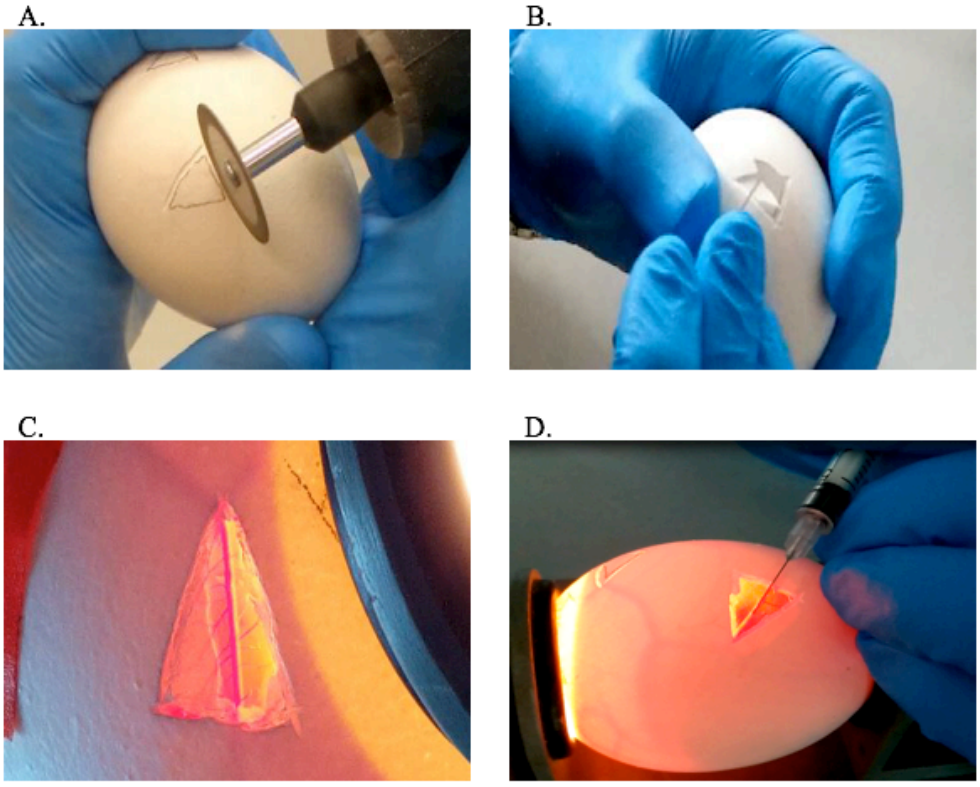


Fig. 12 *i.v.* injection of the chicken embryo in its own shell

Drilling of the triangle (A). Dispatching the triangle from the egg shell (B). View of the vessel after putting a drop of paraffin oil on the membrane (C). *i.v.* in ovo injection (D).

After injection, the piece of the egg shell that was removed (small triangle) was set back over the membrane and sealed with a piece of labelling tape.

Fig. 13 The egg shell is shield with labelling tape after *i.v.* injection

4.3.3 Blood sampling of the chicken embryo

Material

Candling light with fixation system [42]

Multifunction tool (dermal tool) [33]

Insulin one-way syringe 0.3x12mm, 40I.U. [22]

Needle 22G [24]

Paraffin oil [1]

EDTA tube [17]

Labelling tape [28]

In order to take blood from chicken embryos the eggs were prepared the same way as for *i.v.* injections in the chicken's own shell as described in chapter 4.3.2. The maximum amount of blood taken during embryonic development is listed in chapter 5.2.

4.4 ADOPTIVE TRANSFER OF CHICKEN SPLENOCYTES DURING EMBRYONIC DEVELOPMENT

Material

Candling light with fixation system [42]

Ice

SafeSeal Cups 1.5ml [17]

Cell strainer 100µm, Nylon [25]

Cell strainer 50µm, sterile [25]

One-way syringes 1ml [24]

Needle 22G [24]

One-way insulin syringes (0.3x1.2mm needle) 40I.U. [22]

Trypan blue [9]

Labelling tape [28]

Fine preparation set [22]

Hemocytometer, modified Neubauer chamber [29]

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

The adoptive transfer consists of two parts: the first part is the isolation of the desired cells from the donor embryo and the second is their transfer into the host embryo.

4.4.1 Isolation of cells for the adoptive transfer

Eggs were set for incubation for 10 to 14 days under the conditions described in chapter 4.2.

On the day of transfer, the donor eggs were opened and the embryos were sacrificed. With a fine preparation set (scissors and forceps) the spleen was prepared and set directly in 1ml of cold PBS. Depending on the age of the donor embryos, the splenocytes were prepared either individually or in pools of two to five spleens. The homogenization was succeeded by resuspending the spleen in PBS with a 1ml syringe without a needle. After homogenization, the suspension was spun down at 400g for four minutes. The supernatant was discarded, the cells were resuspended in PBS and 1µl of the suspension was used for cell counting in 1 to 10 dilution in Trypan blue. The cell counting was performed by means of a hemocytometer.

4.4.2 Transfer of the cells

In order to be able to transfer 1 to $1.5x10^6$ cells in 50µl solution per embryo the cells were either further diluted in PBS (or other injection substance) or spun down again (400g for four minutes) to reduce the volume of the suspension to 50µl per embryo. The host embryos were prepared for the injection as described in chapter 4.3.2. Prior to the injection of the cells into the embryo, the cells were filtered through a cell strainer (50µm pores). Cells were prepared for three to four transfers each time. For further transfers, cells had to be prepared again.

4.5 AMD3100 TREATMENT

Material

AMD3100 [4], stored at -20°C

Aqua dest.

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

4.5.1 AMD3100 treatment of the embryos

Eggs were prepared as described in chapter 4.3.2. AMD3100 was diluted using aqua dest. in a way that different concentrations of the AMD3100 could be injected maintaining the same volume of 50µl. After dilution AMD3100 was directly injected *i.v. in ovo* as described in chapter 4.3.2. The egg shell opening was sealed and the eggs were set for further incubation (see chapter 4.3.2).

4.5.2 AMD3100 treatment of the cells

Splenocytes of the donor embryos were prepared as described in chapter 4.4.1. After isolation, the cells were incubated with AMD3100 (200µg/ml in PBS) for 15 minutes at room

temperature. The cells were transferred into the host chicken embryos as described in 4.4.2.

4.6 ISOLATION OF LEUKOCYTES

4.6.1 Leukocyte isolation from the embryonic bursa

Material

Ice

SafeSeal Cups 1.5ml [17]

Cell strainer 100µm, nylon [25]

One-way syringe 1ml [24]

Petri dish [17]

Fine preparation set [22]

Biocoll Separating Solution [20]

FACS tubes [36]

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

Bursae were prepared either alone or in pools after decapitation of the embryos, depending on the requirements of each experiment. In case of pool preparation, the bursae were prepared and set on ice until further process with the rest of the pool bursae. With the plunger of one ml one-way syringe and two ml PBS the bursae tissue was passed through a 100µm cell strainer into a petri dish. The cell suspension was collected from the petri dish (around two ml of cell suspension) and carefully layered over the same volume of Biocoll separating solution in a FACS tube. A density gradient centrifugation was performed at 650g, for twelve minutes in a swing out rotor with slow acceleration and no brakes. Leukocytes were found in the interphase, between Biocoll und cell suspensions. The cells were collected with a Pasteur pipette and washed in 4ml PBS (centrifuge at 450g for five minutes).

4.6.2 Leukocyte isolation from embryonic blood

Material

Ice

One-way insulin syringe (0.3x1.2mm needle) 40I.U. [22]

SafeSeal Cups 1.5ml [17]

Fine preparation set [22]

Biocoll Separating Solution [20]

FACS tubes [36]

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

Blood was taken from the embryos as described in chapter 4.3.3 and collected to EDTA tubes. The blood was diluted 1 to 2 in PBS. The cells were then isolated from the suspension as described in 4.6.1.

4.7 FLUORESCENCE ACTIVATED CELL SORTING (FACS)

4.7.1 Extracellular Staining

Material

Leukocytes

96 well plate, U formed bottom [17]

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

Fluo buffer

5gr. Bovine Serum Albumin - Fraction V9 [1]

50mg Sodium azide (NaN3) [1]

Ad 500ml PBS pH 7.2

Primary and secondary antibodies (diluted in Fluo buffer)

Ice

Cells were plated in a 96 well plate ($1x10^6$ cells per well). The cells were then pelleted at 650g for one minute and resuspended in 50µl of the primary antibodies (one or more primaries can be mixed together depending on conjugation, isotypes and specificity, diluted in PBS). After a 30 minutes incubation, the primary antibody solution was washed away (150ul of Fluo Buffer added to the cells, spun at 650g for 1 minute, supernatant discarded). Cells were resuspended and incubated for 30 minutes in the solution of the secondary antibodies (mixed or alone). The cells were washed once more as described above to be finally resuspended in 400µl and transferred to FACS tubes. Before transferring the cell suspension into FACS tubes, the cells were filtered through a 100µm mesh to discard clumps from the suspension. FACS analysis were performed with the *BD FACS Canto II* and *BD Accuri C6*. Analysis of the data was

performed by means of the *FlowJo10.* software program. The antibodies used in this project are listed in Tab. 4 and Tab. 5. All incubation steps were done on ice and in the dark.

4.7.2 Intracellular Staining

Material

SafeSeal Cups 1.5ml [17]

96 well plate [17]

IC Fixation buffer [12]

Permeabilisation buffer [12]

Fluo buffer, stored at 4°C (see 4.7.1)

Primary and secondary antibodies (dilution in Permeabilisation buffer)

Ice

For the detection of antigens that are not localised on the surface of the cells (like for the detection of granulocytes) a cytoplasmic staining was performed. $1x10^6$ cells were plated on a 96 well plate. The cells were pelleted as described in 4.7.1. The fixation and permeabilisation of the cells was performed according to the manufacturer's protocol. The cells were then stained with the primary and secondary antibodies diluted in permeabilisation buffer and as described in 4.7.1.

Antibody	Clone	Isotype	Antigen	Fluorochrome	Dilution	Reference	Supplier
chB6 (Bu1)	AV20	IgG1	Bu1	Unlabelled (UNLB)	1:800	[119]	SBA
chB6 (Bu1)	AV20	IgG1	Bu1	FITC	1:200	[185]	SBA
chB6 (Bu1)	AV20	IgG1	Bu1	Alexa Fluor 647	1:800	[119]	SBA
chCXCR4	9D9	IgG2a	CD184	UNLB	1:1500	[186]	Biorad
GRL1	-	IgG3	-	UNLB	1:10	[187]	DSHB
chIgM	M1	IgG2b	IgM	UNLB	1:200	[188]	SBA
TCR1	TCR1	IgG1	chTCRγδ	UNLB	1:800	[189]	SBA
TCR2	TCR2	IgG1	chTCRαβ-1	UNLB	1:200	[190]	SBA
TCR3	TCR3	IgG1	chTCRαβ-2	UNLB	1:200	[191]	SBA

Tab. 4 Primary antibodies for FACS analysis

Antigen	Biological source	Conjugate	Dilution	Supplier
muIgG (H+L)	Goat	APC	1:800	SBA
muIgG (H+L)	Goat	FITC	1:250	SBA
muIgG1	Goat	Alexa Fluor 647	1:800	SBA
muIgG1	Goat	APC	1:1000	Jackson Immunoresearch

Tab. 5 Secondary antibodies for FACS analysis

4.7.3 Live/dead Staining

Material

SafeSeal Cups 1.5ml [17]

Fluo buffer, stored at 4°C (see 4.7.1)

Fixable Viability Dye eFluor® 780 [12], stored at -80°C

7-AAD Viability Staining Solution [5], stored at 4°C

For the live/dead staining two different dyes were used: Fixable Viability Dye eFluor® 780 or 7-AAD Viability Staining Solution.

For staining with the Fixable Viability Dye eFluor® 780, cells were plated ($1x10^6$ cells/well) and incubated in 50µl of Fixable Viability Dye eFluor® 780 for 30 minutes, on ice, in the dark (1:1000 dilution in Fluo buffer). The cells were then washed as described in 4.7.1. The extra- or intracellular staining was performed after the live/dead staining.

In contrast, the extra- or intracellular staining had to be performed prior to the live/dead staining in case of the 7-AAD Viability Staining Solution. Shortly before FACS analysis the cells were incubated for ten minutes in 50µl of (1:100 in Fluo buffer diluted) 7-AAD, in the dark. After incubation the cells were filtered and transferred in FACS tubes as already described in chapter 4.7.1.

4.7.4 Quantification of cells

In order to quantify cells either 123count eBeads™ were used or the volume of the probes was quantified.

After the extra- or intracellular and the live/dead staining the volume of the probe was adjusted to 300µl. Just before analysis, counting beads were added to the probes in a known concentration (concentration given by producer) and volume (100µl). By means of cross-multiplication the absolute number of the cells was calculated.

$$\text{Absolute Count (cells/µl)} = \frac{\text{Cell Count}}{\text{eBead Count}} \times \text{eBead Concentration}$$

When quantifying by measuring the volume of the probe in which the events were counted, the analysis was performed with the BD FACS Accuri C6. The Accuri C6 has the possibility to specify the probe counted volume. With a cross-multiplication the absolute number of cells could be calculated up to the whole amount for the original probe.

$$\text{Absolute Count (cells/µl)} = \frac{\text{Cell Count}}{\text{Volume Count}}$$

4.8 GENOMIC DNA EXTRACTION FROM EMBRYONIC BLOOD

Material

SafeSeal Cups 1.5ml [17]

TEN medium

10mM Tris-Cl, pH 8 [1]

1mM EDTA [1]

10mM NaCl [1]

STM buffer, stored at 4°C

64mM Sucrose [1]

20mM Tris-Cl, pH 7.5 [1]

10mM $MgCl_2$ [1]

0.5% Triton X-100 [1]

Pronase E [9]

EDTA tubes [17]

96-well-mega block [17]

Seal foil for 96 well plates or blocks [17]

Falcon tubes [17]

Blood was collected to EDTA tubes (see chapter 4.3.3). 200µl of STM buffer were added per well in a 96-well-mega block. After plating the buffer, 2µl of the EDTA blood were added to each well. The probes were then centrifuged at 1000g for 5 minutes. Last remains of fluid were thoroughly discarded by tapping the mega block multiple times on a clean surface. To avoid contamination, a new, clean surface was used for every attempt. Pronase E was mixed in a falcon tube with TEN medium (100µg Pronase E/1ml TEN medium) and 400µl of the mixture was added to each probe. The probes were properly mixed, the block was sealed with a seal foil and then set for one hour for incubation at 37°C and shaking at 300rpm. The reaction was stopped by incubation at 65°C for 15 minutes. One µl of the end product was used for the genotyping of the embryos.

4.9 POLYMERASE CHAIN REACTION

Material

Nuclease free H_2O [1]

Primers, forward and reverse [19]

DNA polymerase, FIREPol MasterMix 7.5 [18]

Template DNA

ThermoCycler [13]

PCR Plates [17]

Seal foil [17]

Polymerase chain reactions were performed in order to genotype embryos.

4.9.1 Genotyping of *JH*-KO$^{-/-}$, *JH*-KO$^{+/-}$ and WT embryos

For experiments with *JH*-KO$^{-/-}$ embryos, the embryos had first to be genotyped. The primers that were used for genotyping are listed below (Tab. 6).

Nr. of primer	Direction	Sequence
13011	Forward	gcccaaaatggccccaaaac
13012	Reverse	agtgacaacgtcgagcacagct
13013	Reverse	atggggccacgggaccgaa

Tab. 6 Primers used for genotyping

The primer pairs used for the *JH*-KO$^{-/-}$ PCR were primer 13011 with the primer 13012, and for the WT PCR the primer 13011 with the reverse primer 13013.

The PCR reaction for the genotyping and the PCR cycling parameters for both PCRs (*JH*-KO$^{-/-}$ und WT PCR) are listed in Tab. 7 and Tab. 8.

Component	Amount	Concentration
Firepol MasterMix	4µl	1x
Template DNA	1µl	1-10ng
Forward primer	4µl	5pmol/µl
Reverse primer	4µl	5pmol/µl
Nuclease free H_2O	7µl	-
	Total: 20µl	-

Tab. 7 PCR reaction

Step	Phase	Temperature	Time	Repeat
1.	Initiation	59°C	15’	x1
2.	Initial Denaturation	95°C	3’	x1
3.	Denaturation	95°C	30’’	x35
4.	Annealing	60°C	30’’	x35
5.	Elongation	72°C	1.5’	x35
6.	Final Elongation	72°C	5’	x1
7.	Cool down	12°C	Unlimited	-

Tab. 8 PCR cycling parameters

4.10 TISSUE HOMOGENIZATION FOR RNA ISOLATION

Material

Tissue, stored at -20°C and transported on dry ice

InnuSpeed lysis tubes, Type P, 0.5ml [41]

Homogenizer [41]

Dry ice

Scalpel, sterile [24]

Petri dishes, sterile [32]

For the homogenization of the bursal tissue, bursae were cut in the middle with a sterile scalpel. In InnuSpeed lysis tubes P, 200µl of lysis buffer mixed with thioglycerol was added and then half of the bursa was added in the tubes and homogenized for 30 seconds. Another 250µl of the lysis buffer/thioglycerol mixture were added to the tissue in the Jena tube and spun again for 30 seconds.

4.11 RNA ISOLATION

Material

Cells or homogenized tissue

SafeSeal Cups 1.5ml [17]

ReliaPrep™ RNA Tissue Miniprep System [39]

NanoDrop Spectralphotometer [40]

RNA was isolated from homogenized tissue according to the product protocol of ReliaPrep™ RNA Tissue Miniprep System from Promega. The concentration of the isolated RNA was defined by NanoDrop Photometer measurement.

4.12 cDNA SYNTHESIS

Material

RNA

GoScript™ Reverse Transcription System [39], stored at -20°C

Nuclease free H_2O

SafeSeal Cups 1.5ml [17]

Ice

cDNA was synthesized according to the product protocol of GoScript Reverse Transcription System from Promega and stored at -20°C.

4.13 QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION

Material

GoTaq qPCR Master Mix [39]

Aqua bidest.

cDNA (1:10 dilution of the previous preparation 4.12)

qRT-PCR primers [19] (working solution of 5pmol/µl)

qRT-PCR analyser [13]

Bio-rad CFX Manager software [13]

All surfaces were cleaned and decontaminated by UV radiation prior to the beginning of the work. The qRT-PCR reaction and the cycling parameters are listed in Tab. 9 and Tab. 10.

Reagent	Amount
GoTaq MasterMix	12.5µl
Primer, forward	1.5µl
Primer, reverse	1.5µl
Nuclease-free H2O	4.5µl
cDNA Template (diluted to 2ng/µl)	5µl
Total:	25 µl

Tab. 9 qRT-PCR reaction

The cycling parameters were set as following:

Step	Phase	Temperature	Time	Cycle
1.	Hot-Start Activation	95°C	2’	x1
2.	Denaturation	95°C	15’’	x40
	Annealing	59°C	30’’	x40
	Elongation	72°C	30’’	x40
3.	Dissociation	95°C	15’’	x1
		57°C	30’’	x1
		95°C	15’’	x1

Tab. 10 qRT-PCR cycling parameters

The primers that were used for qRT-PCR analysis are listed in Tab. 11:

Nr. of primer	Direction	Sequence	Amplification length (bp)
455 CXCR4	Forward	ctgtggctgacctcctctttg	86
456 CXCR4	Reverse	acacaggacatttccgaagtacc	86
457 CXCL12	Forward	ctcaagagcaacagcaagcaa	150
458 CXCL12	Reverse	gcccttaacgttctacccttga	150
459 CXCR5	Forward	cgctcgcctatctgcttatgt	103
460 CXCR5	Reverse	aagttttcggtggttgttcga	103
461 CXCL13.1	Forward	tgccggaggaaagagatcat	116
462 CXCL13.1	Reverse	ttgctgacatccgtctgtgtc	116

Tab. 11 Primers used for qRT-PCR

4.14 HISTOLOGY

4.14.1 Preparation of tissues for histology and histological sections

Material

Cryotubes [3]

Liquid nitrogen [8]

Long forceps [21]

Cryoprotective gloves [21]

Object slides, Superfrost® Plus [25]

Cryostat [32]

Microtome blades (Feather®) [32]

Tissue-Tec® [32]

Paint brush

Formalin 4%, neutral buffered (NBF) [31]

Embedding molds for paraffin sections [25]

Ascending alcohol row [7] **and paraffin row** [31]

- 40% Ethanol for 2 hours
- 50% Ethanol for 2 hours
- 60% Ethanol for 2 hours (alternatively overnight)
- 70% Ethanol for 2 hours
- 80% Ethanol for 2 hours
- 96% Ethanol for 2 hours
- Isopropanol I for 2 hours
- Isopropanol II for 2 hours (alternatively overnight)
- Xylol I for 2 hours
- Xylol for 2 hours
- Paraffin 1 for 2 hours
- Paraffin 3 for 2 hours (alternatively overnight)
- Paraffin 6 for 2 hours
- Paraffin 9 for 2 hours

Descending alcohol row [7]

- 10 minutes in Xylol II
- 10 minutes in Xylol I
- Isopropanol I
- Isopropanol II
- Ethanol 99%
- Ethanol 70%
- Aqua dest.

Acetone 100% [7]

Embedding work station [31]

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

Collected organs either transferred to cryovials and immediately stored in liquid nitrogen or they were fixed in 4% neutral buffered formalin (NBF) for three hours. Formalin was washed off in running tap water for 15 minutes. The tissues were then dehydrated through an ascending alcohol row (see materials). After dehydration they were put to liquid paraffin solutions of different blends (1, 3, 6, 9 paraffin solutions) in order to allow the diffusion of paraffin into the tissue (see materials). The tissues were finally embedded in paraffin at the embedding work station.

The tissues that were stored in liquid nitrogen were taken out of the liquid nitrogen and left for 30 minutes in the chamber of the cryostat (CryoStar NX70 [4]) at -20°C to allow adjustment of the tissue to the chamber temperature. Tissues were taken with forceps out of the cryovials and were fixated with just a drop of cryomatrix (Tissue-Tec) on the specimen chucks. Bursae were then cut with a thickness of 7µm while spleens were cut at 5µm. The sections were then layered on Superfrost® Plus glass slides. Slides were air dried for 24 hours.

4.14.2 Preparation of sections for staining

Before staining the tissues, sections of both types of preparation (Cryo- and paraffin sections) had to be further processed. Cryosections had to be fixated in acetone for two minutes, air dried for ten minutes and rehydrated for 15 minutes in PBS. Paraffin sections on the other hand had to be dewaxed by passing them through a descending alcohol row (see materials).

4.14.3 Histological Stainings - H&E Staining

Material

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

Aqua dest.

Eosin solution [26]

Mayer´s Haematoxylin solution [26]

Ascending alcohol row [7] (see material in chapter 4.14.1)

Eukitt® mounting medium [9]

Cover glasses [25]

The dewaxed and rehydrated slides were put in Mayer´s Haematoxylin solution for ten minutes and then washed shortly in aqua dest. Then they were left for another ten minutes under running tap water. Sections were put in the Eosin staining solutions for 90 seconds and then washed for two minutes in aqua dest. Finally, slides were passed through an ascending alcohol row (see materials) and then mounted with the Eukitt mounting medium and cover glasses (18x18mm).

4.14.4 Immunohistochemical Staining

Material

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

Incubation chamber

Parafilm [9]

Bovine serum albumin [1]

Goat [25] **or horse serum** [27]**, stored at -20°C**

Acetone 100% [7]

0.3% H_2O_2 [1]

Methanol 40% [1]

Primary antibodies (Tab. 12)

Serum from host animal of the biotinylated secondary antibody [25]

Secondary biotin-conjugated antibodies (Tab. 13)

Vector® DAB kit [27]

Mayer´s Hematoxylin solution [26]

Vectastain® ABC kit [27]

Ascending alcohol row [7]

(70% Ethanol, 99% Ethanol, Isopropanol 1, Isopropanol 2, Xylol 1, Xylol 2)

Eukitt® mounting medium [9]

Aqua dest.

Cover glasses [25]

Immunohistochemical staining was performed on dewaxed and rehydrated or fixated and rehydrated sections. In order to block endogenous peroxidase activity, tissues were completely covered with 0.3% H_2O_2 diluted in 40% methanol and a parafilm piece. Sections were incubated in the humid incubation chamber for 30 minutes. Sections were washed three times for five minutes in PBS. In order to block any non-specific binding, the sections were blocked for one hour with serum of the animal in which the biotin-labelled secondary antibody (goat or horse serum) was produced. Therefore, serum was mixed at a 1:40 dilution in PBS with 1% BSA. The blocking solution was washed off again (three times for five minutes with PBS). Sections were then stained with the primary antibody for one hour and then washed off as before with PBS. The labelling of the binding antibodies with peroxidase was performed according to the protocol of the product Vectastain® ABC kit. Peroxidase activity detection was performed with the Vector® DAB kit. For nucleus staining the section were submerged for one minute in aqua dest., one minute in Mayer's Hematoxylin solution and five minutes in running tap water. Finally, the sections were passed through an ascending alcohol row and mounted with Eukitt mounting medium and cover glasses of 18x18mm size. All parts of incubation were performed in a humidity maintaining incubation chamber.

4.14.5 Immunofluorescence Histology Staining

Material

Humid incubation chamber

Phosphate-buffered saline (PBS), pH 7.2 (see 4.3.1)

Bovine Serum Albumin [1]

Serum of the animal in which the secondary antibody was produced [25]

DAPI [1]

Secondary antibody

VectaShield Hard&Set mounting medium (anti fading) [10]

Cover glasses [22]

Goat serum [25]

Mouse serum [25] (only by double staining with antibodies of the same isotype)

Immunofluorescence staining was performed on dewaxed and rehydrated or fixated and rehydrated sections.

4.14.5.1 Single staining

Sections were incubated in a blocking solution consisting of serum of the host animal of the secondary antibody as described in chapter 4.14.4. After blocking, the sections were incubated with the primary antibody for one hour and then the antibody was washed off with PBS as described in 4.14.4. Sections were stained, after washing off, with the secondary fluorescent antibody also for one hour. In the same solution the component for nucleus staining, DAPI (1:500) was added. One hour later secondary antibody and DAPI were washed off with PBS as described before. The mounting of the sections was performed directly after the washing off the secondary antibodies. For the mounting the Hard&Set Mounting Medium was used and cover glasses of 18x18mm size.

4.14.5.2 Double staining with one direct and one indirect staining (by two primary antibodies of the same isotype)

Sections were stained (with unconjugated primary antibody and its secondary antibody) as in the single staining protocol (4.14.5.1) without mounting of the sections. After washing the secondary antibody off, the sections were blocked once more. Since the directly conjugated primary antibodies originate from mice, the blocking solution consisted of mouse serum diluted 1:10 in PBS with 1% BSA. Sections were incubated with the blocking solution for one hour to then be stained with the second primary directly conjugated antibody. The antibody was washed off and sections were mounted with the Vectashield Hard&Set Mounting medium. All incubation steps were performed in the humid incubation chamber, in the dark. Antibodies, primary and secondary, are listed in Tab. 12 and Tab. 13.

4.14.6 Documentation of histological and immunohistochemical stainings

Bright field and fluorescence microscopy and documentation were performed with the use of the microscope Zeiss Axioskop, the camera Zeiss AxioCam MRc5 and the AxioVision microscope software. Pictures of immunohistochemical stainings were partially taken with the M8 digital microscope and scanner from PreciPoint.

4.15 STATISTICAL ANALYSIS AND GRAPHS

Statistical analysis was performed with the SPSS Statistics Software. For the evaluation of data distribution, the normality tests Shapiro-Wilk and Kolmogorov-Smirnov were used. Significance was evaluated with the independent t-Test or ANOVA One-way Test for normally distributed data or by non parametric tests (Kruskal-Wallys) for independent samples in case of not normally distributed data.

Graphics were designed with Windows Excel or with the GraphPad software.

Antibody	Antigen	Clone	Host	Conjugate	Isotype	Dilution IHC/IF[1]	Reference	Supplier
chB6(Bu1)	Bu1(a+b)	AV20	Mouse	FITC	IgG1	- / 1:100	[185]	SBA
chB6(Bu1)	Bu1(a+b)	AV20	Mouse	UNLB	IgG1	1:200 / 1:100	[119]	SBA
GRL1	Granulocyte	Polyclonal	Mouse	UNLB	IgG3	1:100 /-	[187]	DSHB
chIgM	IgM	M1	Mouse	UNLB	$IgG2_{bκ}$	1:500 / 1:500	[188]	SBA

Tab. 12 Primary antibodies for histology

Antigen	Host	Conjugate	Dilution	Supplier
muIgG1	Goat	Biotin	1:200	SBA
muIgG1	Goat	AlexaFluor 488	1:200	Jackson
muIgG (H+L)	Horse	Biotin	1:200	Vector Biozol
muIgG (H+L)	Goat	AlexaFluor 568	1:200	Fischer

Tab. 13 Secondary antibodies for histology

[1] **IHC: Immunohistochemistry, IF: Immunofluorescence histology**

5. RESULTS

A method for intravenous manipulation of the chicken embryo had to be established in order to perform several experiments that demanded the intravenous treatment of the embryo.

5.1 ESTABLISHING A METHOD FOR INTRAVENOUS MANIPULATION OF THE CHICKEN EMBRYO

Two different approaches were tested and compared to each other. Firstly, a method of *i.v.* injection after transfer of the embryo in a surrogate egg shell was examined. The second method examined was the *i.v.* injection of the embryo directly in its own egg shell.

5.1.1 Transfer of chicken embryos to surrogate egg shells

For the examination of the intravenous manipulation after transfer of the embryo to a surrogate egg shell, embryos were transferred as described in chapter 4.3.1. Out of a total of 903 embryos that were transferred in surrogate egg shells, 285 embryos died after transfer, without reaching the stage for intravenous manipulation. The average mortality rate was at 28% (from 5% to 76%) (see Tab. 14). These mortality rates do not apply to the method of direct injection in the embryo's own egg shell since embryos do not need transfer into a surrogate egg shell.

5.1.2 Survival of embryos after transfer into surrogate egg shells and *i.v.* injection

After transfer of the embryos to surrogate egg shells, PBS was injected *i.v.* (see chapter 4.3.2) and the survival rate of the embryos was calculated. In total, 158 embryos were transferred into surrogate egg shells, 45 embryos survived after their transfer to surrogate egg shells, and 21 of them also survived after *i.v.* injection. The method of intravenous manipulation of the chicken embryo after its transfer to a surrogate egg shell shows an average survival rate of 13.6% (from 6% to 18% as shown in Tab. 14).

Transferred embryos	Dead embryos after transfer	Mortality rate in %
21	7	33%
60	45	75%
63	48	76%
60	45	70%
36	7	19%
35	7	20%
40	15	37.5%
42	14	35%
77	22	28%
30	7	23%
25	10	40%
19	8	42%
51	11	21%
46	8	17%
32	3	9%
31	4	13%
62	12	19%
61	5	8%
54	4	7%
58	3	5%
Total: 908	Total: 285	Average: 28%

Tab. 14 Mortality rates of embryos after transfer to a surrogate egg shell

Transfers	Embryos after transfer	Embryos after *i.v.* injection	Total survival rate
35	15	6	17%
63	15	4	6%
60	15	11	18%
Total: 158	Total: 45	Total: 21	**Average:** 13.6%

Tab. 15 Survival rate of transferred and *i.v.* injected embryos

5.1.3 Intravenous injection of embryos in their own egg shell. Survival of the embryo depends on the age of embryo on the day of injection and the volume of the injected substance

In order to examine the method of intravenous manipulation of the embryo directly in its own egg shell, embryos were injected as described in chapter 324.3.2. In order to define the appropriate volume for injection, embryos were injected *i.v.* with 50µl and 100µl of PBS on various embryonic days. Ten embryos were injected *i.v.* on ED10. Half of them received 50µl while the other half received 100µl PBS. In both cases, the survival rate of the embryos was 60%. Additionally, 50 ED11 embryos were injected *i.v.* with either 50µl or 100µl PBS. 91% of the ED11 embryos that received 50µl PBS survived, while of those embryos that received 100µl 60% survived. Furthermore, 45 ED12 embryos were *i.v.* treated again with either 50µl or 100µl PBS. Out of 40 embryos that received 50µl, all 40 survived the injection whilst of five embryos that received 100µl, four survived (survival rate 80%). Finally, embryos were also injected on ED14. 100% of the ten embryos survived in the case of those embryos that were injected with 50µl PBS. Out of 16 embryos that were injected with 100µl, eleven embryos survived (survival rate 69%).

Day of injection	Volume of injection	Number of embryos	Survival rate
ED10	50µl	5	60%
ED10	100µl	5	60%
ED11	50µl	45	91%
ED11	100µl	5	60%
ED12	50µl	40	100%
ED12	100µl	5	80%
ED14	50µl	10	100%
ED14	100µl	16	69%

Tab. 16 Survival rates after *i.v.* injection in association with the embryonic age and volume of injection

The survival of the embryos is much higher with the method of direct injection into the embryos in their own egg shell than with the method of injection after transfer of the embryo to a surrogate egg shell (see Tab. 15). Furthermore, the embryo survival depends on the age of the embryo but also on the volume of the injected substance as shown in Tab. 16.

5.2 BLOOD SAMPLING FROM EMBRYOS

For all experiments that required blood sampling from chicken embryos, blood was collected as described in chapter 4.3.3. It was examined which amount of blood could be deprived from a single embryo. The amount of blood depends on the age of the embryo as shown in Tab. 17. The blood sampling volumes as listed in Tab. 17 apply to the highest possible blood sampling volumes. The blood sampling of theses volumes led to the death of the embryo. On ED8 the maximum amount of blood that could be collected was 40µl. On ED9 the amount rose up to 50µl. On ED10 the collection of 100µl was possible. On ED11 200µl could be collected, while one day later, on ED12 500µl were collected. On ED14 650µl could be collected, whilst on ED16 800µl could be collected. Finally, on ED18 the volume of the blood sample rose to 1ml.

Embryonic day	ED8	ED9	ED10	ED11	ED12	ED14	ED16	ED18
Volume of sample	40µl	50µl	100µl	200µl	500µl	650µl	800µl	1000µl

Tab. 17 Highest possible blood volume retrieval from a single embryo regardless of survival

For experiments which demand the survival of the embryos (as in case of blood sampling for genotyping for the performance of subsequent experiments), blood was taken on ED11, at the earliest and blood sample volume was limited to 10 to 20µl per embryo. That way the survival of the embryos was only marginally influenced.

Blood sampling was performed and 10 to 20µl were taken on ED11 from each embryo. From 119 embryos from which blood was taken, 99 survived the blood sampling, which confers to a survival rate of 83%.

5.3 MIGRATION PATTERN OF B CELLS DURING EMBRYONIC DEVELOPMENT

After the intravenous manipulation method was established, it was possible to examine the migration of B cells through the blood stream by taking blood samples on subsequent embryonic days. Isolated leukocytes were double stained either with the AV20 and the anti-chIgM or with the AV20 and the anti-chCXCR4 antibodies (see chapter 4.7.1, Tab. 4 and Tab. 5). $AV20^+$ cell, IgM^+ B cell and the $CXCR4^+$ B cell populations were examined and compared throughout the examination interval from ED8 to ED18. That way a better understanding of the migration course was achieved. Blood was taken from embryos on ED8,

10, 12, 14, 16 und 18 and leukocytes were isolated by density gradient centrifugation (as described in chapter 4.6.2). Due to the small cell populations, live/dead staining was included to focus on living cells.

On ED8 the mean absolute number of detected B cells was 2.22/µl, while on ED10 the number was reduced to 0.25/µl. On ED12 the number of B cells rose to 1.93/µl and reached its peak on ED14 with 16.23/µl. On ED16 the number of B cells started to decrease to level 3.07/µl. Finally, on ED18 the number of B cells reached its lowest with 0.2/µl. When examining the relative amounts of the population, a similar curve is seen though the peak of the B cell is detected on ED12 with 0.7% (see Fig. 14). On ED8 the level of the B cells is 0.127% and on ED10 0.033%. After reaching its peak on ED12 with 0.7%, the number of B cells decreases again towards hatch and the amount of B cells on ED14 is 0.55%, on ED16 0.47% and on ED18 0.044%.

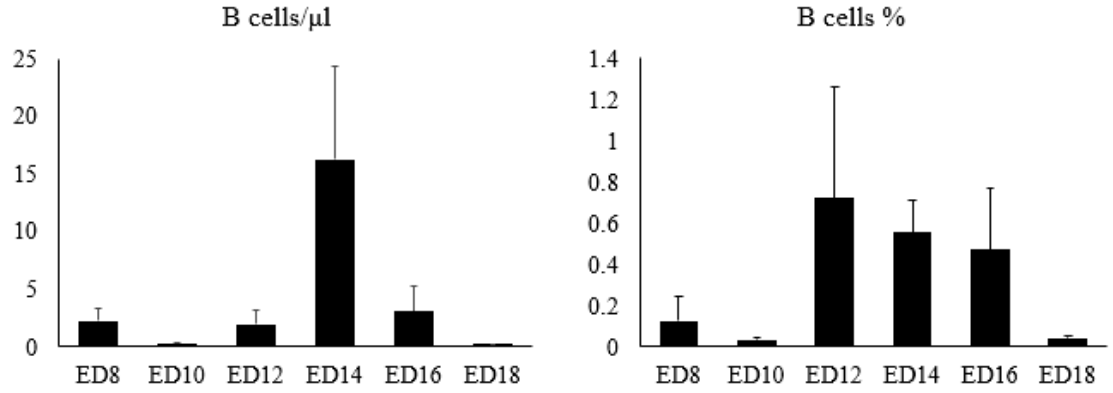


Fig. 14 B cells detected in the embryonic blood on ED8 to ED18

Embryonic blood was taken and density gradient centrifugation was performed. The isolated cells were then stained with the B cell marker AV20 for FACS analysis. Beside the percentages (right diagram), also absolute numbers (left diagram) of the cell populations were calculated and compared between the embryonic days ($n \geq 3$, not normal distribution per Kolmogorov-Smirnov and Shapiro-Wilk tests, non-parametric analysis, Kruskal-Wallis, $p<0.05$)

5.3.1 IgM expression by B cells detected in the blood between ED8 and ED18

B cells that were detected by AV20 staining were gated to examine if they express the surface immunoglobulin IgM. On ED8 the amount of the B cells expressing IgM on their surface was at 0.12% and vastly increased to 16.6% on ED10. On ED12 the level fell to 2.1%, but increased again on ED14 to 2.34%. The level slowly increased until ED16 (2.5% of the B cells were IgM^+) to radically increase on ED18 to the level of 23% (Fig. 15).

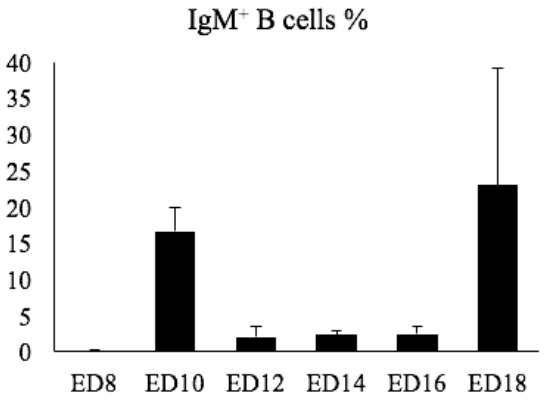


Fig. 15 IgM expression by B cells detected in the embryonic blood on ED8 to ED18

Embryonic blood was taken and density gradient centrifugation was performed. Isolated cells were stained with the B cell marker AV20 and the anti-chIgM antibody for FACS analysis. $AV20^+$ cells were gated and the gated B cell population was examined for IgM expression. Percentages were calculated and compared between the embryonic days ($n \geq 3$, not normal distribution per Kolmogorov-Smirnov and Shapiro-Wilk tests, non-parametric analysis, Kruskal-Wallis, $p<0.05$)

5.3.2 CXCR4 expression by B cells detected in the blood between ED8 and ED18

The expression of the CXCR4 chemokine receptor was detected in the spleen but also in the bursa on ED11 (by the time of B cell migration). The expression increased in the bursa while B cell colonized the bursa and the majority of B cells migrates towards the bursa [155]. However, the expression of the receptor by migrating cells in the blood has not been studied yet. Therefore, blood was taken from embryos on the same days as for the IgM expression analysis and leukocytes were double stained with the anti-chCXCR4 and the AV20 antibody [192] as described in chapter 4.7.1 for FACS analysis. Due to the small cell populations, live/dead staining was included to focus on living cells.

On ED8 2.38% of the B cells were expressing the CXCR4 on their surface. On ED10 the percentage of B cells expressing CXCR4 rose massively to 38.96% and on ED12 it remained at the same level with 39%. On ED14 there was a further increase and $CXCR4^+$ expressing B cells made 72% of the B cell population. After ED14 the percentage started to decrease towards hatch and on ED16 it was 66.6% and on ED18 35.9% (Fig. 16).

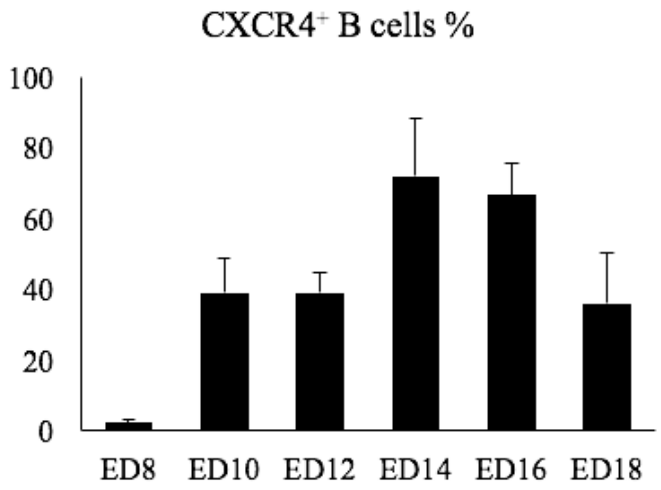


Fig. 16 CXCR4 expression by B cells detected in the embryonic blood on ED8 to ED18

Embryonic blood was taken and density gradient centrifugation was performed. Isolated cells were double stained with the B cell marker AV20 and the anti-chCXCR4 antibody for FACS analysis. Detected AV20 cells were gated and the gated B cell population was examined for CXCR4 expression. Percentages but also absolute numbers were calculated and compared between embryonic days ($n \geq 3$, $CXCR4^+$ B cells % data normally distributed per Kolmogorov-Smirnov and Shapiro-Wilk tests, independent t-Test analysis, $p<0.05$)

5.4 BLOCKING OF THE CXCR4 CHEMOKINE RECEPTOR BY MEANS OF ITS ANTAGONIST, AMD3100

AMD3100 is a specific CXCR4 antagonist [159] and by blocking the binding pocket of the CXCR4 [160] it interferes with the interaction of CXCR4 with its ligand CXCL12. AMD3100 was used to block the CXCR4-CXCL12 interaction *in vivo* in chicken embryos.

5.4.1 Blocking of the CXCR4 chemokine receptor by treating the embryos with the AMD3100

Embryos were treated with AMD3100 on different embryonic days and with different dosages. All AMD3100 embryo treatments are listed in the following overview table (Tab. 18), though all experiments will be presented in detail in the present chapter.

Experiment	Dose	Day of injection	Day of analysis	Method of analysis
1a	20µg/kg	ED11	ED16	Histology, H&E
1b	60µg/kg	ED11	ED16	Histology, H&E
1c	80µg/kg	ED11	ED16	Histology, H&E
2a	150µg/kg	ED11	ED17	Histology, H&E and IHC
2b	150µg/kg	ED11, 12	ED17	Histology, H&E and IHC
2c	150µg/kg	ED11, 12, 14	ED17	Histology, H&E and IHC
2d	480µg/kg	ED11	ED17	Histology, H&E and IHC
3	480µg/kg	ED11, 12, 14	ED18	FACS
4	1000µg/kg	ED11, 12, 14	ED18	FACS

Tab. 18 Treatment of embryos with AMD3100 on diverse embryonic days

5.4.1.1 Histological analysis of embryonic bursae with or without AMD3100 treatment

Due to lack of literature on AMD3100 effects in the chicken, firstly trials had to be performed in order to find the appropriate dosage of AMD3100 to block the CXCR4 receptor.

Therefore, embryos of the first experiments (Tab. 18, 1a-1c) received a relatively low dose of AMD3100 [164]. The embryos were separated in three groups and different doses were tested in each group on ED11. Embryos were treated *i.v.* with 20µg, 60µg or 80µg/kg AMD3100. The treatment was performed as described in chapter 4.5.1. Bursae were prepared on ED16 and embedded in paraffin. Paraffin sections were prepared and stained with H&E (see chapter 4.14.1 to 4.14.3).

In the H&E staining of the embryos treated with a low dosage of AMD3100 a similar cell population was detected as in the control groups (one control group in which embryos were neither treated with AMD3100 nor injected and one mock treated control group) (see Fig. 17). The cells seen in the H&E staining showed a similar organization in the bursa. Cells were organized in follicles to the same number and size. The follicles were distributed evenly throughout the bursa as in the control groups. Number, size and density of the follicles were also similar to the follicles of the control bursae.

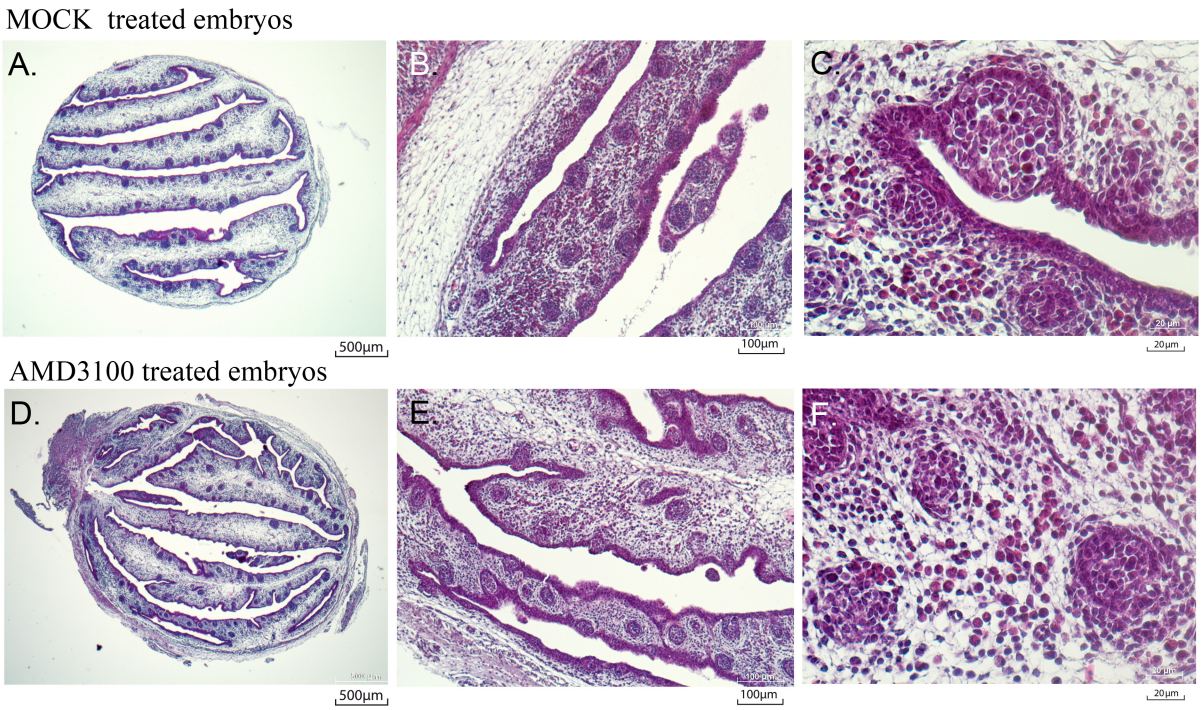


Fig. 17 H&E staining of ED16 bursae without and with AMD3100 treatment

Bursae H&E staining on paraffin sections of ED16 control (A.-C.) and AMD3100 treated (80µg/kg) (D.-F.) embryos. In both groups bursal follicles are formed in the same extent. Follicles are evenly distributed in both bursae. Representative pictures out of at least three examined organs per group are shown.

Since low dosage AMD3100 treatment did not have an effect on the development of the embryonic bursa higher doses were tested.

Therefore, in the following AMD3100 experiment embryos were treated with 150µg/kg or 480µg/kg of AMD3100 (see Tab. 18, Experiment 2a-2d). Since after the first injection, all embryos survived the higher dosage of AMD3100 without problems and in order to achieve an effect by the AMD3100 treatment, embryos were again treated on subsequent days to provide the inhibitor during the entire period of B cells migration. All embryos survived the repeated *i.v.* AMD3100 injections (ED11, 12 and 14) and the higher doses of AMD3100. The bursae were prepared on ED17. Bursae were partially embedded in paraffin and partially cryopreserved in liquid nitrogen (see chapter 4.14.1). Bursae were stained again with H&E to examine if the higher dosage and repeated treatments had an effect on the development of the bursa.

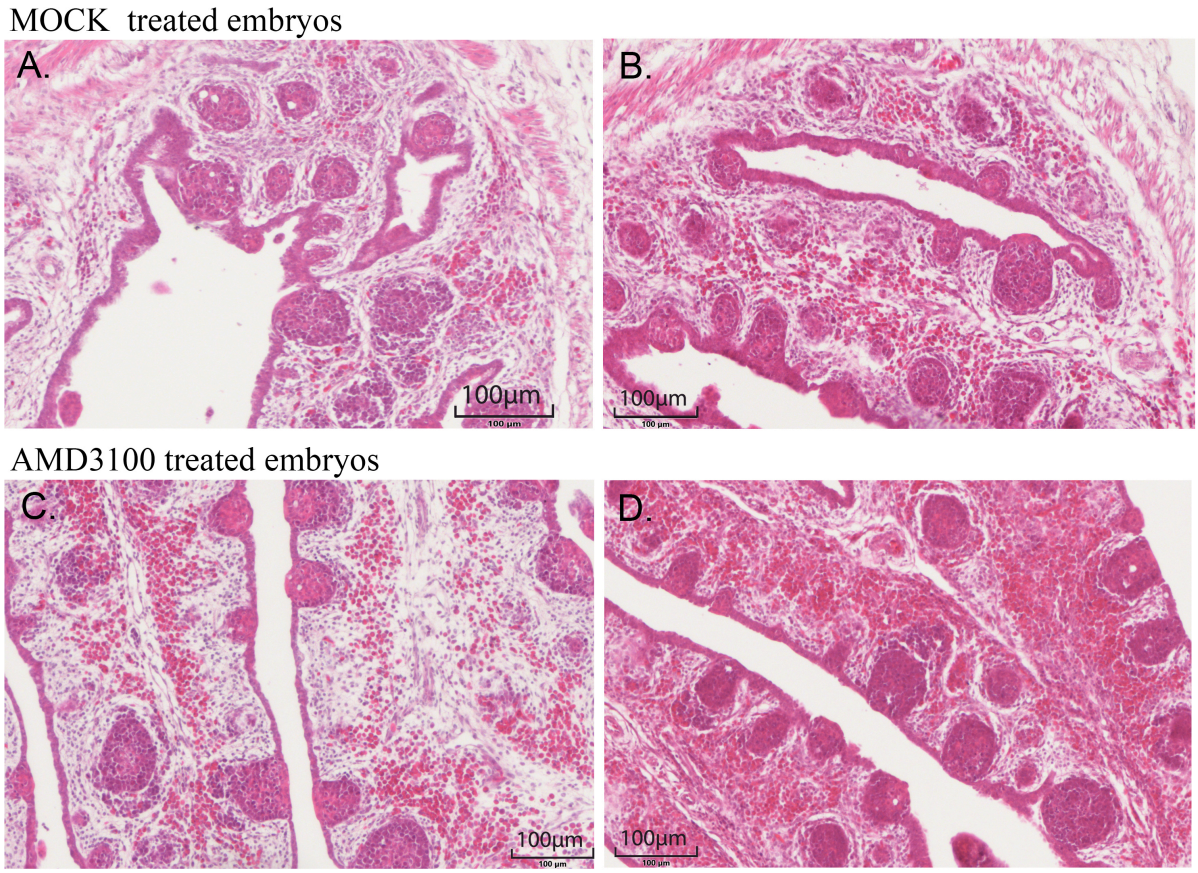


Fig. 18 H&E staining of ED17 bursae with or without AMD3100 treatment

H&E Staining of control bursae (A and B) and AMD3100 treated bursae (C and D) on ED17. C and D: A cell population is seen in the connective tissue, surrounding the follicles and making the histological picture of the AMD3100 treated bursae look denser. Representative pictures out of at least three examined animals per group are shown.

Follicle formation in both groups (AMD3100 treated and MOCK treated embryos) was indistinguishable. Follicles were found throughout the bursae in similar distribution. The number, size and density of the follicles was similar in both AMD3100 treated and control embryos. Interestingly, the H&E staining revealed a population of cells located in the connective tissue surrounding the follicles of the treated embryos. This cell population was also found in the control bursae but in much smaller numbers. Since it was the first time having an effect of the AMD3100 treatment on the embryonic bursa, additional staining was performed to evaluate the composition of the follicles. Bursae of both groups were stained with the AV20 antibody to examine if the follicles were filled with B cells (see Fig. 19).

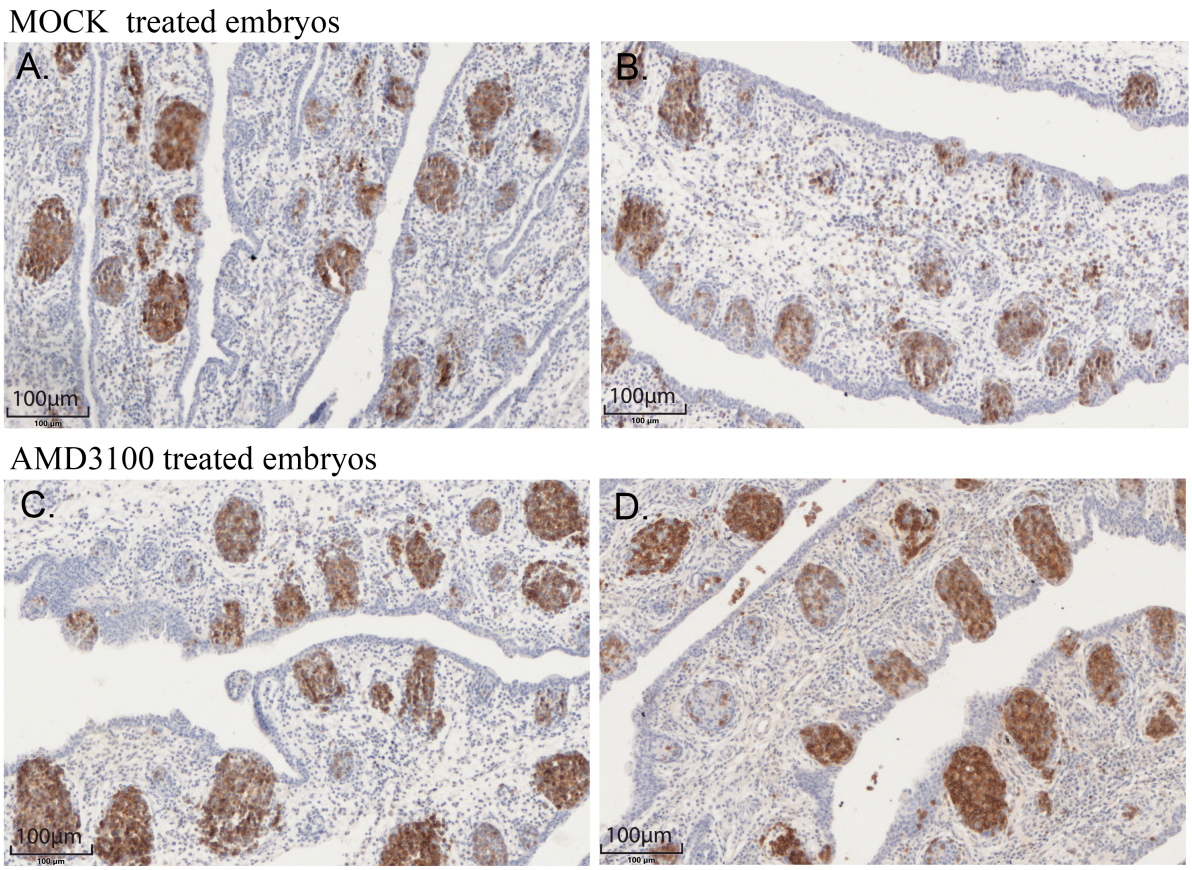


Fig. 19 B cell staining of the control and AMD3100 treated bursae, ED17

A. and B.: Bursae of control embryos stained with the B cell marker, AV20. C. and D.: B cell staining of bursae of embryos that were treated with AMD3100. There is clear formation of B cell follicles in all bursae. The distribution of the follicles and of B cells inside the follicles is comparable in all bursae. B cells colonized the bursa despite AMD3100 treatment and blocking of the CXCR4 receptor. Representative pictures out of at least three examined animals per group are shown.

The staining of the bursae with the B cell marker AV20 showed that the follicles in the bursae of the treated embryos were filled with B cells. The distribution of the follicles throughout the AMD3100 treated embryos' bursa is similar to the bursae of the control group. Furthermore, the composition of the follicles is indistinguishable between the groups. A small amount of B cells is also found in the connective tissue between the follicles in bursae of both groups.

In order to examine the assumption that the cell population found in the bursal connective tissue of the AMD3100 treated embryos might be granulocytes, a granulocyte staining was performed with the anti-granulocyte antibody GRL1 [193] (see Fig. 20).

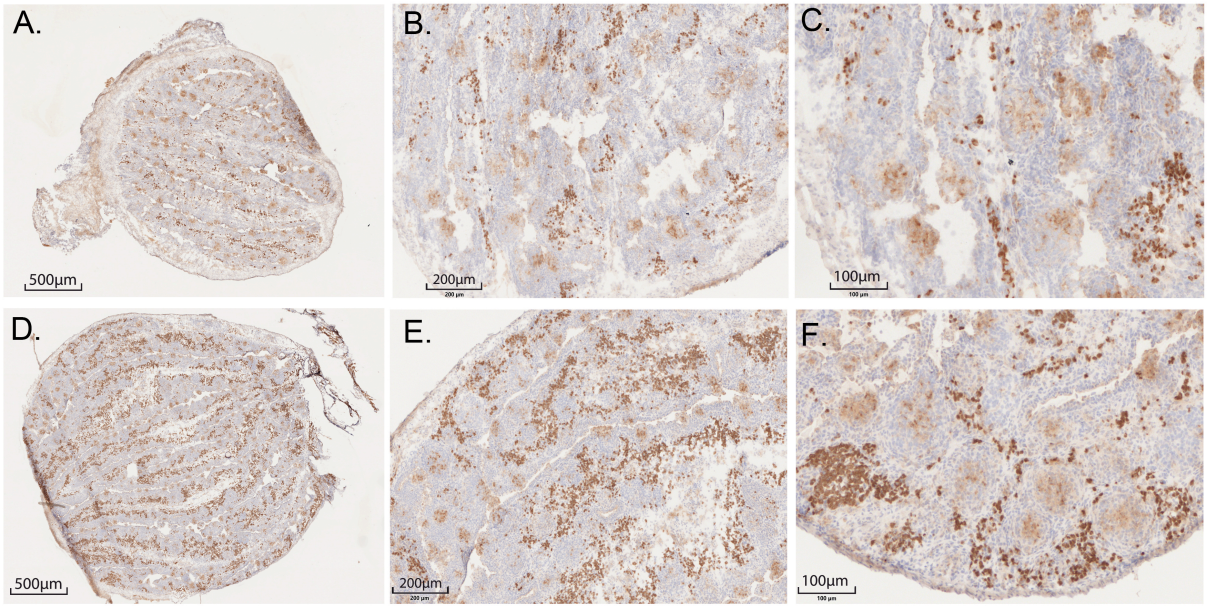


Fig. 20 Granulocyte staining of the control and the AMD3100 treated bursae, ED17

A.-C.: Granulocytes were found throughout the bursae of the control embryos but mainly in the connective tissue between follicles. D.-F.: Granulocytes were also detected in the bursae of the AMD3100 treated embryos. Immunohistochemistry shows that more granulocytes were present in bursae of treated embryos. Representative pictures out of at least three examined animals per group are shown.

The staining of the sections with the GRL1 antibody confirmed the assumption of having an increased number of granulocytes in the bursae of AMD3100 treated embryos. These granulocytes were found solely distributed in the bursal connective tissue but seemed to form mainly clusters. Granulocytes were also found in the control bursae either as single cells or in small clusters but in much lower numbers. In both groups granulocytes are primarily localized, as also seen in the H&E staining, in the connective tissue surrounding the B cell follicles.

In order to better evaluate the histological findings, FACS analysis was performed. Furthermore, a macroscopic examination of the embryos was performed.

5.4.1.2 Macroscopic analysis of bursae of embryos with or without AMD3100 treatment

Prior to staining and FACS analysis all embryos underwent macroscopic examination. The organs were inspected but no abnormal findings observed. Additionally, the embryos were weighed and the bursa and the spleen of the embryos were removed. Bursae and spleens were also weighed. The weights of the embryos were compared. Furthermore, the ratios of the bursal weight to the embryonic weight and the splenic weight to the embryonic weight were calculated and compared. The mean weight of the control embryos was 28.82gr, while the 480µg/kg AMD3100 treated embryos weighed on average 26.39gr. The 1000µg/kg

AMD3100 treated embryos weighed on average 27.04gr. The mean bursal to embryonic weight ratio of the control group was 0.0017, identical to the mean ratio of the 480μg/kg AMD3100 treated embryos. The mean bursal to embryonic weight ratio of the 1000μg/kg AMD3100 treated embryos was a bit lower with 0.0015 but this was not statistically significant. Finally, the ratio of the splenic to embryonic weight of the control group was 0.00038, of the 480μg/kg AMD3100 treated group 0.00041 and that of the 1000μg/kg AMD3100 treated group 0.00039.

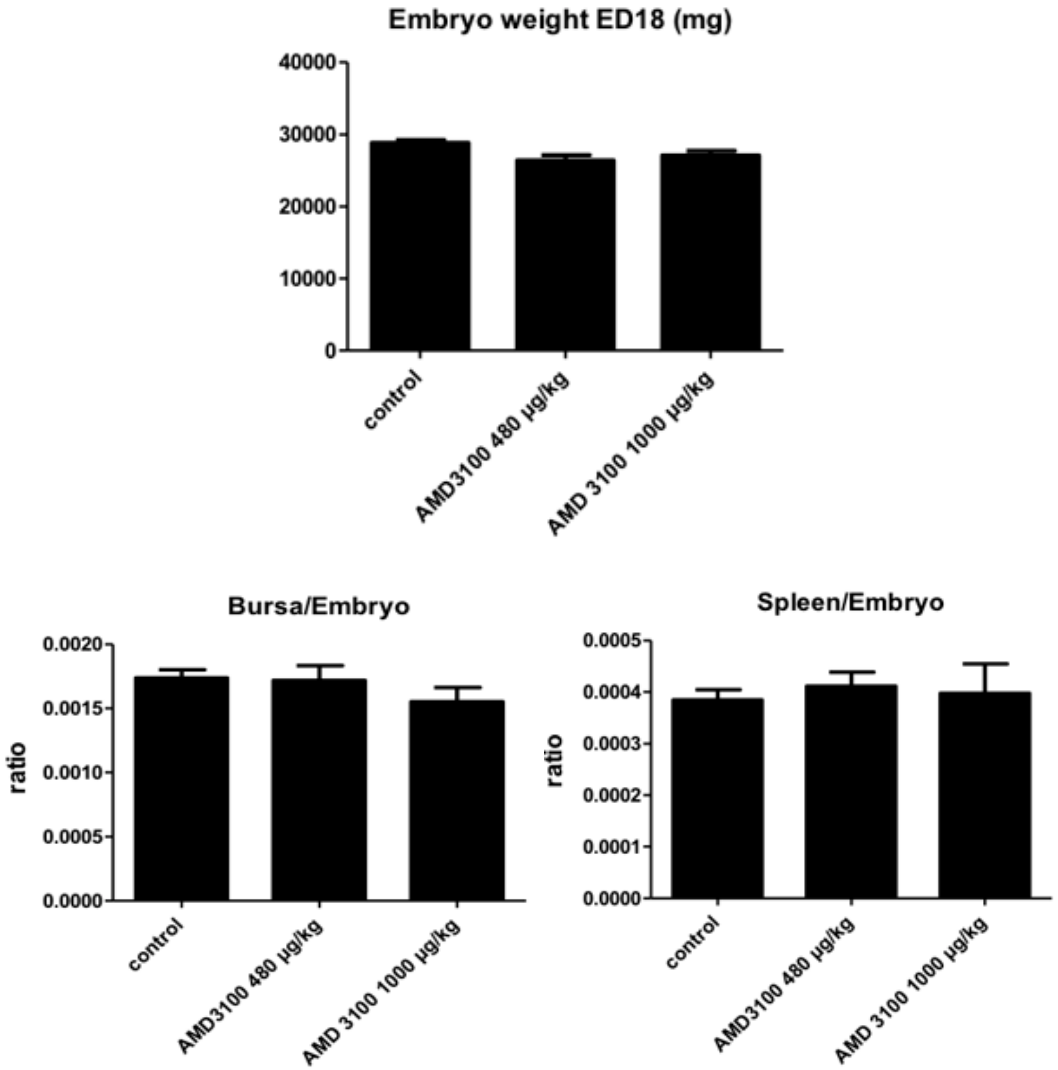


Fig. 21 Comparison of the weight of the embryos and the ratios of bursal and splenic weight to the embryonic weight.

The embryos and their organs -spleen and bursa- were weighed and compared. The ratio of the bursal and splenic to the embryonic weight were also evaluated in order to examine if blocking of the CXCR4 receptor by AMD3100 embryo treatment affects the growth of the embryos and their organs. No statistically significant difference was detected (normally distributed per Shapiro-Wilk and Kolmogorov-Smirnov tests, independent t-test, $p>0.05$).

The treatment of the embryos with the AMD3100 had no effect on the size of the embryo nor on the size of the organs and their proportion to the embryo's development (Fig. 21).

5.4.1.3 FACS analysis of the embryonic bursal cell populations with or without AMD3100 treatment

Embryos were treated again with AMD3100 (Tab. 18, experiment 3 and 4) on ED11, 12 and 14 with the highest dosage that was used in the second experiment (480µg/kg), but also with an even higher dosage of 1000µg/kg. The 1000µg/kg dosage was used in order to examine if with a higher dosage an effect on B cell migration would be visible. Bursae were prepared on ED18 and stained with the B cell marker, AV20.

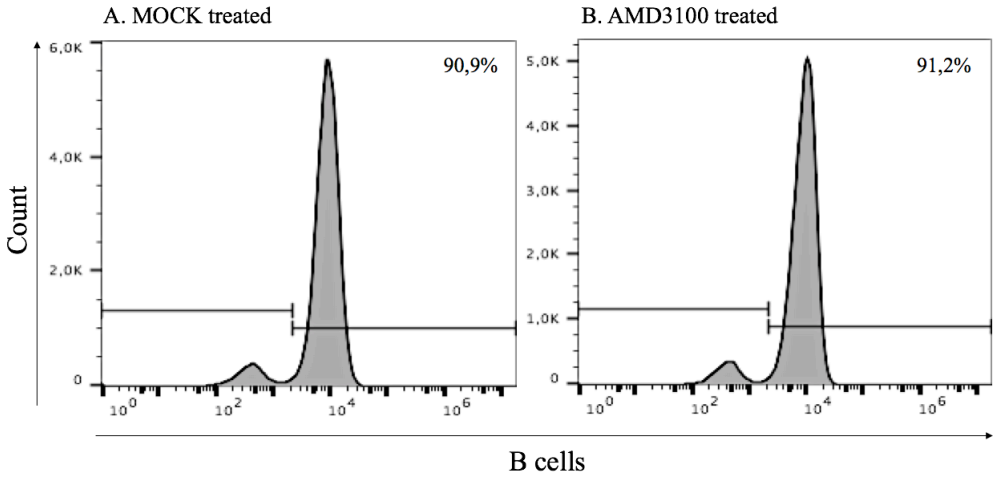


Fig. 22 Bursal B cells of control and AMD3100 treated embryos

Bursae of control (A.) and 1000µg/kg AMD3100 treated (B.) embryos were stained with the B cell marker, AV20. B cells were detected in both groups of bursae. Representative results out of at least three examined animals per group are shown.

AV20 positive cells were detected in the bursae of the embryos treated with the CXCR4 receptor antagonist in similar percentages as in the control bursae. The blocking of the CXCR4 receptor did not seem to inhibit the migration of the B cells into the bursa. To quantify these results bursae of embryos were treated with the highest dose of AMD3100 (1000µg/kg). Bursae of AMD3100 treated and mock treated embryos were prepared for quantitative FACS analysis.

5.4.1.4 Quantitative FACS analysis of the bursal cell population of embryos with or without AMD3100 treatment

Bursal cells were isolated as described in chapter 4.6.1 and stained with the B cell marker (AV20) and also with the granulocyte marker GRL1 (see intracellular staining in chapter 4.7.2). Furthermore, 123count eBeads were added to the probes for cell quantification (see

chapter 4.7.4). The absolute numbers of B cells and granulocytes were calculated and compared. The mean absolute number of bursal B cells was 297.7 cells/µl in the mock treated control group, while in the AMD3100 treated embryos was 638 cells/µl.

The mean absolute count of granulocytes in the treated bursae was 1759 cells/µl while in the control embryos the granulocytes detected were on average 1119 cells/µl. The difference between the two groups was not statistically significant for both cell populations. However, the increase in the granulocyte population seen in the FACS analysis did confirm the histological picture of higher numbers of cells mainly detected in the connective tissue and the impression of denser bursae of the treated embryos.

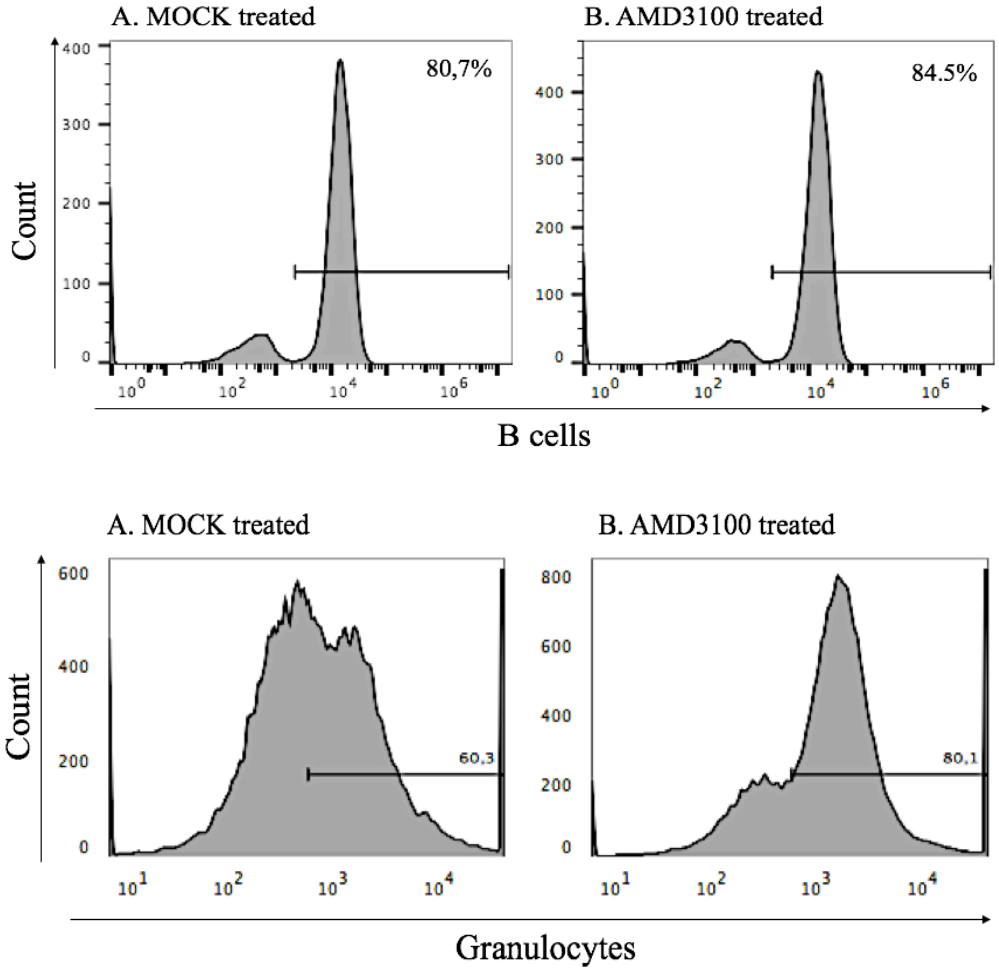


Fig. 23 FACS analysis of bursal B cells and granulocytes

Bursal B cells of control (A. top panel) and AMD3100 treated (B. top panel) embryos stained with the AV20 antibody. Bursal granulocytes of control (A. bottom panel) and AMD3100 treated (B. bottom panel) embryos stained with the GRL1 antibody. Representative results out of at least three examined animals per group are shown.

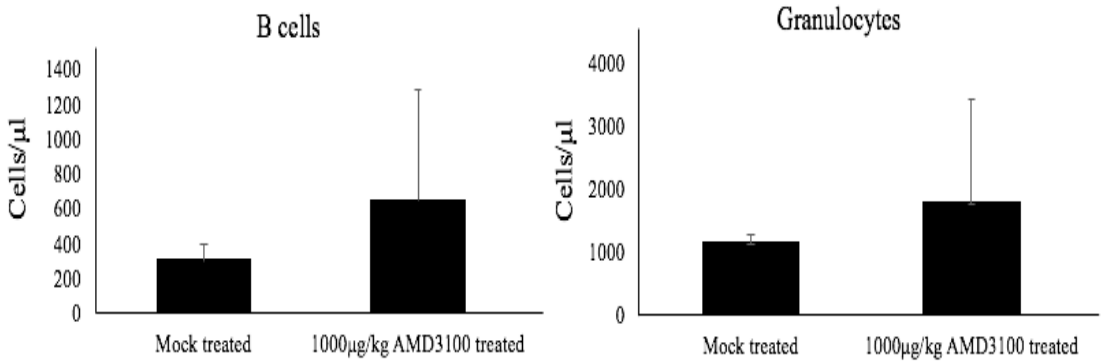


Fig. 24 Quantification and comparison of the bursal B cell and granulocyte populations of control and AMD3100 treated embryos

After quantification of the bursal cell population -B cells and granulocytes- B cells found in the bursae of the AMD3100 treated embryos were increased in comparison to the control embryos (on the left). Granulocytes showed also an increase in the treated embryos (on the right). Even though the results were not statistically significant they confirm the histological picture from previous experiments (n≥3, not normally distributed per Shapiro-Wilk and Kolmogorov-Smirnov tests, non-parametric test, p>0.05).

5.4.2 CXCL12 expression in the bursa

In order to investigate the possible reasons of higher B cell and granulocyte numbers in the bursae of the AMD3100 treated embryos of the experiments described in chapter 5.4, CXCL12 expression in the bursa was analysed by qRT-PCR. Therefore, the CXCL12 expression of the WT embryos was examined and then compared to that of the bursae of AMD3100 treated embryos. LSL embryos were set for incubation and treated *i.v.* on ED11, ED12 and ED14 as described in chapter 4.5.1, with 1000µg/kg AMD3100. AMD3100 was diluted in aqua dest. and as in all experiments, 50µl volume were injected in the embryos. As a control, embryos received on the same days 50µl of aqua dest. intravenously. On ED19 the bursae were prepared, RNA was isolated and cDNA was synthesized (see chapter 4.11 and 4.12) for qPCR analysis (described in chapter 4.13).

Furthermore, at the same time all probes were examined for the expression of the CXCL12 ligand CXCR4 but also for another chemokine axis, the CXCR5-CXCL13 axis. The results are shown in the following figure.

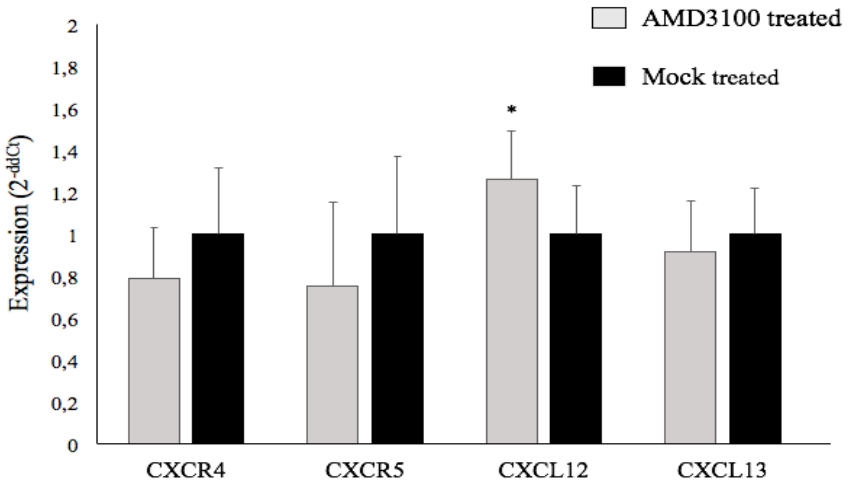


Fig. 25 Expression of CXCR4, CXCR5, CXCL12 and CXCL13 in the embryonic bursa of AMD3100 treated embryos in comparison to mock treated embryos, ED19

Embryos have been treated with AMD3100 on ED11, 12 and 14 while a second group of embryos were mock-treated. Bursae were prepared on ED19 and the expression of CXCR4, CXCR5, CXCL12 and CXCL13 was analysed per qRT-PCR. Expression of the genes is shown in comparison to the level of expression in the mock treated embryos (mock treated expression =1). $n \geq 3$, data normally distributed per Kolmogorov-Smirnov and Shapiro Wilk tests, significance examined per independent t-Test, $p \geq 0.05$ for CXCR4, CXCR5 and CXCL13, $p < 0.05$ for CXCL12.

The expression of the CXCR4 in the bursa of the AMD3100 treated was 0.2 fold decreased, while the expression of the CXCL12 chemokine was 0.2 fold increased. The receptor-chemokine pair CXCR5-CXCL13 showed a small decrease, both for the CXCR5 receptor expression as well as the expression of the CXCL13 chemokine in the AMD3100 treated embryos. The CXCR5 was 0.3 fold decreased, while the CXCL13 was in 0.15 fold decreased in comparison to the mock treated embryos.

5.5 ADOPTIVE CELL TRANSFER

Adoptive cell transfer is the transfer of cells from an organism in another or back to the same, after *ex vivo* treatment or transformation of the cells. For the purposes of this project, the method of adoptive cell transfer in the chicken embryo *in ovo* was established and performed in order to enable further *in vivo* studies on B cell migration and its regulation.

5.5.1 Establishment of the method of adoptive cell transfer in the chick embryo

The method of intravenous substance application in the embryo's own egg shell was used to inject cells *i.v. in ovo* (see chapter 4.3.2). Different amounts of cells were transferred in embryos to test which number of cells is well tolerated by the embryos. Cells between $1x10^6$

and $20x10^6$ were diluted in 50µl PBS and transferred to host embryos as shown in the following table. In seven host embryos $1x10^6$ cells were transferred per embryo and six of them survived (86% survival rate). When $1.5x10^6$ cells were transferred, the survival rate decreased to 57%. After transferring $2x10^6$ cells, the survival rate decreased to 40%. Cell transfer of $3x10^6$ cells led to 33% survival rate while after $5x10^6$ cell transfer the survival rate fell radically to 6%. Transfer of $10x10^6$ cells led to 25% survival rate. Finally, after transfer of $20x10^6$ cells per embryo none of the six host embryos survived.

Transferred cells	Embryos injected	Embryos survived	Survival rate
$1x10^6$	7	6	86%
$1.5x10^6$	38	22	57%
$2x10^6$	35	14	40%
$3x10^6$	9	3	33%
$5x10^6$	30	2	6%
$10x10^6$	4	1	25%
$20x10^6$	6	0	0%

Tab. 19 Survival rate of embryos in association to adoptive cell transfer of various amounts of cells

The more cells were transferred, the less embryos survived. The best tolerated cell amount was that of $1x10^6$ cells per embryo as shown in Fig. 26. Therefore, in all further experiments of adoptive cell transfer $1x10^6$ cells were transferred.

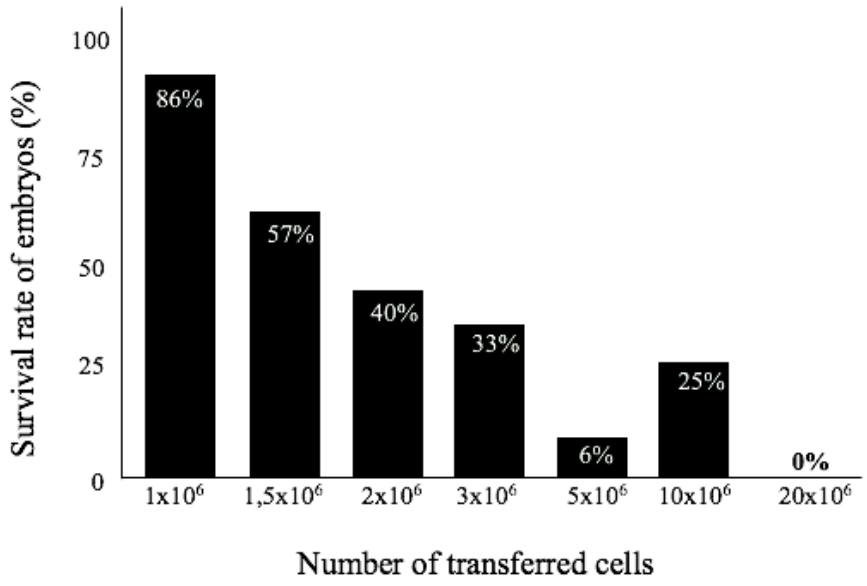


Fig. 26 Correlation of the number of transferred cells to embryos' survival rates

The survival rate of the embryos after adoptive transfer depends on the number of transferred cells. The best survival rate was seen by transfer of $1x10^6$ cells per embryo ($n \geq 3$).

5.5.2 Adoptive cell transfer experiments

Adoptive cell transfer experiments are listed in the following table Tab. 20 and detailed presented in this chapter. All adoptive cell transfer experiments were performed as described in 4.4 and either analysed by histology and/or FACS analysis.

Host	Donor	Transferred Cells	Day of transfer	Day of analysis
WT	GFP^+	Splenocytes	ED12	ED18
***JH*-KO$^{-/-}$**	WT	Splenocytes	ED14	ED19

Tab. 20 Adoptive cell transfer experiments overview

5.5.2.1 GFP^+ splenocytes in WT host embryos

The first experiments of adoptive cell transfer were carried out to examine whether it is possible to transfer cells from one embryo to another with the cells surviving the isolation and transfer while, most importantly, maintaining their capability to migrate. Therefore, GFP^+ were transferred in GFP^- host embryos. This way GFP^+ donor cells can be detected in the GFP^- host embryo. The analysis of the experiment was performed on ED18 by FACS. Bursae were examined for GFP^+ donor cells and were stained with the B cell marker AV20. In some of the GFP^- embryos, GFP^+ cells were detected. As shown in Fig. 27, part B., Q2 and Q3 population, a representative probe out of at least three examined animals per group, 0.74% of the cells consisted of GFP^+ cells. Most of them (0.65% out of the total 0.74%) were GFP^+ B cells. These GFP^+ cells can only originate from the donor embryos.

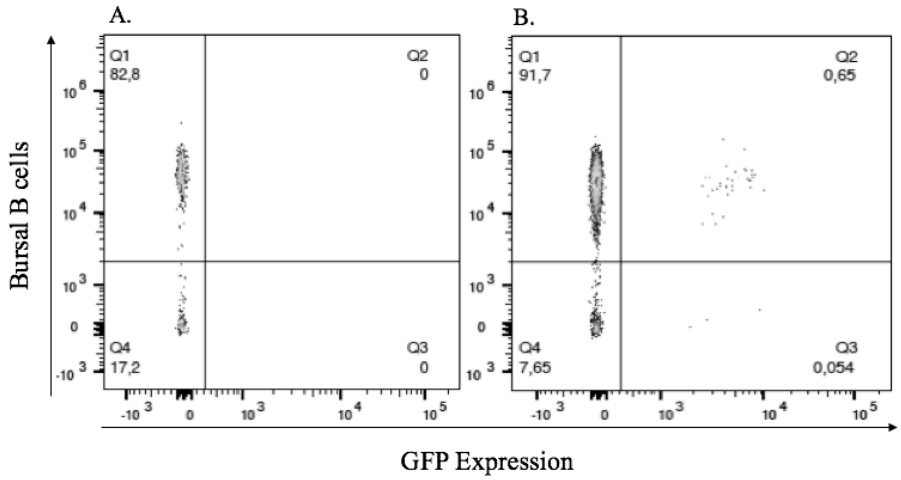


Fig. 27 FACS analysis of a control WT bursa and a WT bursa after adoptive transfer of GFP^+ cells

FACS analysis after adoptive transfer of GFP^+ cells into GFP^- embryos. Bursae of control embryos (A.) and the host embryos after the cell transfer were examined for GFP expression and stained with the anti-chB6 (Bu1)/anti-muIgG1-APC antibodies. In the host embryos GFP^+ cells were detected (cells in B., Q2 and Q3). Most of the GFP^+ cells were B cells (Q2). Representative results out of at least three examined animals per group.

In order to examine which, type of cells represented the GFP^+ cells $AV20^-$ population (Fig. 27, Q3 population in B.), two further stainings were performed, for αβ- and γδ-T cells, with the TCRαβ1 and TCRαβ2 marker and TCRγδ marker respectively. The small population of GFP^+ cells that were not B cells were not T cells either. In the control embryos around 0.52% of the cells were αβT cells and only 0.04% γδT cells. In the embryos that received donor splenocytes 0.27% cells were αβT cells and 0.09% γδT cells. However, none of the T cells expressed GFP^+ in the host embryos. The GFP^+ cells in the host embryos made 0.5% of the cell population but none of these cells were T cells (Fig. 28).

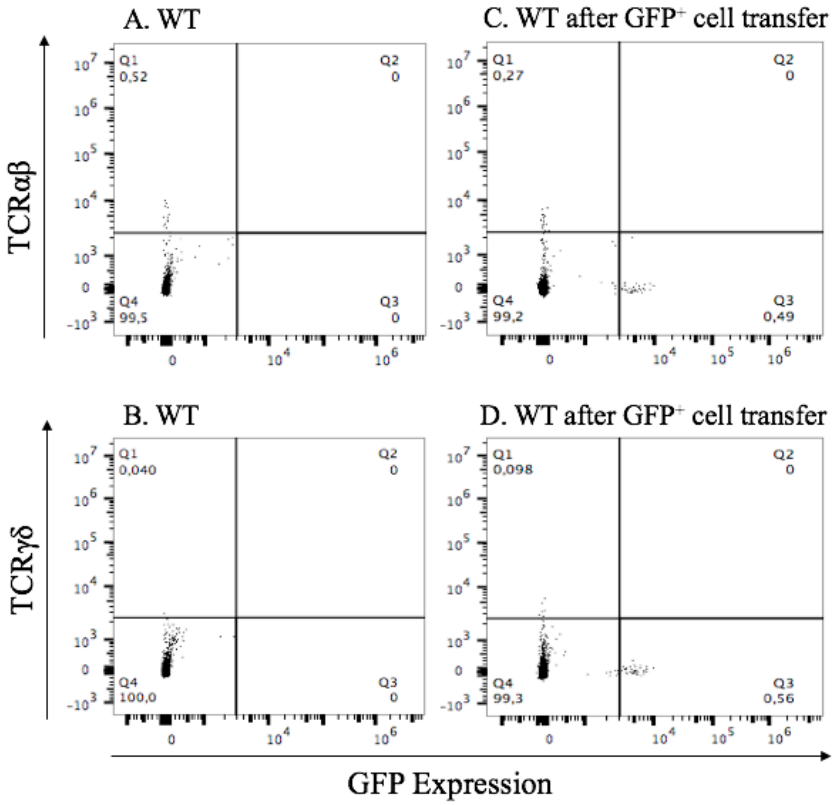


Fig. 28 TCRαβ, TCRγδ and GFP expression in the bursa after transfer of GFP^+ cells into WT embryos

Bursae were stained with the T cell markers (for both TCRαβ and TCRγδ). A: In the control bursa (left panels) very few cells were αβ T cells and even less cells were γδT cells. B: In the embryos which received donor cells (right panels) very few cells were αβT cells but none of them originated from the transferred GFP^+ splenocytes. Representative results out of at least three examined animals per group are shown.

5.5.2.2 WT splenocytes in *JH*-$KO^{-/-}$ host embryos

Furthermore, splenocytes isolated from WT (LSL) donor embryos were transferred into *JH*-$KO^{-/-}$ embryos.

B cells, as mentioned before, colonize the bursa of *JH*-$KO^{-/-}$ embryos and even organize themselves in follicles, but do not express the B cell receptor on their surface [125]. B cells of

the *JH*-KO$^{-/-}$ bursa can be detected with the AV20 antibody but since they do not express IgM on their surface, there should be no detection of IgM when staining with the anti-chIgM antibody. This was confirmed after fluorescence histology with the anti-chIgM/anti-muIgG-AlexaFluor 568 on cryosections of bursae of all three genotypes (WT, *JH*-KO$^{+/-}$ and *JH*-KO$^{-/-}$). Cells in the bursal follicles of WT and *JH*-KO$^{+/-}$ embryos express the IgM surface immunoglobulin, while in the *JH*-KO$^{-/-}$ bursal tissue there is no IgM detection (Fig. 29).

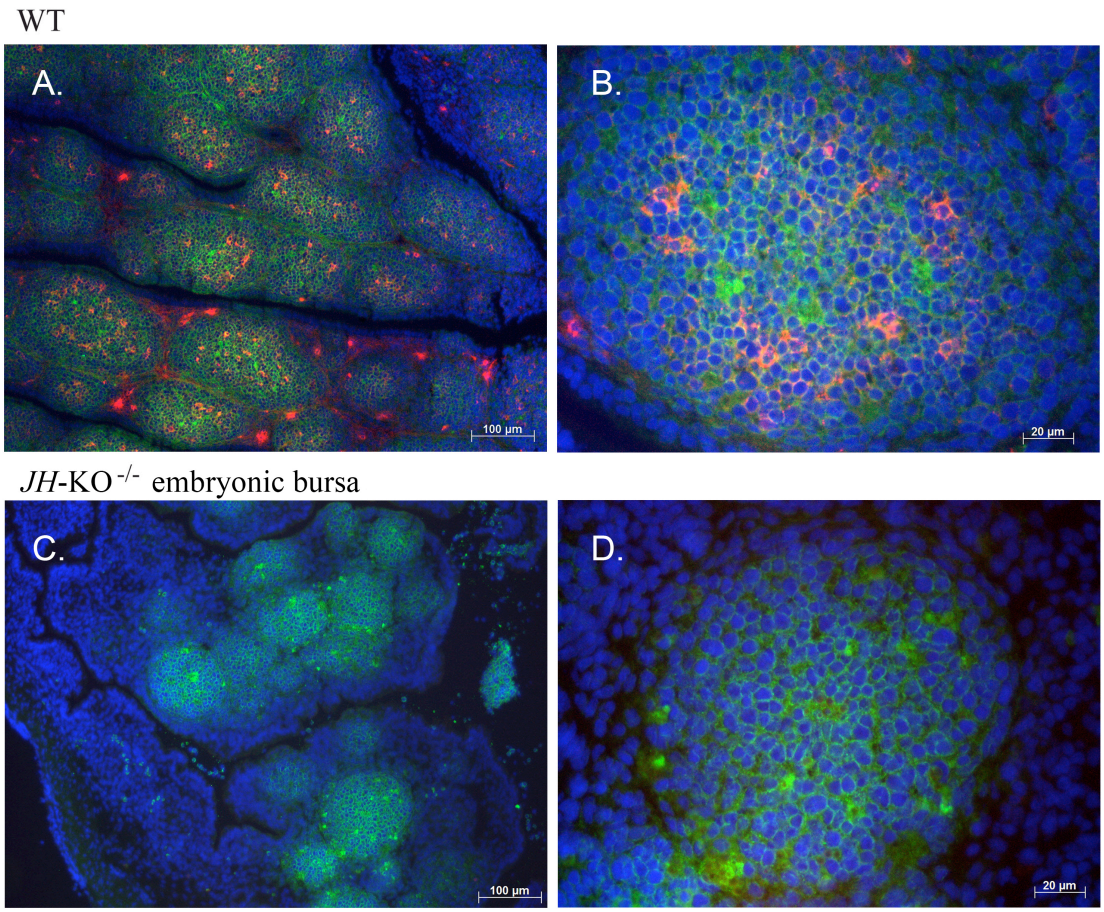


Fig. 29 Double staining with the B cell marker AV20 and with the IgM marker, embryonic bursa

Nucleus (in blue), B cells (in green) and IgM (in red) staining of a WT (A. and B.) and a *JH*-KO$^{-/-}$ (C. and D.) bursa. WT embryo's bursa in x100 (A.) and in x400 (B.) magnification. Bursa of *JH*-KO$^{-/-}$ embryo x100 (C.) and in x400 (D.) magnification. Representative results out of at least three examined animals per group are shown.

First of all, the embryos had to be genotyped in order to identify the *JH*-KO$^{-/-}$ embryos. The genotyping of the embryos was performed as described in chapter 4.9.1, after blood sampling (described in chapter 4.3.3) and extraction of gDNA (see chapter 4.8).

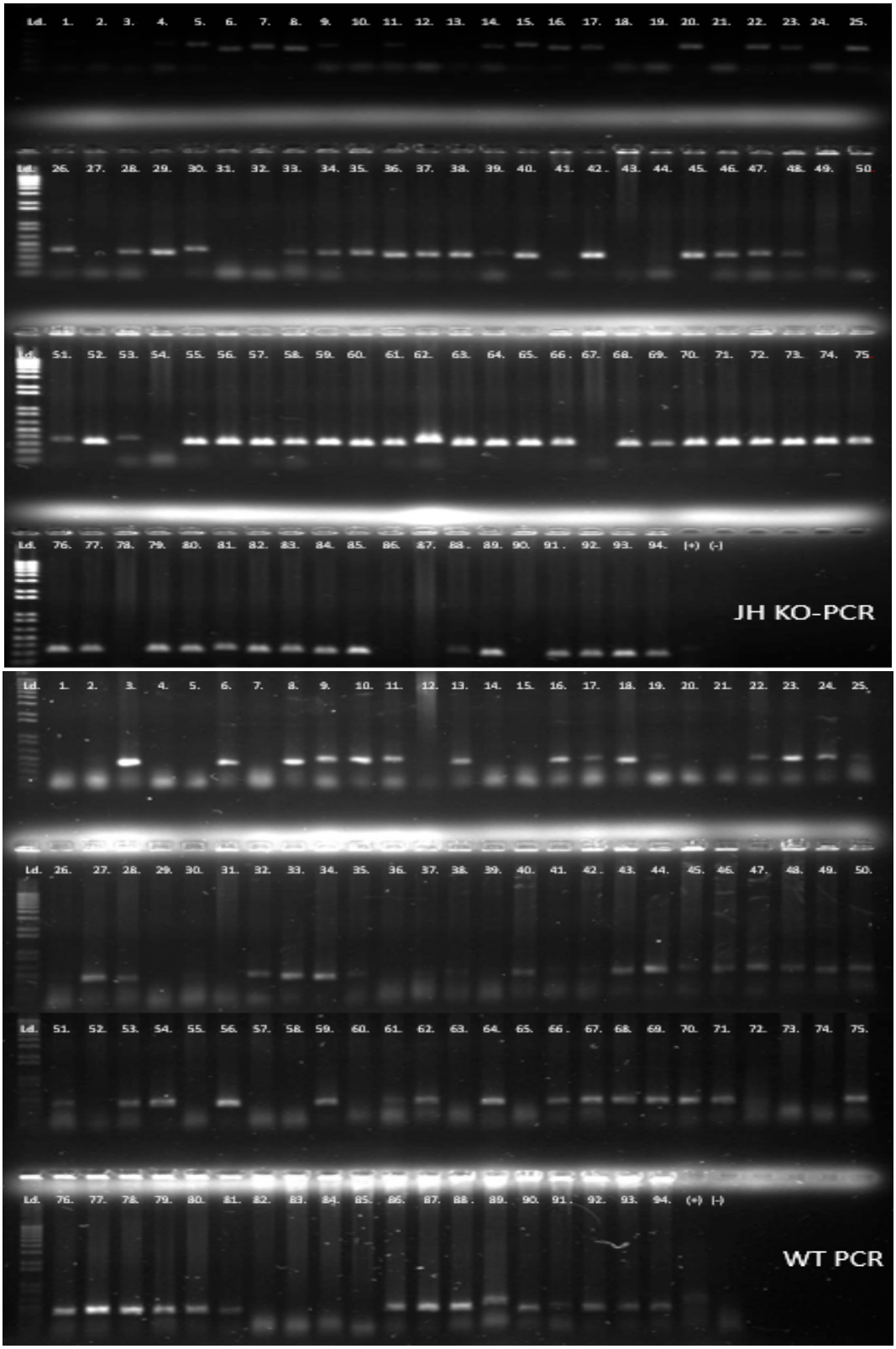


Fig. 30 Genotyping of embryos to identify the *JH*-KO$^{-/-}$ embryos

To identify the *JH*-KO$^{-/-}$ embryos, two PCRs were run, the WT PCR and the *JH* PCR. The PCRs were evaluated and embryos with a band in the WT PCR but none in the *JH* PCR were accounted as WT embryos. Embryos with a band both in the WT PCR and *JH* PCR were considered to be *JH*-KO$^{+/-}$. Finally, embryos with a band only in the *JH* PCR were accounted as *JH*-KO$^{-/-}$. The positive and negative control probes are marked as (+) and (-) respectively, at the end of each PCR.

To identify the *JH*-KO$^{-/-}$ embryos, two PCRs were run, the WT PCR and the *JH* PCR. The WT PCR amplifies a part of a region which has been deleted in the *JH*-KO$^{-/-}$ line. Therefore, a band in the WT PCR can only be detected in the WT and *JH*-KO$^{+/-}$ embryos, while in the WT PCR of homozygous KO birds there is no band detected, since the target sequence for amplification is deleted. The PCRs were evaluated and the embryos that had a band in the WT PCR but none in the *JH* PCR were accounted as WT embryos (i.e. embryo Nr. 67). The embryos that had a band both in the WT PCR and the *JH* PCR were considered to be *JH*-KO$^{+/-}$ (like embryo Nr. 56). Finally, embryos that had a band only in the *JH* PCR were accounted as *JH*-KO$^{-/-}$ (like embryo Nr. 52).

After genotyping the embryos, WT embryos were sorted out and used as splenocyte donors. WT splenocytes were transferred in the *JH*-KO$^{-/-}$ embryos (see chapter 4.4). On ED19 the bursae of the embryos were collected. Cryosections were prepared (see chapter 4.14) and stained with the mouse-anti-chIgM followed by anti-mouse IgG-AlexaFluor 568 (in red in Fig. 31) antibodies. The nucleus of the cells was counterstained with DAPI (in blue).

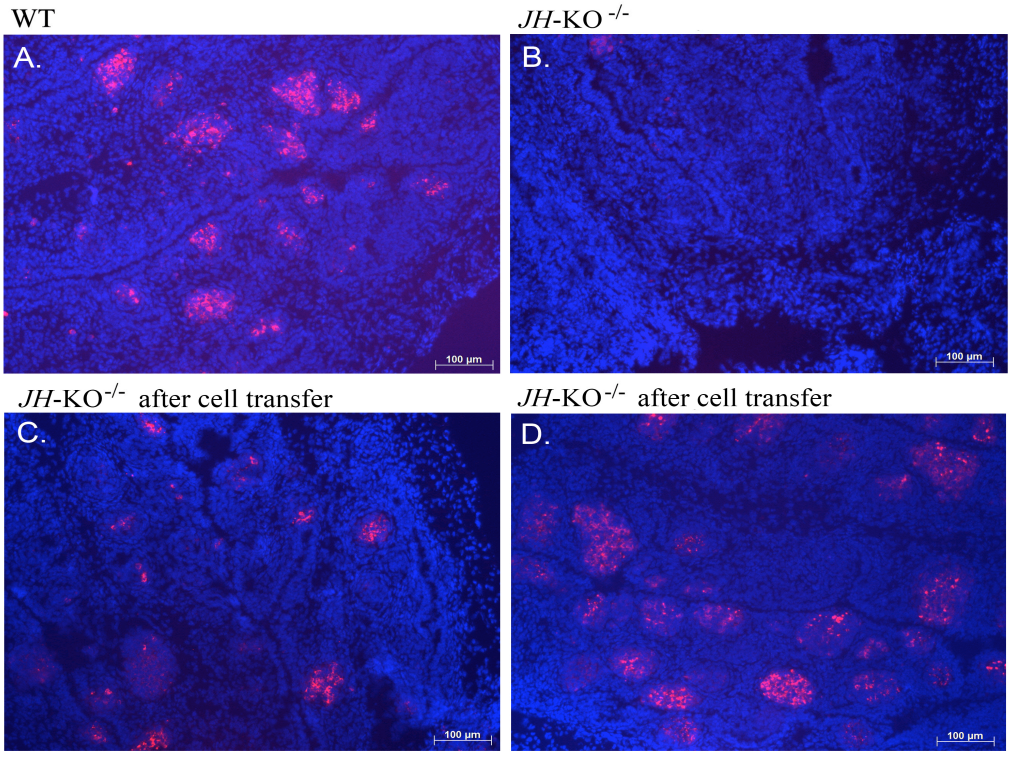


Fig. 31 Bursal B cell staining of WT, *JH*-KO$^{-/-}$ and *JH*-KO$^{-/-}$ embryos after cell transfer

Embryos were stained with the anti-chIgM1/AlexaFluor 568 (in red) and nucleus staining with DAPI (in blue). As expected, the sIgM was detected in the WT bursa (A.) while in the *JH*-KO$^{-/-}$ bursa (B.) there is no staining to see. However, in the bursa of two *JH*-KO$^{-/-}$ embryos in which WT splenocytes were transferred (C. and D.), there are definitely IgM^{+} cells to see. Representative results out of at least three examined animals per group are shown.

IgM^{+} follicles were detected in the bursae of embryos into which WT splenocytes were transferred. IgM^{+} positive cells were detected mostly in the bursal follicles. Once again it was

shown that transferred cells maintain their ability to move towards the bursa, colonize the target organ and form follicles. Some of the bursae of this experiment were used for FACS analysis and were stained with the anti-chIgM antibody. GFP expression was also tested in the bursae as shown in the following figure (Fig. 32).

In a WT embryo around 25% of the bursal B cells expressed IgM while in the *JH*-KO$^{-/-}$ embryo there was no IgM expression at all. However, about 2% of the total bursal B cell population of the *JH*-KO$^{-/-}$ embryos, into which WT splenocytes were transferred, were found to be IgM^{+} and GFP^{-} at the same time proving their origin from the donor embryo.

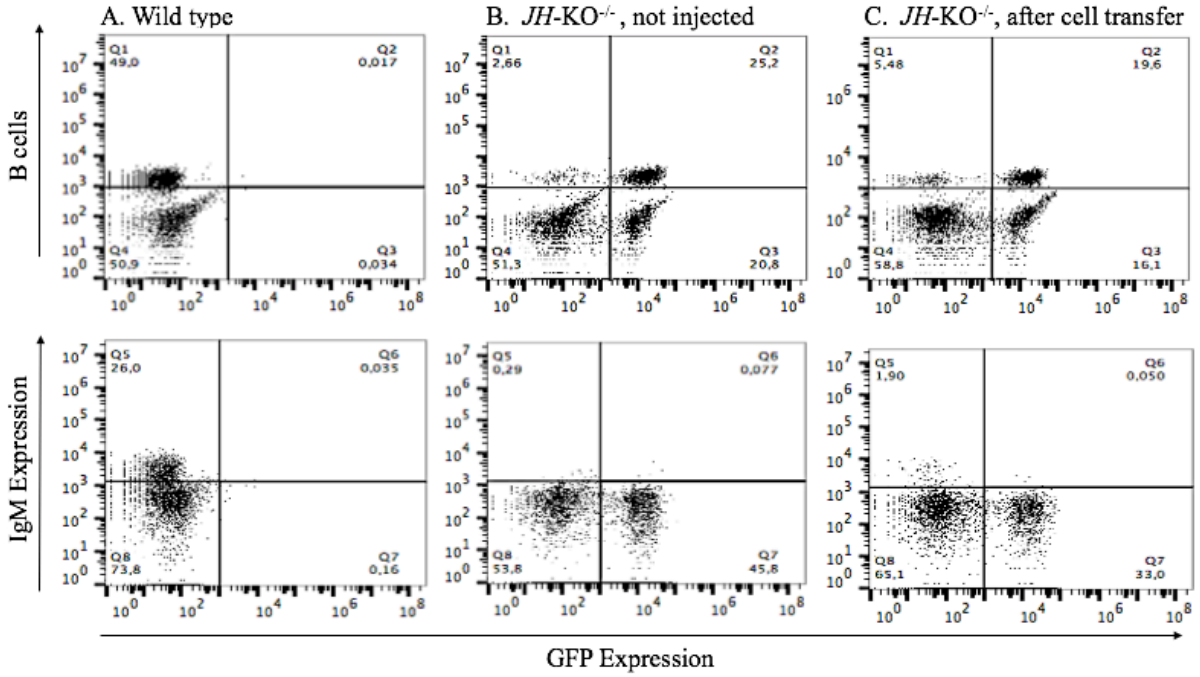


Fig. 32 IgM and GFP expression by bursal B cells in WT, *JH*-KO$^{-/-}$ and in *JH*-KO$^{-/-}$ embryos after WT cell transfer

Top panel: Bursal cells were stained with AV20. At the same time GFP expression was also examined (top panel). After gating B cells, the cells' IgM expression was examined. IgM^{+} cells were detected in WT embryos with the anti-chIgM Ab at 25% of the total B cells. All cells are GFP^{-}. In the bursae of *JH*-KO$^{-/-}$ embryos (bottom panel, middle fig.) none of the GFP^{+} were IgM^{+}, AV20^{+} cells. Finally, in the *JH*-KO$^{-/-}$ embryos in which cells were WT splenocytes were transferred, about 2% of the gated B cells were IgM^{+} cells. The cells expressing IgM on their surface were GFP^{-}, confirming their origin from the WT donor embryos. Representative results out of at least three examined animals per group are shown.

5.5.3 Inhibition of the migration of transferred B cells in the host embryo by cell AMD3100 treatment prior to transfer

Adoptive cell transfers were planned to investigate if AMD3100 treatment of cells prior to their transfer into host embryos, would maintain their ability to migrate into the bursa or if the blocking of their CXCR4 receptor would disable their migration into the bursa. For that

approach, two adoptive cell transfer experiments were planned. The first experiment was the transfer of WT splenocytes into *JH*-KO$^{-/-}$ embryos and the second was the transfer of GFP^{+} cells into WT embryos. Both experiments are presented in detail in this chapter.

5.5.3.1 WT cells transferred in *JH*-KO$^{-/-}$ embryos after AMD3100 treatment

WT embryos were used as donors and *JH*-KO$^{-/-}$ as host embryos. On ED11 blood was taken from embryos of *JH*-KO$^{+/-}$ to *JH*-KO$^{+/-}$ chicken breeding and gDNA was extracted to further genotype the embryos (see chapter 4.9.1).

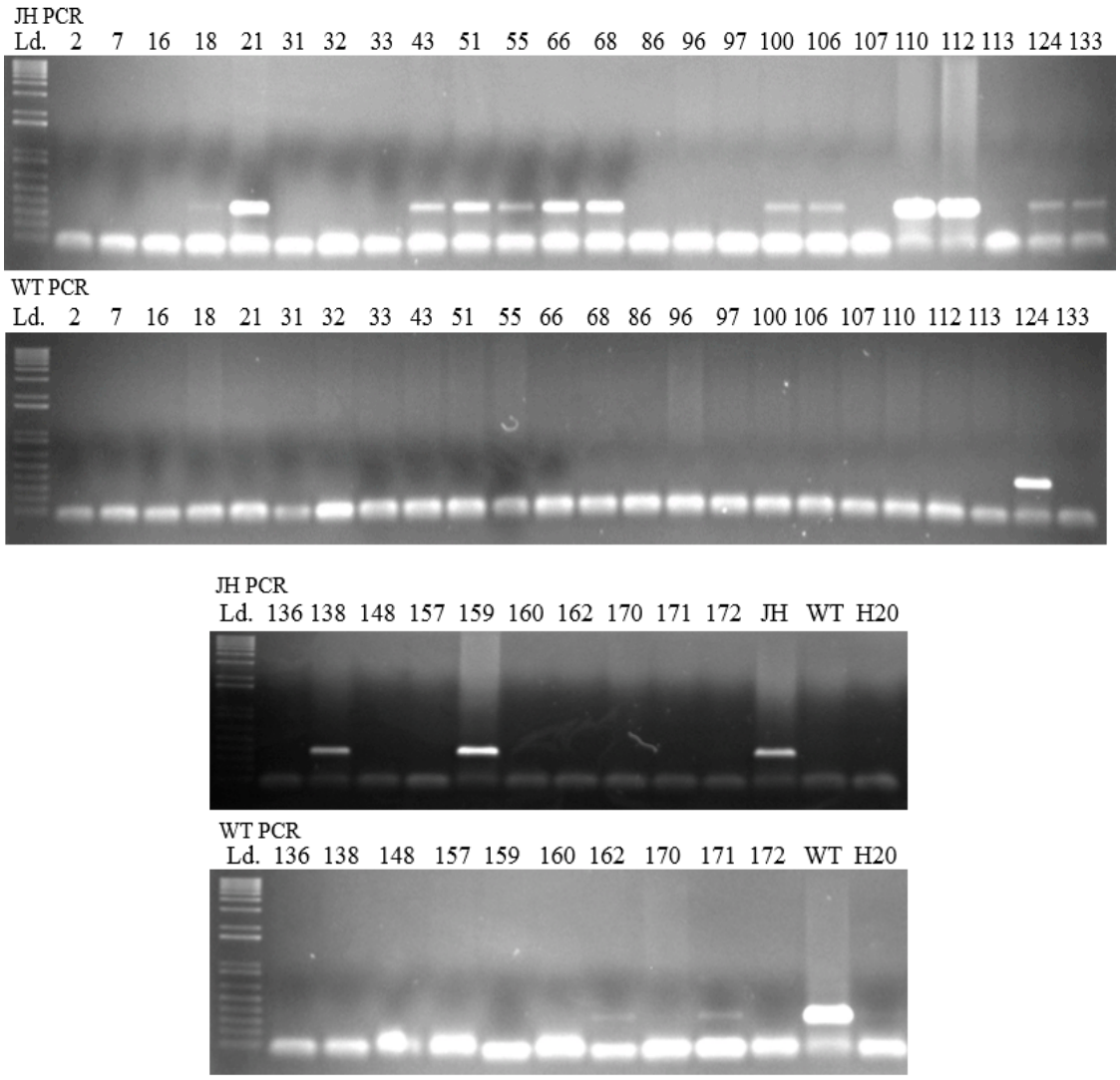


Fig. 33 Genotyping of embryos stemming from *JH*-KO$^{+/-}$ to *JH*-KO$^{+/-}$ breeding

As described before, to genotype the *JH*-KO$^{-/-}$ embryos, two PCRs have to be run, one WT and one *JH*-PCR. The PCR gels seen here include some of the probes that were tested. The *JH* and WT probes in the *JH* PCR are the positive and negative control for the *JH* PCR respectively while for the WT PCR, for the positive control a WT DNA probe was used. As a negative control *JH*-KO bird DNA was used.

Splenocytes from LSL donor embryos were isolated on ED14 and transferred into the homozygous KO embryos. The *JH*-KO$^{-/-}$ host embryos were separated in two groups, one

control group, in which the cells were transferred with the standard procedure (see chapter 4.4) and one group in which the cells were transferred after they were treated with AMD3100 to block the CXCR4 receptor on the cells' surface.

On ED19 bursae were prepared and then stained with the anti-chIgM antibody for FACS analysis.

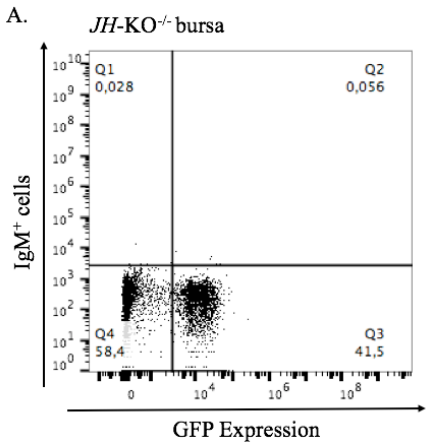


Fig. 34 IgM expression in a *JH*-KO$^{-/-}$ embryo on ED19

FACS analysis of a *JH*-KO$^{-/-}$ embryo for IgM expression on ED19 (A.)

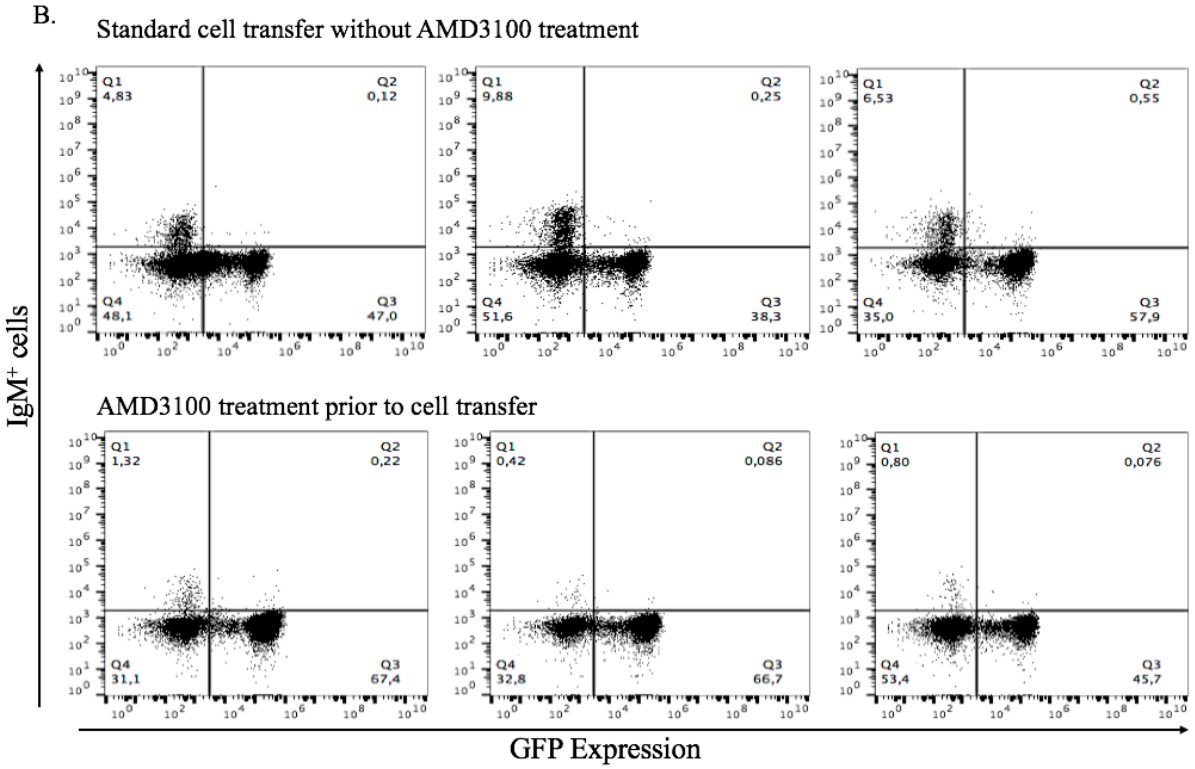


Fig. 35 IgM and GFP expression by bursal cells after transfer of WT splenocytes into *JH*-KO$^{-/-}$ embryos

Bursae of the embryos were tested for IgM$^+$, GFP$^-$ cells after WT cell transfer into *JH*-KO$^{-/-}$ embryos on ED19. In the bursae of *JH*-KO$^{-/-}$ embryos in which WT splenocytes were injected (B., top panel), up to 17% IgM$^+$, GFP$^-$ cells (B., top panel, population in Q1) were detected, while this cell population was limited to 1% in the embryos in which WT cells were injected after the cells were treated with AMD3100 (B., bottom panel). In contrast, in the *JH*-KO$^{-/-}$ the bursal cell consists of both GFP$^-$ and GFP$^+$ cells but none of the cells express the IgM (Fig. 34) Representative results out of at least three examined animals per group are shown.

By absolute counting the IgM^+/GFP^- cells were estimated per bursa and statistical analysis was performed.

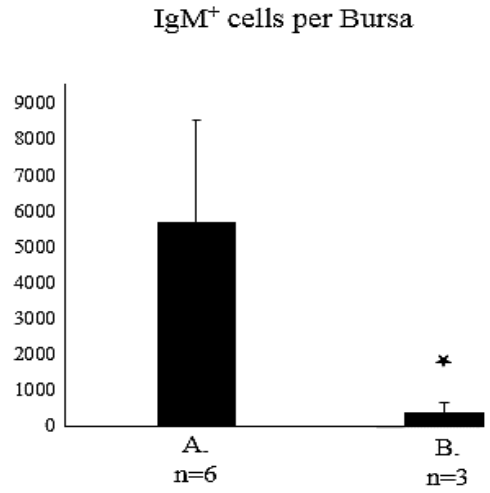


Fig. 36 Detection of WT, IgM^+ cells in the *JH*-$KO^{-/-}$ embryos after adoptive cell transfer

After detection of WT IgM^+ cells in the bursae of the *JH*-$KO^{-/-}$ embryos per FACS analysis and absolute counting, the amount of the transferred cells detected in the bursa were compared. In the bursae of the embryos, in which AMD3100 treated cells were transferred (B.) were significantly less IgM^+ cells detected than in the bursae of the embryos in which splenocytes were transferred without AMD3100 treatment (A.) ($n \geq 3$, normally distributed per Shapiro-Wilk and Kolmogorov-Smirnov tests, independent T-test, $p<0.05$).

In the embryos, in which adoptive cell transfer took place without prior AMD3100 treatment, the mean number of IgM^+ cells detected in the bursa was 5692 donor IgM^+ cells while in the embryos which received cells that were treated with AMD3100, only 378 cells made it on average to the host's bursa, which means that the number of transferred cells migrating to the bursa was reduced radically.

6. DISCUSSION

In the developmental process of lymphocytes, the migration of the cells between organs is essential. Specifically, as described in chapter 2.3, the rearrangement of the heavy and light chain immunoglobulin loci takes place independently from the bursal microenvironment. However, for further diversification, proliferation and development of the cells the bursal microenvironment is essential and therefore, B cell precursors need to migrate into the bursa. This project focused on the migration of the B cell precursors from the spleen into the bursa, in order to better understand the mechanisms and signals driving the migration of the cells.

6.1 EARLY INTRAVENOUS MANIPULATION OF THE DEVELOPING CHICKEN EMBRYO

Intravenous manipulation of the chick embryo has been performed by different groups in the past [175, 194]. Although blood sampling or intravenous injections have been commonly performed, the requirements of the current study underlined the need for a method which enables the intravenous manipulation of the chicken embryo for *i.v.* drug administration, blood sampling but also for adoptive transfer of cells into the embryos, without compromising their viability. Most importantly, the method should enable multiple intravenous manipulations in single embryos, which has not been described previously. Thus, elements of different methods have been examined and adapted to the demands of this project. A method by which the requirements of the study are fulfilled was successfully established.

Two different approaches have been used to approach the chicken embryo for intravenous manipulations (described in chapter 4.3.1 and 4.3.2). Both approaches were investigated and evaluated in their efficiency. Manipulation of the embryo directly in its own egg shell was proved to be more efficient for different reasons. The intravenous injection after transfer of the embryo into a surrogate egg shell is connected to lower time efficiency and higher mortality rates. It demands double as much time as needed for injections directly into the embryo, in its own egg shell, since several steps are involved. On ED3 embryos need to be transferred into the surrogate egg shell, followed by the intravenous manipulation, starting from ED11. In contrast, direct intravenous manipulation of the embryo in its own egg shell requires just one day of manipulation. However, the most important disadvantage of the method is the lower survival rate of the embryos (see chapter 5.1.1 and 5.1.2, Tab. 15 and Tab. 16). The lower survival rates are explained by the fact that the embryos need to be

transferred twice (transfer from own egg shell to the weight boat and from the weight boat into the surrogate egg shell) before they even reach the day of intravenous manipulation. The embryos on the day of transfer (ED3) are in their very early developmental stages [184] and therefore, very vulnerable. The finest contact might damage them. Consequently, some embryos do not survive the transfers. The number of these embryos add up to the embryos that do not survive the manipulation and thereby, the survival rate of this method is lower.

On the other hand, the direct approach in the embryo's own egg shell, beside the better survival rate, offers the advantage of being handier to perform. Due to higher stability of the vessels to be injected since they are attached at the inner shell membrane, but also due to the more superficial localization of the vessels the approach to the vessel is easier. Moreover, the costs for direct intravenous manipulation of the embryos in their own egg shell are much lower. For the method of manipulation after transfer into surrogate egg shells, extra material is required such as the turkey eggs, weight boats, plastic wrap and penicillin/streptomycin. For the method of direct intravenous injection in the embryos own egg shell none of this material is needed.

Due to the advantages of the intravenous manipulation of the embryo in its own egg shell, all further experiments were performed by the direct manipulation in this way.

For the intravenous application of substances into the embryo, experiments were performed to determine the appropriate volume of *i.v.* administration (see chapter 5.1.3, Tab. 16). In most studies *i.v.* administration of substances has been performed with a volume of 100µl [175, 176] on ED11, but none of the studies described survival rates of the embryos. In the current study, embryos that were injected with 50µl have consistently shown better survival rates than embryos of the same age that were injected with 100µl (see 5.1.3, Tab. 16). Thus, for all experiments application of substances was limited to the volume of 50µl. Furthermore, it was shown that the older the embryos are, the better they tolerate the injection (see 5.1.3, Tab. 16).

As mentioned previously, it was of great significance to standardize the intravenous manipulation in order to enable experiments which require multiple manipulations of the embryo. Prior studies for blood analysis have been performed, but no data are presented neither on the method and the volume of the blood samples nor on the efficiency of the method and the survival rates of the embryos [195]. It is assumed that blood samplings have been terminating experiments for the embryos. In the current study, in order to perform most of the planned experiments, it was essential that the embryos survive after blood sampling. In the experiments presented in chapters 5.5.2 and 5.5.3, blood sampling was used to genotype

the embryos prior to adoptive transfer. A volume of 10µl is sufficient to perform genotyping after gDNA extraction. Therefore, the survival rates of the embryos were examined after blood sampling of this volume on ED11 (see chapter 5.2). It was shown that the restriction to 10 to 20µl, minimally affects the survival of the embryos. Preliminary studies have shown, that blood sampling of the same volume on ED10 led to much worse survival rates due to strong bleedings after blood sampling.

On the other hand, for the analysis of the B lymphocyte migration pattern, higher blood sample volumes were required. Therefore, efforts were made to retrieve as much blood from a single embryo as possible in order to minimize the number of embryos sacrificed for these experiments. Thereby, the highest blood sample volumes that can be collected from a single embryo were defined. In contrast to blood sampling for genotyping, in this experiment further survival of the embryos was impossible. The size of the blood samples, highly depends on the age of the embryo. The older the embryos are, the more blood can be collected (see chapter 5.24.3.3, Tab. 17). The size and the stability of the vessels increase until around ED16 and therefore it is easier to perform blood sampling in older embryos. To conclude, for blood sampling it is important to define the purpose of the procedure and to determine if blood sampling is terminal or not. When the survival of the embryos is required, the blood sampling volume should be restricted to the minimum.

The method of adoptive cell transfer is widely used for various purposes since many years. Some examples of the method's application are gene-, stem cell- and immunotherapy. *In vivo* treatment of cells is sometimes not possible or might be critical for the organism. A substance might be deactivated or excreted before reaching its target. Besides, substances may have a different effect *in vivo* than *in vitro*, such as cytotoxicity for other cells. On the other hand, cells can be treated directly *ex vivo* and then adoptively transferred into the host carrying new properties, without risking the safety of the host organism. For these reasons the method of adoptive cell transfer is an important tool in both research and therapy applications.

In the chicken, similar approaches have been used before. For example, diverse types of cells have been transferred to the chorioallantoic membrane of the embryo [170]. In other studies, the method has been used to transfer metastatic tumor cells [175]. For the transfer of poor or non-metastatic tumor cells 1 to 5 million cells per embryo and for highly metastatic cells 5 thousand cells per embryo have been transferred *i.v.* on ED11 [175]. Survival rates are not presented in this study. In another study, adoptive transfer was used to transfer bursal cells into four days old age-matched embryos [177]. The embryos received *i.v.* 1.25 to 20 million cells. In the same study, splenocytes of 32 days old donors were transferred into 4 days old

host embryos. In this case, 12.5 to 50 million cells were transferred *i.v.* per embryo [177]. Finally, in another study, adoptive transfer was performed to transfer spleen or bone marrow cells into ED14 embryos. In this case $2.5x10^5$ to $2x10^6$ were adoptively transferred *i.v.* into ED14 embryos (suspension of 150µl medium) [148]. As noticed, there is high variance in the number of transferred cells between the studies and both studies do not present data on the efficiency of the method and the survival rates of the embryos. In order to be able to perform adoptive transfer experiments and to properly evaluate the results, it was important to optimize and standardize the method.

In the present study different number of cells were transferred from donor embryos to age-matched host embryos as described in 5.5.1. The survival of the host embryos clearly depends on the number of cells transferred (chapter 5.5.1, Fig. 26). In order to determine the optimal number of cells per transfer, 0,5 to 20 million cells per embryo were transferred *i.v.*. The more cells were transferred, the less host embryos survived the procedure. When 20 million cells were transferred, none of the embryos survived and already on the next day the embryos deceased. A possible explanation, is that the more cells are transferred, the more likely it is that cells build clots which compromise the survival of the embryo. Transfer of one to 1.5 million cells was very well tolerated by the embryos. Furthermore, in contrast to the study of Wilson *et al*. in which cells were kept up to one hour at 4°C before transfer [175], through the performance of several experiments, the experience was gained that cells need to be prepared and transferred quickly. New cells had to be prepared every fourth to fifth embryo to avoid longer standing of the cells prior to injection. It was concluded that for the best survival rates of the host embryos of adoptive cell transfer experiments one to 1,5 million cells should be injected per embryo and only shortly after preparation of the cells. Cells should be diluted in a volume of 50µl of the appropriate medium (i.e. PBS).

Adoptive transfers were performed according to the guideline above: transfer of splenocytes from GFP^+ embryos into WT embryos (see 4.4 and 5.5.2), and the transfer of WT splenocytes into *JH*-$KO^{-/-}$ age-matched embryos. In the first case, it was seen that GFP^+ B cells were detected in the bursa of Fabricius of the host WT embryos. The cells maintained their capacity to migrate and colonize the bursa. The cells did not undergo apoptosis and migrated, like the endogenous B cell population into the bursa. Furthermore, it was examined if some of the GFP^+ donor cells found in the host bursae were coming from the T cell lineage, however, none of the donor GFP^+ cells that migrated into the bursae were T lymphocytes. The few cells that were T lymphocytes, were not of donor origin. Though the number of donor cells detected in the host bursae was relative low, the method of adoptive cell transfer was

successful and it was confirmed that it is possible to transfer donor splenocytes into a host embryo and to detect them in the host bursa. However, it was taken into consideration that it might be possible that the donor cells need to compete with the endogenous B cells colonizing the bursa, leading to low efficiency of the transfer.

In order to bypass this problem, a further experiment was performed and splenocytes were isolated from WT donor embryos. Age-matched *JH*-KO$^{-/-}$ embryos were used as hosts for the WT splenocytes. As described in chapter 2.6, *JH*-KO$^{-/-}$ embryos have B cell precursors which migrate into the bursa and even colonize bursal follicles but without expressing surface immunoglobulin. This enables the detection of WT B cells of donor origin and the differentiation from the host embryo's own B cells by their IgM expression. This experiment offers the opportunity to examine if donor WT cells, expressing the surface IgM, have an advantage over the host cells (not expressing surface immunoglobulin) and therefore, colonizing the bursa in higher numbers than *JH*-KO$^{-/-}$ B cells. Moreover, it was possible to test the origin of the IgM^{+} B cells by further analysing the GFP expression of the cells. IgM^{+} cells deriving from the WT embryo have to be GFP^{-}, while the host embryo cells are mostly GFP^{+}. Though most of the cells of the *JH*-KO$^{-/-}$ embryos are GFP^{+}, some of the cells are GFP^{-}, therefore the evaluation of the origin of the cells can not only rely on the GFP expression, but if cells express GFP, then they definitely originate from the *JH*-KO$^{-/-}$ embryos.

In order to ensure that the anti-μ antibody, which was intended to be used for histological analysis after adoptive transfer, does not bind to the regions of the heavy chain loci still maintained in the *JH*-KO$^{-/-}$ cells, histological analysis was performed. WT bursae were positively stained with both AV20 and anti-chIgM antibodies (see Fig. 29), while in *JH*-KO$^{-/-}$ embryos positive staining for B cells with the AV20 but not with the anti-chIgM (see Fig. 29) was seen.

After adoptive transfer of the WT splenocytes into *JH*-KO$^{-/-}$ embryos, single staining was performed to detect IgM expression in WT embryos, in *JH*-KO$^{-/-}$ embryos and in *JH*-KO$^{-/-}$ embryos which had received splenocytes of age-matched WT embryos. IgM^{+} cells were detected in the bursae of the *JH*-KO$^{-/-}$ host embryos (Fig. 31). Not all follicles in the *JH*-KO$^{-/-}$ bursae after cell transfer were positive for IgM expression. Some follicles were highly colonized with IgM^{+} cells, and other were completely negative for IgM expression. Finally, there were follicles which seemed to contain less IgM^{+} cells than others. This result is in agreement with the knowledge that follicles are colonized by two to five B cell precursors which proliferate in the follicle [109]. Some of the follicles were colonized just by donor IgM^{+} cells, others by both host IgM^{-} cells and donor IgM^{+} cells and finally some of the

follicles were only colonized by host IgM^- cells. The adoptive cell transfer result was also examined by flow cytometry and the presence of IgM^+ cells in the bursae of *JH*-$KO^{-/-}$ embryos after cell transfer (2% of the B cells were IgM^+, GFP^- shown in Fig. 32) was confirmed by a second method.

At this point, it would be very interesting to hatch *JH*-$KO^{-/-}$ embryos after adoptive cell transfer of WT splenocytes to investigate the progress of the IgM^+ cells in the host chicks and to analyse the phenotype of the chicks after hatch. Endogenous bursal B cells in *JH*-$KO^{-/-}$ embryos undergo apoptosis and do not emigrate out of the bursa to the periphery. Since, IgM expressing donor cells are detected in the *JH*-$KO^{-/-}$ host embryos bursae, it is likely that these cells of WT origin have the capacity to further develop and even migrate out of the bursa. However, it should also be taken into consideration that eventually immunological competent lymphocytes will respond to the mismatched major histocompatibility complex (MHC) molecules of the transferred donor cells and the host's own cells. This might cause the destruction of the donor cells and lead to strong immune responses in the host chicken. Since fully competent alloimmune responses are expected in the chicken few days post hatch [196], a further question would be if the cells survive and further develop, how long these cells are detectable and what their capacity is in immunological responses. If transferred cells survive post hatch in the recipient embryos, the method of adoptive cell transfer of WT splenocytes into *JH*-$KO^{-/-}$ will be a valuable tool for a better insight into the emigration process of the B cells out of the bursa back to the periphery.

6.2 MIGRATION PATTERN OF B LYMPHOCYTES DURING EMBRYONIC DEVELOPMENT

Most of the experiments analysing the embryonic blood have been performed by histological examination of blood smears [170]. The early blood sampling method standardized in the current study enables the analysis of the blood samples by flow cytometry. It was possible to examine the early appearance of B cell precursors in the embryonic blood and the surface immunoglobulin and chemokine receptor CXCR4 expression.

$AV20^+$ cells were detected already on ED8 and absolute number analysis showed the highest numbers of $AV20^+$ cells on ED14. When examining the relative numbers of $AV20^+$ cells it was interesting to see that 0.7 % was the maximum percentage of $AV20^+$ cells detected in the blood (reached on ED12). The percentage seems to be low. AV20 binds the Bu1 antigen

during this period, since there is data which shows that the Bu1 antigen is expressed in pre-bursal B cells already by ED14 [192] while in the present study the $AV20^+$ cells were already detected on ED8. Consequently, on the question why the percentage is so low it is assumed that during the same period many other cells also migrate through the blood stream. These cells might be of the myeloid lineage such as dendritic cell precursors, granulocytes and monocytes, but also thrombocytes and a very small number of T lymphocytes. Precursor B cells are migrating from the spleen in the bursa between ED10 and ED14 [120, 197], but the first hematopoietic $CD45^+$ cells (such as granulocytes, macrophages and dendritic cells) start migrating into the bursa already on ED8 and until ED15 [198]. This would explain the low levels of B cells. To examine this further stainings with different markers are required. In contrast to mammals, the identification and clear separation of the chicken embryonic cells is still very difficult due to lack of specific markers. Finally, during the whole migration period of the cells into the bursa, around ten to twelve thousand cells colonize the bursa. Taking this into consideration, the absolute number of $AV20^+$ cells detected in the blood at one time point is considered to be valid.

On ED8 a minimal percentage of the $AV20^+$ cells expressed IgM. This amount increased radically on ED10 and decreased afterwards. From ED12 to ED16 the percentage of IgM expressing B cells remained on the same level. On ED18 the highest level of IgM expressing B cells was achieved. It seems that on ED10 rearrangements of the immunoglobulin loci have taken place and allow the expression of the surface immunoglobulin which suddenly increases the level of the detectable IgM significantly. This is compatible with rearrangement of the heavy and light chain loci between ED10 and ED12 [115]. It is considered as possible that IgM^+ cells migrate into the bursa and thereby, since the rearrangements occur during ED10 and ED12, the IgM^+ level decreases after ED12. Moreover, these results are compatible with the knowledge that B cell precursors express the surface immunoglobulin independent of the bursal microenvironment and prior and during their migration into the bursa.

A very interesting option for further evaluation of the signals that might initiate the migration of the B cell precursors is the generation of double knockout chickens, for both heavy and light immunoglobulin chains. Characterization of the *JH*-$KO^{-/-}$ embryos showed that expression of the heavy chain on the cell surface is not required for the B cell precursors migration into the bursa [125]. However, in the heavy chain knockout line, the light chain of the immunoglobulin was not knocked out, which means that it was unknown if essential signals for the migration of the B cell precursors are initiated by the light chain of the immunoglobulin. In 2014 a further knockout line was generated, namely the IgL knockout

line [199]. The light chain of the immunoglobulin was targeted by homologous recombination in primordial germline cells to knockout the light chain locus. The light chain knockout line had a completely different phenotype than the heavy chain knockout line. Homozygous *IgL*-KO embryos possess B cells precursors which, in contrast to the B cells of the *JH*-KO$^{-/-}$ embryos, express the heavy chain on their surface. The heavy chain though presents a deletion of the C_H1 constant region. Moreover, they migrate into the bursa and emigrate back to the periphery. From the *IgL*-KO line characterization it was concluded that the expression of the heavy chain molecule on the surface of the cells supports the development of the B cells [199]. It would be of great interest to generate a double knockout of both heavy and light chain locus and thereby completely knockout the immunoglobulin molecule and completely eliminate the rearrangement of the immunoglobulin loci. The complete absence of rearrangement in both loci and by analysis of these embryos would give answers to the question if rearrangement of the immunoglobulin chains might signal and initiate the migration of B cell precursors. Generating birds with the double knockout, will be easy, since only appropriate pairing of the two lines is needed. The analysis of the B cell migration and development in double knockout birds will give important insights in essential signals for the developing process of the cells in the complete absence of the immunoglobulin signals.

Since there are data indicating a significant role of the CXCR4 chemokine receptor driving the migration of the B cells, it was important to examine if the receptor is actually expressed by the B cells found in the blood. It is already known that the receptor is expressed in the spleen and also in the bursa [155, 156] but until now there was no study investigating its expression in the actual migrating B cells. It would not be possible that migrating cells that do not express the CXCR4 receptor would migrate under regulating signals of the CXCR4-CXCL12 interaction. Therefore, it was essential to examine the expression of the CXCR4 chemokine receptor in B cells in the embryonic blood. The expression of CXCR4 receptor is detected already in blood B cells on ED8. The highest numbers of CXCR4 expressing cells fit perfectly with the migration period of B cells. It was confirmed that B cell precursors express the CXCR4 during their migration period but even after this period many of the B cells detected in the blood still express the receptor on their surface, which might suggest the significance of the receptor also on later stages.

6.3 *IN VIVO* BLOCKING OF THE CXCR4 CHEMOKINE RECEPTOR BY TREATMENT OF THE EMBRYOS WITH AMD3100

Splenic B cell precursors express the CXCR4 receptor. Bursal B cells also express the receptor and while the B cell population in the bursa grows towards hatch, the expression of the receptor in the bursa also rises [155]. Experiments of the current study have shown that CXCR4 expressing B cell precursors are detected in the blood during the migration period of B cell precursors (5.3.2). With the ability to intravenously manipulate the embryo, the opportunity arose to block the CXCR4 receptor in the embryo and examine its role *in vivo* and if blocking of the CXCR4 receptor would inhibit the migration of the B cell precursors into the bursa of Fabricius. According to *in vitro* results, blocking of the CXCR4 receptor with the inhibitor AMD3100 in a migration assay led to inhibition of the DT40 cell (chicken B cell line) migration towards the ligand CXCL12 [155]. Therefore, it was considered possible that CXCR4 blocking *in vivo* might also inhibit the migration of the cells towards CXCL12 *in vivo* and thereby results in an empty bursa or, more likely, a less colonized bursa with fewer follicles or the same amount of follicle filled with less B cells.

Due to lack of previous *in vivo* studies with AMD3100 in the chicken, the appropriate dose of the inhibitor had to be tested. Different doses were tested and well tolerated. It was important to examine the embryonic development after AMD3100 treatment of the embryos macroscopically since studies in CXCR4 deficient mice have shown lethal effects of the deficiency of the receptor on the development of the embryos [69, 186, 200]. After triple administration of the highest dose of AMD3100 on ED11, ED12 and ED14 embryos were macroscopically examined. The macroscopic examination as well as the comparison of the embryonic weights and the weight ratios (splenic weight to embryonic weight and bursal weight to embryonic weight ratios) did not reveal abnormalities in the development of the embryos and their organs. The fact that blocking of the CXCR4 receptor in the chicken embryo did not cause abnormalities or death of the embryo was interpreted by the physiology of the chicken embryonic development. On ED11 (starting point of AMD3100 treatment) organogenesis of the embryo is fully completed and the embryo grows meanwhile in size [184]. On the other hand, homozygous CXCR4 deficient mice, have to deal with the deficiency of the receptor from the beginning of organogenesis therefore, having more severe effects.

Since embryos tolerated well the highest dose of AMD, the dose of 1000µg/kg was chosen for

further experiments in order to achieve a clear effect on the bursal colonization. The administration of AMD3100 on ED11, ED12, and ED14 was performed on these specific days in order to cover as much as possible of the B cell migration period but also taking into consideration that *i.v.* injection is a stressful procedure for the embryo and there are limitations in the frequency of manipulations. Each injection requires scoring and opening of a new window on the egg shell (4.3.2). Moreover, preliminary experiments have shown that injections prior to ED11 are possible but go along with very high mortality rates due to probable high fragility of the vessels at this early stage. Injections damage the vessels and lead to massive bleeding and therefore, beside the high mortality rate also to uncertainty in the efficiency of the drug application.

Histological analysis was performed and the H&E staining (see chapter 5.4.1) did not reveal the expected effect on colonization of the bursa with B cells and the B cell follicle formation. The bursae of the treated embryos seemed to be better colonized than the bursae of the mock treated embryos. Furthermore, a cell population in the connective tissue between the follicles, mainly localized in the middle of the bursal folds, was detected in high numbers (see chapter 5.4.1, Fig. 18). The B cell staining revealed that follicles detected in the bursa of the AMD3100 treated embryos were filled with $AV20^+$ cells. A difference between the groups was not visible by microscopic examination of the B cell stainings (see chapter 5.4.1, Fig. 19).

As mentioned before, H&E staining revealed changes regarding a cell population in the connective tissue surrounding the follicles. In mammals, granulocytes express the CXCR4 chemokine receptor [201] and the receptor regulates the trafficking of these cells. Blocking of the CXCR4 receptor in mammals leads to mobilization of granulocytes from the bone marrow [86, 202]. Consequently, it was taken into consideration that the cells seen in abundance in the H&E stained tissues are granulocytes. In order to examine this hypothesis, bursae were stained with the granulocyte marker GRL1 [193]. The staining confirmed the presence of a larger $GRL1^+$ cell population in the AMD3100 treated embryos. Most of the cells were mainly gathered around follicles in larger groups whereas in the mock treated embryos the cells were localized in much smaller groups or even solely.

Lymphocyte populations were also analysed by flow cytometry to better quantify the results of the histological analysis of the AMD3100 treated bursae. FACS analysis was performed and absolute numbers of both B cells and granulocytes were calculated for both groups of embryos. Opposite to initial expectations, both populations of cells increased in the treated embryos. The results were statistically not significant, though they did confirm the histological picture of more dense and colonized bursae of the treated embryos with both

increased numbers of B cells and granulocytes.

It was expected that blocking of the CXCR4 chemokine receptor would interfere with the CXCR4-CXCL12 axis and B cell precursors migration from the spleen into the bursa would be inhibited. However, that was not the case since B cells were found in the bursae of the treated embryos in even higher numbers than in the mock treated embryos. In order to examine the reasons of these results further experiments were performed. Several explanations have been considered.

In the absence of binding or in the absence of B cells in the bursa, it is possible that CXCL12 expression is upregulated in the bursa. Moreover, upregulation of the CXCL12 might be considered as a result of hypoxic conditions as described in 2004 by Ceradini *et. al.* [203]. Multiple intravenous manipulations of the embryos are most likely a stress factor for the developing embryo and might cause clot formation in the vessels of the embryo. By qRT-PCR analysis of the bursal CXCR4 and CXCL12 expression, a small but significant difference in the CXCL12 expression was detected between mock treated and AMD3100 treated group. Expression of the CXCL12 increased in the AMD3100 treated group (see chapter 5.4.2, Fig. 25). Independently of the reason of CXCL12 upregulation, it is not clear if such a small increase in the expression (0.2 fold) has an actual biological effect. However, the higher expression of the CXCL12 still might explain the increased numbers of B cells and granulocytes in the bursa. Under the effect of the upregulated CXCL12, available $CXCR4^{+}$ cells might migrate in higher numbers than in the control group.

Additionally, another chemokine ligand-receptor system was analysed, namely the CXCL13-CXCR5. From studies in mammals, the CXCL13-CXCR5 axis seems to regulate the migration of the lymphocytes within the germinal centers zones [76, 77, 204]. Prior studies in the chicken have shown that the pair is also expressed in the bursal follicles and in the germinal centers and might also have a regulatory role in the trafficking of the lymphocytes within these structures [143, 155]. The expression analysis of this chemokine receptor-ligand pair was performed to examine if the AMD3100 treatment had an effect on the expression of this set of chemokines or if the changes in the bursal cell populations might have been caused in response to changes in the CXCR5-CXCL13 interaction. However, this does not seem to be the case. Small changes were seen but without statistical significance (see chapter 5.4.2, Fig. 25).

As mentioned before, there are no prior studies in which AMD3100 has been used in the chicken *in vivo*. This meant for the planning of the experiments that doses used in mammals

had to be tested. As described in chapter 2.5, there are studies examining the AMD3100 safety and pharmacokinetics during which 10µg to 260µg/kg were applied *i.v.* and *s.c.* [164]. In $CD34^+$ cell mobilization study, patients were treated with a single application of 1000µg/kg AMD3100 *s.c.* [165]. In the current study the doses of AMD3100 varied from 20µg up to 1000µg/kg. First effects of the AMD3100 embryo treatment were seen after *i.v.* injection of 480µg/kg AMD3100. Up to 1000µg/kg have been used to treat embryos with the same effect. Nonetheless, it is not known if higher doses would have a different result in the migration of B cell precursors. It is possible that a drug requires a much higher dose in one species and lower doses in others. Maybe in the chicken an even higher dose might be required to block the receptor and show biological activity and the expected effect.

In a study of immature B cell mobilization from the bone marrow, 80µg/kg AMD3100 were injected *i.v.* in mice and within one to two minutes disruption of the cell migration was documented at the same time with minor morphological changes of the cells [205]. Three hours after treatment the morphological changes were reversible, which speaks for the rapid decoy rate *in vivo* [164, 205]. In the study of pharmacokinetics and safety of AMD3100, after *i.v.* treatment of probants, an up to 3.1 fold increase of the leukocytes was documented. The blood cell count returned to normal after 24 hours [164]. Though pharmacokinetics of the inhibitor in the chicken *in vivo* have not been examined and it is not clear how fast the agent is degrading *in vivo*, it was assumed that similar rates apply in the case of the chicken.

Taking into consideration the suggested short half live of the AMD3100, AMD3100 was administered three times, on ED11, ED12 and ED14. These specific time points were chosen in order to block the CXCR4 as efficiently as possible during the whole B cell migration period (from ED10 to ED14). Three intravenous manipulations are possible to be performed, thus two of the migration days (ED10 and ED13) could not be covered. It is taken into consideration that B cells have colonized the bursa during these days. Without AMD3100 treatment on ED10 and ED13, B cells can migrate into the bursa and eventually in the absence or under lower competition the cells might have proliferated at a higher rate.

Moreover, a study on neovasculature after myocardial infarction, in which the AMD3100 was used for the blockage of the CXCR4 receptor, indicated different effects of AMD3100 treatment depending on acute or chronic administration. Single AMD3100 treatment of mice led to elevated circulating endothelial progenitor cells (EPCs) and incorporation of these cells in the ischemic border zone. In contrast, after continuous infusion of AMD3100, though increasing EPCs in the circulation, the EPCs were not similarly incorporated [206]. The purpose of this example is to take into consideration that a different biological effect might be

associated with a longer treatment with AMD3100, in comparison to the acute treatment.

In 2005 an alternative chemokine receptor for the CXCL12 chemokine was identified. The receptor is known as CXCR7, atypic chemokine receptor 3 (ACKR3) or RDC-1. CXCR7 has ten times higher affinity towards CXCL12 than CXCR4 [78] but lacks intrinsic chemotactic activity towards its ligand as most decoy receptors. It has been shown that CXCR7 can, independently from the CXCR4 receptor, mediate T cell chemotaxis through the CXCL12 chemokine [78]. Binding of the CXCR7 to CXCL12 initiates signalling through β-arrestin mediated MAPK pathway (see Fig. 5**)** [207]. Furthermore, it has to be considered that AMD3100 has been shown to also bind to CXCR7 and has allosteric agonistic properties. AMD3100 induces the recruitment of β-arrestin to CXCR7 at concentrations from 10 μM [208]. Finally, AMD3100 increased CXCL12 binding and CXCL12-triggered β-arrestin recruitment to CXCR7 [208]. CXCR7 is expressed by B cells in the marginal zone in the spleen [207]. In the chicken genome the CXCR7 gene is located on chromosome 7, but it has not been characterized. Though it is unknown whether the receptor is expressed in the chicken or in which cells this alternative receptor might be expressed, the presence of CXCR7 in mammals is a proof of existing regulatory mechanisms *in vivo* which complicate the interpretation of the regulatory mechanisms of the CXCR4-CXCL12 signalling and its biological effects. It is important to consider the chemokine ligand – receptor axis as part of an extended network of regulating and signalling components.

There are numerous studies in which chicken embryos were treated with testosterone in order to inhibit bursal development [103, 209]. The bursa develops normally up to ED10 but further development is severely affected by the treatment. Treated chickens do not form follicles due to the absence of follicle bud inducer cells [210]. It was seen that some $CD45^+$ hematopoietic cells which migrated into the bursa, do not make it to the surface of the bursal surface epithelium and differentiate into GRL1 and GRL2 granulocytes [100, 211]. The capacity of $CD45^+$ hematopoietic cells to differentiate into granulocytes when they do not reach the surface epithelium, might explain the elevated numbers of granulocytes in the AMD3100 treated embryos bursae. It is considered that $CD45^+$ hematopoietic cells, due to interrupted signalling from the CXCR4-CXCL12 signalling, might not reach the surface epithelium of the bursa and thereby differentiate into $GRL1^+$ cells, increasing the number of granulocytes in the treated embryos bursae.

Finally, it has to be considered that though histological and flow cytometry analysis lead to conclusions on the composition of the bursal cell population they do not give information on

the B cell receptor repertoire of the B cells which were detected in the AMD3100 treated embryos.

6.4 *EX VIVO* BLOCKING OF THE CXCR4 PRIOR TO ADOPTIVE CELL TRANSFER, INHIBITED THE MIGRATION OF THE TRANSFERRED CELLS

The *in vivo* blocking of the CXCR4 receptor in embryos with AMD3100 (see chapter 5.4) had an effect on the bursal population (B cells and granulocytes increased in the treated embryos bursae) which was not expected. Possible explanations were presented in the previous chapter (6.3) and it is possible that other signals or factors in the embryo might interfere to balance or overcome the initial blocking of the CXCR4 receptor. Beside all possible interpretations of the results, it should not be ignored that blocking of the CXCR4 receptor, did have an effect on the colonization of the bursa and the bursal cell populations. Therefore, it was required to further investigate the role of CXCR4 on bursal colonization. With the ability to successfully perform adoptive cell transfers with a standardized method, the opportunity arose to further investigate the role of the CXCR4-CXCL12 interaction through a different approach.

Adoptive cell transfers were performed and *JH*-KO$^{-/-}$ embryos received WT donor splenocytes. As shown in 5.5.2, donor cells can be detected in the host bursae by their IgM expression and the absence of GFP expression. At the same time, adoptive cell transfers were performed and *JH*-KO$^{-/-}$ embryos received WT donor splenocytes, which were first treated with the CXCR4 antagonist AMD3100 (5.5.3). The treatment of the cells with AMD3100 was performed by incubation of the cells with the agent for 15 minutes. As mentioned before, preliminary studies have shown that transfer of the donor cells should be performed as quickly as possible after preparation of the cells to avoid the damage or death of the donor cells prior to transfer. Damaged or dead donor cells would not be able to migrate into the host bursa. Thus, in order to avoid a misleading result due to incubation of the cells and transfer of damaged or dead donor cells which are anyway unable to migrate, the control group embryos received donor cells which were also incubated for 15 minutes under the same conditions, in the same medium but without AMD3100. Examination and analysis was performed by flow cytometry (see Fig. 35). Adoptive cell transfer in *JH*-KO$^{-/-}$ embryos which received mock treated WT donor splenocytes led to the migration and detection of WT donor cells, expressing IgM in the bursa. In the *JH*-KO$^{-/-}$ embryos which received AMD3100 treated WT donor splenocytes, there were also some IgM^{+} cells detected, though in much lower numbers.

In order to quantify this result, absolute counting was performed and a statistical significant difference was found (see Fig. 36). A single treatment of the cells with AMD3100, blocked their CXCR4 receptor and significantly inhibited the migration of the cells in the host bursa (see chapter 5.5.3).

Though *ex vivo* blocking of the CXCR4 receptor in the cells is sufficient to inhibit the migration of the cells towards the ligand, the result of this experiment indicates that it is most likely that the blocking of the receptor *in vivo* is overcome through other mechanisms which might interfere and lead to a different biological effect or are able to further drive the migration of the B cell precursors into the bursa in the absence of the CXCR4-CXCL12 interaction.

Though the two different approaches of the experiments led to controversial results, both approaches speak for the regulatory interaction of the chemokine ligand-receptor: first, the inhibition of the donor B cell migration after *ex vivo* treatment of cells with AMD3100 and adoptive transfer and second, the fact that *in vivo* CXCR4 blocking also caused changes in the bursal cell population (even though with the opposite result than expected). Consequently, there are still questions to be answered related to their function. To better understand the role of the regulatory mechanism, further experiments need to be performed.

First of all, the clonal diversity in the follicles should be examined. Bursal follicles are normally colonized by two to five B cell progenitors [109], which means that the B cells composing each follicle are not monoclonal. Follicles need to be isolated from histological sections and sequenced to investigate if the follicles consist of B cells derived from one unique cell. Thereby, it can be examined if CXCR4 blocking did inhibit the migration of B cell progenitors in the bursa and compromised the colonization of the follicles *in vivo*, leading to formation of follicles from much fewer B cell precursors which highly proliferated though with a less diverse surface immunoglobulin repertoire.

Additionally, analysis of other cell populations in the bursa should be performed. Besides B lymphocyte progenitors, a wide variety of cells also express the CXCR4 chemokine receptor. Granulocytes, T lymphocytes (both $CD4^+$ and $CD8^+$ cells) and $Kul01^+$ myeloid cells express the CXCR4 receptor [155]. T lymphocytes are detected in the bursa in very small populations as shown in chapter 5.5.2 in Fig. 28. $Kul01^+$ cells belong also to the normal bursal cell population and are localized in the interfollicular connective tissue [100]. Therefore, it is meaningful to examine the bursa for possible changes in the other cell populations after inhibition of the CXCR4 chemokine receptor *in vivo*. Finally, the composition of the spleen

and thymus populations should also be examined, as important lymphoid organs in which hematopoietic cells, B and T lymphocyte precursors need to migrate.

Moreover, it would be interesting to examine the B cell capacity of the AMD3100 treated embryos. Studies in $CXCR4^{-/-}$ or $CXCL12^{-/-}$ deficient mice demonstrated that absence of the CXCR4-CXCL12 interaction, leads to a severe B cell deficit and a clear deficit in the colonization of the bone marrow by hematopoietic cells and developing B cells [69]. Thus, it would be interesting to analyse the B cell population post hatch, in case the biological effect of the AMD3100 treatment is seen in the post hatch B cell development stage. It was shown that the treatment of the B cell with the AMD3100 did not result in a less colonized bursa, but it is unknown what properties and capacities the migrated B cells carry.

Another option to further investigate the CXCR4-CXCL12 role includes blocking of the CXCR4 with other inhibitors. The decision to use AMD3100 to block the CXCR4 receptor was made based on the fact that AMD3100 was used in *in vitro* studies and it has successfully inhibited the migration of cells of the chicken B cell line DT40 towards the ligand CXCL12 in migration assays [155]. Meanwhile, due to the results of the direct embryo treatment with AMD3100 and due to the short term activity of the AMD3100, other options for blocking of the CXCR4 should be considered. There is a wide variety of CXCR4 antagonists available (POL5551 [212], T140 and analogs [213, 214] and LY2510924 [215]). Beside one study in which beads soaked in T140 and TN14003 were implanted in embryos after 50 hours of incubation [216], there is no available literature in the chicken. However, a long term active antagonist of the receptor would probably be more efficient in this type of experiments.

In order to completely block the CXCR4 receptor, the generation of CXCR4 deficient chicken line is also an option. However, due to the severe side effects and perinatal deaths of homozygous CXCR4 deficient mice, it is assumed that similar problems would rise from a deficiency in the chicken embryo development. Therefore, a conditional CXCR4 deficiency would be meaningful, in order to be able to knockout the CXCR4 gene either in specific tissues or cells or in a specific time period, after the organogenesis has completed and during B cell migration to the bursa. The much promising CRISPR/Cas9 (Clustered regularly interspaced short palindromic repeats and Cas9 endonuclease) tool enables fast and accurate genome editing [217], so that the generation of the conditional CXCR4 deficient chicken line can actually be considered. Moreover, another option would be the generation of a chicken line carrying the Cre protein, which when paired with a line where the CXCR4 gene is targeted with loxP sites would delete the targeted sequence of the CXCR4 receptor.

Beside the role of CXCR4, to generally better understand the early B cell development performance of further experiments is required. Some experiments that are enabled by the methods standardized in this current study or arose by the results of this study, are presented here.

As described in 6.1, it would be interesting to further examine the development of *JH*-KO$^{-/-}$ embryos after adoptive transfer of WT splenocytes to examine the fate of the donor cells. The phenotype of the hatched chicks should be analysed, including analysis of whether the transferred cells survive till hatch and can be detected after hatch in the periphery. Moreover, in case the cells are still present after hatch it has to be examined whether they function as mature B cells. In other words, if adoptive transfer of WT splenocytes during early B cell development can reconstitute the B cell population in heavy chain knockout chickens and bypass immune responses due to mismatched MHC molecules.

Additionally, it would be interesting to perform experiments to block the L-selectin cell surface adhesion molecule. While B cell progenitors and dendritic cells migrate into the bursa through the blood stream, they express at the same time on their surface the sialyl-LewisX carbohydrate [118, 120, 121], otherwise known as sCD15. The expression of sialyl-LewisX suits with the theory that migration of the cells might be mediated by the cell surface adhesion molecule L-selectin or otherwise known as CD62L. Rabbits together with chickens, sheep and cattle, belong to the species that require a gut associated lymphoid tissue -GALT- for the proliferation and diversification of the B cells antibody repertoire [218]. Rabbit's pro- and pre-B cells are found in the bone marrow and in order for the cells to further develop and diversify, they have to migrate into the appendix (GALT). B cell precursors, expressing CD62L on their surface, migrate towards the appendix where the peripheral lymph node addressin (PNAd), a receptor for the CD62L, is expressed on the high endothelial venules (HEV). In adoptive transfer experiments mostly CD62L^{+} cells migrated towards the appendix while blocking of the PNAd by means of an anti-PNAd antibody, the migration of the cells was reduced to half [219]. Moreover, CD62L ligation is connected to the induction of a 'rolling' movement of the cells on the endothelial surface. The binding enables the interaction of the cells with chemokines and also integrin binding with final result the extravasation of the cells. In the appendix the formation of proliferative regions in the follicles seems to be regulated by the CXCR4-CXCL12 interaction in response to commensal-derived signals. Similar to the chicken, CXCR4 is expressed by B cells while CXCL12 is expressed by stromal cells (in the rabbit's appendix) [218]. The theory of selectin-mediated migration in the chicken B cell development can be examined by performance of principally similar

experiments as in this project, trying to block the interaction between L-selectin and its receptor. Therefore, two approaches can be used. The first as direct treatment of the embryos with blocker of the CD62L or its receptor PNAd or second, by adoptive transfer experiments after cell treatment with anti-CD62L specific antibodies or CD62L antagonists and following examination of the colonization of the bursa. Furthermore, an even more interesting experiment would be to block in embryos both CXCR4 and CD62L at the same time *in vivo* and examine the colonization of the bursa.

7. SUMMARY

Although B cells were first found and identified as antibody producing cells in the chicken and numerous studies have been performed to analyse B cell development in mammals, the knowledge on the early B cell development in the chicken is still limited.

In order for B cell precursors to differentiate and develop, they need to migrate from the spleen, where they are first localized, into the bursa of Fabricius, where they further diversify and proliferate. However, the signals that drive the migration of the pre-bursal B cells from the spleen into the bursa have yet to be discovered. In this project the factors involved in B cell precursor immigration into the bursa were analysed *in vivo*.

Methods for intravenous manipulation and adoptive cell transfer were adapted to the needs of the project and standardized. The method of intravenous manipulation directly in the embryos while in its own egg shell through scoring of a window over a vessel proved to be the optimal choice. The method of intravenous manipulation enabled blood sampling and further survival of the chicken embryo, allowing further manipulations during embryonic development. Small volumes of blood sampling from ED11 or older embryos do not compromise the survival of the embryos. Intravenous injections were also performed by means of the same method. Administration of substances was possible up to three times in the same embryo without severe effects on the embryo survival rates.

Adoptive cell transfer experiments were performed to standardize the method. It enabled the analysis of the colonization of the bursa by donor cells. Through adoptive cell transfer of splenocytes from donor embryos to age-matched host embryos, it could be shown that donor splenocytes maintain their capacity to migrate into the bursa and colonize follicles while expressing IgM on their surface.

The migration pattern of B cells during different development time points was analysed by flow cytometry between embryonic day 8 and 18. The first B cells were already detected on ED8, while the highest amount of B cells in the blood was detected on ED12. Expression of the surface immunoglobulin and the CXCR4 receptor was also analysed by flow cytometry. Both surface immunoglobulin and CXCR4 receptor are expressed during the time period of the examination in the embryo.

Embryos were treated with the specific CXCR4 antagonist, AMD3100, to investigate the effect of blocking the CXCR4-CXCL12 interaction on the colonization of the bursa of Fabricius. However, intravenous treatment of the embryos with the CXCR4 antagonist did not

inhibit the migration of the B cells, but had an effect on the migration of the cells since more B cells and granulocytes were detected in the bursae of the AMD3100 treated embryos.

Adoptive transfer of splenocytes from a donor embryo to a host embryo was successful: transferred cells maintained their capacity to migrate in the host bursa and colonize follicles. With this knowledge, further experiments were performed and donor cells were treated with the CXCR4 inhibitor prior to their transfer while another group of embryos received donor cells without AMD3100. AMD3100 treatment prior to transfer significantly reduced the migration of transplanted cells into the bursa compared to mock treated cells.

In contrast to the experiments of direct AMD3100 treatment of the embryos, it was shown that *ex vivo* blocking of the CXCR4 receptor on the surface of the donor cells prior to their transfer into the host embryo does lead to inhibition of the migration of the transferred cells.

The successful blockage of bursal colonization with B cells after treatment with the CXCR4 inhibitor AMD3100 confirmed the significance of the CXCR4-CXCL12 interaction *in vivo* in the regulation of migration during early B cell development.

8. ZUSAMMENFASSUNG

B Zellen wurden zuerst im Huhn als Antikörper produzierende Zellen beschrieben. Zwar wurde die Entwicklung von B Zellen in Säugetieren in den vergangenen Jahren im Detail erforscht, jedoch sind unsere Kenntnisse bezüglich der Entwicklung der B Zellen im Huhn limitiert.

Die B Vorläuferzellen werden zuerst in der Milz gefunden. Von dort aus wandern sie weiter in die Bursa Fabricius, um sich dort weiter zu differenzieren und zu entwickeln. Im Rahmen dieser Arbeit wurde die frühe B Zellen Entwicklung in Bezug auf die Signale, welche für die Kolonisierung der Bursa Fabricius notwendig sind, analysiert.

Methoden zur intravenösen Manipulation und des adoptiven Zelltransfers zu verschiedenen Entwicklungszeitpunkten des Hühnerembryos wurden etabliert und an die Anforderungen dieser Arbeit angepasst und standardisiert. Die direkte intravenöse Manipulation des Embryos in seiner eigenen Eischale hat sich als beste Methode herausgestellt. Bei dieser Methode wird ein geeignetes Blutgefäß lokalisiert und durch Entfernen der Eischale über diesem Bereich freigelegt. Die Blutentnahme von geringen Mengen ab Embryonaltag 11 hat minimalen Einfluss auf das Überleben der Embryos. Intravenöse Injektionen wurden mit der gleichen Methode durchgeführt. Bis zu drei aufeinanderfolgende intravenöse Manipulationen pro Embryo wurden in dieser Arbeit durchgeführt, ohne massiv das Überleben der Embryos zu schädigen.

Der adoptive Zelltransfer in Hühnerembryonen wurde standardisiert, um die Analyse der Kolonisierung der Bursa von Spender-Zellen zu ermöglichen. Nach adoptivem Zelltransfer von Milzzellen in gleichaltrige Embryos konnte gezeigt werden, dass die transferierten Zellen ihre Fähigkeit zur Kolonisierung der Bursa und Ausbildung von B Zellfollikeln beibehalten.

Die Migration der B Zellen mit dem Blut wurde zwischen Embryonaltag 8 bis 18 mittels Durchflußzytometrie untersucht. Die ersten B Zellen wurden am Embryonaltag 8 detektiert, während die höchste Anzahl am Embryonaltag 12 erreicht wurde. Desweiteren, wurde die Expression von Oberflächen Immunoglobulin der B Zellen analysiert sowie die Expression des CXCR4 Chemokinrezeptors. B Zellen, die IgM exprimieren sowie B Zellen, die CXCR4 Rezeptor exprimieren, wurden in minimaler Anzahl am Embryonaltag 8 detektiert. Beide, IgM und CXCR4 Rezeptor, sind während der angegebenen Zeit exprimiert.

Um die Bedeutung der CXCR4-CXCL12 Interaktion *in vivo* zu untersuchen, wurde die Wirkung des CXCR4 Inhibitors AMD3100 auf die Kolonisierung der Bursa mit B Zellen

untersucht. Die Behandlung der Embryos durch intravenöse Injektion des CXCR4 Inhibitors AMD3100 führte nicht zu einer Hemmung der Migration der B Zellen.

Nachdem B Zellvorläufer aus der Milz nach dem adoptiven Transfer ihre Fähigkeit zur Kolonisierung der Bursa und Ausbildung von Follikeln beibehielten, war es möglich, die Bedeutung von CXCR4 in einem zweiten System zu analysieren. Hierfür wurden die Spenderzellen vor deren Transfer mit AMD3100 behandelt. Im Vergleich zu der Kontrollgruppe wurden in den Embryos, die AMD3100 behandelte Spenderzellen erhielten, signifikant weniger Spender-B Zellen in der Bursa Fabricius detektiert.

Obwohl der exakte Mechanismus der Bursakolonisierung noch nicht geklärt ist und weiterer Untersuchungen bedarf, konnte eindeutig die Signifikanz der CXCR4-CXCL12 Interaktion in der Kolonisierung der Bursa Fabricius gezeigt werden.

9. LITERATURE INDEX

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10. ATTACHMENT

A. Chemicals and Reagents

References have been noticed as high numbers next to the chemicals and reagents mentioned in chapter 4. (Material and Methods) according to the following list:

1. AppliChem GmbH, Darmstadt
2. Invitrogen GmbH, Karlsruhe
3. Nunc GmbH & Co. KG, Wiesbaden
4. Sigma GmbH, Deisenhofen
5. BioLegend GmbH, Fell
6. Southern Biotechnologies Inc., Birmingham/USA
7. CLN GmbH, Freising
8. AIR LIQUIDE Deutschland GmbH, Düsseldorf
9. Sigma Aldrich, Saint-Louis, United States
10. Linaris, Wertheim
11. Merck KGaA, Darmstadt
12. eBioscience Inc., Frankfurt
13. Bio-Rad AbD Serotec GmbH, Puchheim
14. Jackson ImmunoResearch Europe Ltd., New Market/UK
15. Developmental Studies Hybridoma Bank, Iowa City, Iowa, United States
16. Qiagen GmbH, Hilden
17. Sarstedt AG, Nümbrecht
18. Solis BioDyne OÜ, Estonia
19. Eurofins GmbH, Hamburg
20. Biochrom GmbH, Berlin
21. Carl Roth GmbH, Karlsruhe

22. Henry Schein VET GmbH, Hamburg
23. Brüterei GmbH Thole, Bösel
24. MarMed GmbH, Cölbe
25. VWR International GmbH, Ismaning
26. Medite GmbH, Burgdorf
27. Biozol Diagnostica Vertrieb GmbH, Eching
28. Lyreco, Marly France
29. neoLab Migge GmbH, Heidelberg
30. SAV Liquid Production GmbH, Flintsbach am Inn
31. Friendly provided by the Institute for Anatomy, Histology and Embryology of the Department for Veterinary Sciences, LMU Munich
32. Thermo Fischer Scientific Messtechnik GmbH, Munich
33. Conrad GmbH, Munich
34. HEKA Brutgeräte GmbH, Rietberg
35. Grumbach Brutgeräte GmbH, Aßlar
36. BD Biosciences, Germany
37. Siepmann GmbH, Herdecke
38. Saran, United States
39. Promega GmbH, Germany
40. Peqlab Biotechnologie GmbH, Erlangen
41. Analytik Jena GmbH, Jena
42. Tobias Caratiola, Munich

B. Laboratory instruments in alphabetical order

Instrument	Product type	Company
Analysis Scale	Mettler PJ400	Mettler-Toledo GmbH, Gießen
Autoclave	Varioklav Type 500E	Thermo Scientific, Munich
Centrifuge	Sigma 4K15C	Sigma, Deisenhofen
Centrifuge	Sigma 2-6 Small	Sigma, Deisenhofen
Centrifuge	Centrifuge 5810	Eppendorf, Cologne
Dermal tool	Dermal® 3000-15	Conrad Electronic GmbH, Hirschau
Egg candler	LED egg candler	Siepmann GmbH, Herdecke
Egg candler with fixation system	Egg candler with fixation system	Tobias Caratiola, Munich
Incubator	BSS 200/8203	Grumbach Brutgeräte GmbH, Asslar
Incubator	BSS 300/8203	Grumbach Brutgeräte GmbH, Asslar
Incubator	HEKA-Favorit-Olymp Customized order	HEKA Brutgeräte GmbH, Rietberg
Flow Cytometer	FACS CantoII	Becton Dickinson, Heidelberg
Flow Cytometer	FACS Accuri C6	Becton Dickinson, Heidelberg
Flock ice maker	Scotsman AF100	Scotsman Ice Systems, Milan, Italy
Gel Documentation Software	Image Quant Capture 300	GE Healthcare, Garching
Hemocytometer	Modified, Neubauer	Brand, Wertheim
Hand centrifuge	Rotilabo®-Mini-Centrifuge	Carl Roth GmbH + Co.KG, Karlsruhe
Heating cabinet	neoLab Universal Wärmeschrank Basic 39l	neoLab Migge Laborbedarf-GmbH
Homogenizer	SpeedMill Plus	Analytikjena GmbH, Jena
Horizontal shaker	KL-2	Edmund Bühler GmbH, Hechingen
Liquid nitrogen tank	Locator 6 Plus	Barnstead Thermolyne, Dubuque/USA
Microscope	Axioskop	Zeiss GmbH, Jena
Microscope	Leica DMIL Camera DF340XF	Leica GmbH, Bensheim
Microscope	M8, digital microscope and scanner	PreciPoint, Freising GmbH
Microwave S2	MS1987U	LG Electronics, Ratingen
Micro centrifuge	5415R	Eppendorf AG, Hamburg
NanoDrop Spectralphotometer	NanoDrop ND-100	Peqlab GmbH, Erlangen
PCR-Workstation	Ultraviolet Sterilising PCR Working station	Peqlab, Erlangen
pH-meter	Sartorius PB-11 with Glas electrode PY-P10	Sartorius AG, Göttingen

Safety working hood	Microflow	Nunc, Wiesbaden
Shaker	Combi shaker KL-2	Johanna-Otto GmbH, Hechingen
Shaking incubator with hood	Shaker KS15A with Incubator hood TH15	Edmund Bühler GmbH, Hechingen
Thermo cycler	MJ Mini	Bio-Rad, Munich
Thermo shaker	TS 1 Thermoshaker	Biometran GmbH, Göttingen
Transportable incubator	Modell 400	Siepmann GmbH, Herdecke
Vacuum centrifuge	Speed Vac Concentrator	Bachofer, Reutlingen
Ultra centrifuge	Avanti J-25	Beckmann Coulter, Krefeld
Vortex-Mixer	GVLab®	Gilson Inc., Middleton, USA

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